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Inhibition of Mammalian Target of Rapamycin Signaling by CCI-779 (Temsirolimus) Induces Growth Inhibition and Cell Cycle Arrest in Cashmere Goat Fetal Fibroblasts (*Capra hircus*)

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The mammalian target of rapamycin (mTOR) is a Ser/Thr kinase. It plays an evolutionarily conserved role in regulating cell growth, proliferation, survival, and metabolism via different cellular processes. The purpose of this study was to explore the inhibitory effects of CCI-779 (temsirolimus), a specific mTOR inhibitor, on mTOR signaling, and examine the mechanism of cell growth suppression by CCI-779 in Cashmere goat fetal fibroblasts (GFb cells). GFb cells were sensitive to CCI-779 and the survival rate of cells treated with $>3.0\,\mu M$ of CCI-779 was significantly reduced compared with the control ($p<0.01$). CCI-779 inhibited the phosphorylation of mTOR (at Ser2448) and S6 (at Ser240/244), and the expression of mTOR, p70S6K, and S6. Thus, CCI-779 was toxic to GFb cells, and it induced a dose-dependent decrease in cell proliferation and caused G1/S cell cycle arrest. Taken together, these data show that CCI-779 can inhibit mTOR signaling and proliferation in GFb cells *in vitro*. Therefore, mTOR is an important regulator for GFb cell growth and proliferation.

Introduction

The mammalian target of rapamycin (mTOR) is a Ser/Thr kinase. It forms two complexes with additional proteins, namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The best understood of these proteins is mTORC1; studies have demonstrated that mTORC1 is implicated in many human diseases, including diabetes (Fraenkel *et al.*, 2008; Zoncu *et al.*, 2011), obesity (Um *et al.*, 2004; Chakraborty *et al.*, 2010), and certain cancers (Shaw and Cantley, 2006; Jiao *et al.*, 2011). In addition, the mTORC1 signaling pathway is important in the nervous system (Li *et al.*, 2010; Neasta *et al.*, 2010), ageing (Selman *et al.*, 2009; Kapahi *et al.*, 2010), and immune cells (Powell and Delgoffe, 2010). It can balance self-renewal and differentiation of hematopoietic stem cells (Kobayashi *et al.*, 2010) and induce differentiation of pluripotent human embryonic stem cells (Easley *et al.*, 2010). mTORC1 signaling plays an evolutionarily conserved role in the regulation of cell growth, proliferation, survival, and metabolism via different cellular processes.

In the past few years, significant advances in our understanding of the regulation and functions of mTOR have revealed the crucial involvement of this signaling pathway in protein synthesis and degradation (Qian *et al.*, 2010; Bajotto *et al.*, 2011). mTORC1 also controls important functions in peripheral organs, such as muscle oxidative metabolism, white adipose tissue differentiation, and β cell–dependent insulin secretion (Catania *et al.*, 2010). mTORC1 activation is sufficient to stimulate specific metabolic pathways, including glycolysis, the oxidative arm of the pentose phosphate pathway, *de novo* lipid biosynthesis (Duvel *et al.*, 2010), and even cholesterol trafficking and fasting-induced ketogenesis (Sengupta *et al.*, 2010; Xu *et al.*, 2010). mTOR is an evolutionarily conserved nutrient-sensing protein kinase that regulates growth and metabolism in all eukaryotic cells (Wullschleger *et al.*, 2006). Cell metabolism is tightly coupled to energy status and nutrient levels by mTOR signaling.

Progress has been made in the identification of mTOR signaling pathway components and their particular functions in humans and mice (Wullschleger *et al.*, 2006; Selman *et al.*, 2009), but little is known about the role of this pathway in small ruminants. Specifically, little knowledge is available regarding the role of mTOR in goat cells. In this current study, in order to identify the mTOR signaling pathway and explore its functions and regulatory mechanisms in goat cell growth, Cashmere goat fetal fibroblasts (GFb cells) were

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treated with CCI-779 {also named temsirolimus or rapamycin 42-[2,2-bis(hydroxymethyl)propionate]}, which is a specific mTOR inhibitor. The mTOR signaling pathway was found to be functional in GFb cells and acted as an important regulator of cell growth and proliferation.

Materials and Methods

Cell culture conditions

Inner Mongolian Cashmere goats were bred on a natural diet in Inner Mongolia, China. A 40-day-old fetus was removed by caesarean from a pregnant ewe and cut into small pieces. The tissue pieces were cultured as primary culture and fetal fibroblasts were isolated. The GFb cells were cultured in DMEM/F12 (Gibco) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc.), 100 U/mL penicillin G, and 100 mg/mL streptomycin (Sigma-Aldrich, Inc.), and maintained in monolayer culture at 37°C in humidified air containing 5% CO_2. P2 to P6 generation cells were used in the experiments. Cellular morphology was observed using a light microscope (Nikon Eclipse Ti-S; Nikon Corporation).

Reagents and antibodies

CCI-779, a bioavailable derivative of rapamycin, was synthesized by Wyeth Pharmaceuticals, Inc., and presented by Dr. Naomoto (Okayama University, Japan). CCI-779, from a TORISEL injection (25 mg/mL), was stored at 4°C and then diluted to appropriate concentrations with culture media before use. The following primary antibodies were purchased and used in this study: anti-S6 and anti-phosphor-S6 (Ser240/244; Cell Signaling Technology, Inc.), anti-p70S6K (Santa Cruz Biotechnology, Inc.), anti-β-actin (Sigma-Aldrich, Inc.), and phosphor-mTOR (Ser2448; Abcam). The anti-mTOR antibody used herein is a mouse serum polyclonal antibody that was raised against in our laboratory against the C-terminus of the mTOR kinase of Cashmere goat.

Trypan blue exclusion assay

The antiproliferative activity of CCI-779 on GFb cells was determined using a trypan blue exclusion assay. GFb cells were seeded into 24-well culture plates at 1×10^4 per well and incubated for 24 h before drug treatment. For sensitivity experiments, subconfluent cells were treated for 48 h with 0.01–20 μM of CCI-779 or vehicle only (0.5% ethanol [v/v]) or no treatment at all, and then cells were harvested and stained with trypan blue. For growth curve experiments, subconfluent cells were treated for 48 h with CCI-779 (1–20 μM), and then cells were harvested with trypsin on the indicated day after treatment and stained with trypan blue. Cell numbers were calculated using a hemocytometer.

Cell cycle analysis by flow cytometry

For cell cycle analysis, GFb cells were seeded into six-well tissue culture plates at 3×10^5 cells per well and incubated for 24 h at 37°C. Subconfluent cells were treated for 48 h with 11 μM of CCI-779, and then harvested. Cells were washed with cold phosphate buffered saline and stained with propidium iodide (50 mg/L). DNA content was analyzed by flow cytometry (FACS; Calibur, Becton-Dickinson Co.).

Western blotting

GFb cells were seeded into six-well tissue culture plates at 3×10^5 cells per well and incubated for 24 h at 37°C. Subconfluent cells were treated for 48 h with CCI-779 (0.1–10 μM), and then harvested with trypsin. Cells were lysed and processed as previously described (Wang *et al.*, 2010). The concentrations of protein lysates were measured using the Bio-Rad protein determination method (Bio-Rad Laboratories). Equal amounts (40 μg) of protein were electrophoresed in 8% (w/v) or 12% (w/v) sodium dodecyl sulfate polyacrylamide gels. Then the proteins were transferred onto a PVDF transfer membrane and incubated with the primary antibody overnight at 4°C. This was followed by incubation with IRDye 800CW conjugated goat (polyclonal) anti-rabbit/mouse IgG secondary antibody (1:5000; LI-COR, Inc.) at room temperature for 1 h. The Odyssey Two-Color Infrared Imaging System (LI-COR, Inc.) was used for signal detection.

Statistical analysis

Descriptive statistics were generated for all quantitative data and these are presented as mean ± one standard deviation. Proliferation of the cells exposed to CCI-779 was compared with the negative control. Statistical significance was defined as $p<0.05$.

Results

CCI-779 inhibited proliferation of GFb cells

The effect of CCI-779 on the proliferation of GFb cells was examined by trypan blue exclusion assay. GFb cells were sensitive to CCI-779 and the survival rate of cells treated with >3.0 μM CCI-779 was significantly reduced compared with the control ($p<0.01$). There was no significant difference between the survival of cells treated with vehicle (0.5% ethanol) and the untreated control (Fig. 1). As shown in Figure 2, the growth curves demonstrated that 5, 10, and 20 μM of CCI-779 clearly suppressed GFb cell growth from 6 to 7 days after treatment.

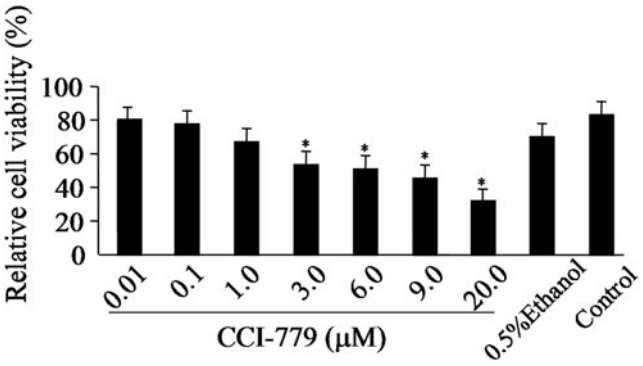

FIG. 1. Effect of CCI-779 and vehicle (0.5% ethanol; v/v) on cell proliferation of GFb cells examined by trypan blue exclusion assay. Survival rate of cells treated with >3.0 μM of CCI-779 was significantly reduced compared with the vehicle only and untreated controls. GFb, goat fetal fibroblasts. *p<0.05.

CCI-779 induced G1/S cell cycle arrest in GFb cells

To analyze the cell cycle after CCI-779 treatment, GFb cells were incubated with CCI-779 (11 μM for 48 h) and FACS analysis was performed. Inhibition of cell cycle progression occurred due to CCI-779 treatment, as demonstrated by a decrease in the proportion of cells in S phase (Fig. 3). Thus, CCI-779 induces G1/S cell cycle arrest in these cells.

CCI-779 inhibited activation of mTOR *and its downstream targets p70S6K and S6*

To explore the mechanism of GFb cell growth inhibited by CCI-779, the expression levels of mTOR, p70S6K, and S6 were investigated with western blotting, while the phosphorylated activities of phospho-mTOR (Ser2448) and phospho-S6 (Ser240/244) were examined. CCI-779 inhibited the activity of these kinases and slightly suppressed the expression of mTOR, p70S6K, and S6 (Fig. 4). CCI-779 induced a dose-dependent decrease in the expression and activities of mTOR and S6 in GFb cells 48 h after treatment.

Discussion

The mTOR has emerged as a major effector of cell growth and proliferation by regulating protein synthesis. The mTOR signaling pathway also affects the development of early embryos and newborns, and even adult growth. mTORC1 and mTORC2 mediate both intracellular and extracellular signals and form an intersecting biochemical network with other signaling pathways (Laplante and Sabatini, 2009; Caron *et al.*, 2010). It is believed that mTOR regulates basic biological processes and acts as a central regulator of cell growth.

The mTOR signaling pathway has been investigated extensively in humans and some model animals, whereas this is the first report to examine this pathway in the goat. This is largely due to the lack of basic genetic, gene expression and function data for these ruminants. In this current study, the mTOR signaling pathway was found to be functional in GFb cells. CCI-779 inhibited the activity of the mTOR signal pathway and cell proliferation, it blocked the cell cycle, and it was also cytotoxic to GFb cells in a dose-dependent manner. CCI-779, a rapamycin ester analogue with the molecular formula $C_{56}H_{87}NO_{16}$ and a molecular weight of 1030.30 Da, is a water-soluble synthetic rapamycin ester inhibitor of the mTOR protein that has been developed as both oral and intravenous formulations. It exhibits antitumor effects *in vitro* and *in vivo* (Frost *et al.*, 2004; Ma and Jimeno, 2007), and demonstrates dose-dependent antitumor activity in some carcinoma models. Moreover, CCI-779 induces G1 cell cycle arrest and apoptosis (Teachey *et al.*, 2006; Gridelli *et al.*, 2008). At present, CCI-779 is undergoing phase III evaluation as an antitumor agent.

The morphologies and densities of GFb cells after mTOR inhibition were observed using an optical microscope and confocal cell imaging (using a combination of fluorescent dyes for F-actin). GFb cells were sensitive to CCI-779, as some cells changed to a more round shape, while the structure of the cytoskeleton was seen to change under confocal laser scanning microscopy after treatment with CCI-779 (28, 29). Previously, mTOR was shown to regulate the expression of slit diaphragm proteins and cytoskeleton structure (Vollenbroker *et al.*, 2009). Further, activated mTOR is enriched at the actin arc and EGF stimulation induces actin arc formation

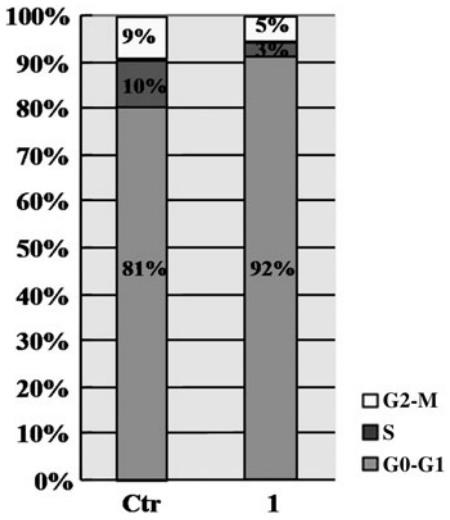

FIG. 3. Cell cycle analysis was performed by flow cytometry. CCI-779 induced G_1/S cell cycle arrest in GFb cells. Ctr: control (48 h without CCI-779); 1: treatment with 11 μM of CCI-779 for 48 h.

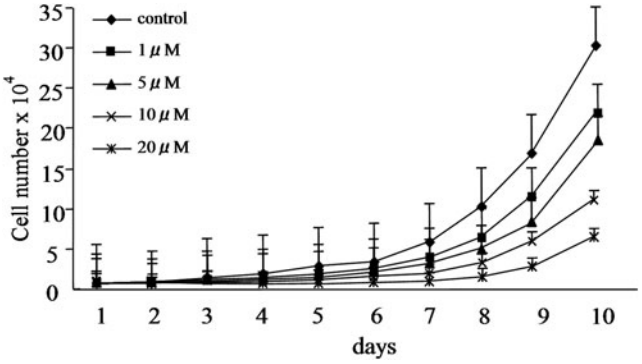

FIG. 2. CCI-779 suppresses the proliferation of GFb cells. CCI-779 treatments were started at day 1 and cell numbers were assessed every 24 h until day 10. ◆: control; ■: 1 μM; ▲: 5 μM; ×: 10 μM; *: 20 μM.

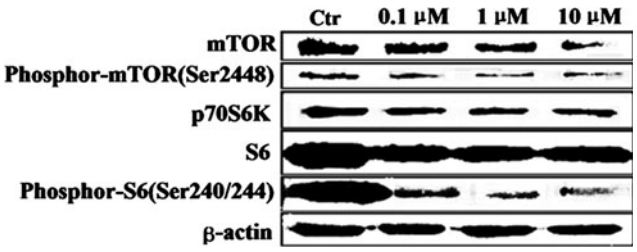

FIG. 4. Activities of mTOR and its downstream targets were inhibited by CCI-779 in GFb cells. CCI-779 inhibited the activity of the mTOR and S6. Expression levels of the mTOR, p70S6K, and S6 were slightly suppressed in GFb cells at 48 h after treatment. CCI-779 induced a dose-dependent decrease in the expression and activities of mTOR and S6. mTOR, mammalian target of rapamycin.

in Swiss 3T3 fibroblasts (Berven *et al.*, 2004). These data suggest that mTOR may be a key regulator in the organization of the cell cytoskeleton.

Recently, mTOR was identified to play a role in protein synthesis in pigs (Suryawan *et al.*, 2010; Torrazza *et al.*, 2010). In cows, nutrient and growth factor signals are associated with the mTOR signaling pathway to regulate mammary protein synthesis and milk protein yield (Burgos *et al.*, 2010; Toerien *et al.*, 2010). In contrast to porcine and bovine studies, investigations of mTOR functions in sheep have focused on the effects of the mTOR signaling pathway on the proliferation and migration of trophectoderm cells (Gao *et al.*, 2009; Kim *et al.*, 2010; Kim *et al.*, 2011). The development of sheep fetal skeletal muscle stimulated by nutrient is associated with the mTOR signaling pathway (Zhu *et al.*, 2004; Brown *et al.*, 2009). In additional experimentation, the genes that code important mTOR signaling pathway–related kinases were cloned, including *mTOR* (HM114224), *p70S6K* (GU144017), *S6* (GU131122), *Akt* (HM130679), *Rheb* (HM569224), *FKBP38* (JF714970), *TSC2* (HQ684023), *ERK*, and *4EBP1*, and the expression patterns of these genes were detected in different tissues. The cloned Cashmere goat genes share conserved nucleotide sequences with data available in GenBank, and their tissue distribution of mRNA accumulation was different. Thus, the mTOR signaling pathway is evolutionarily conserved in the goat.

Previously, inhibition of mTOR was shown to induce decreased phosphorylation activities of p70S6K and S6, which are downstream targets of mTOR. In this current study, inhibition of mTOR not only decreased the phosphorylation activity of the proteins, but it also suppressed the expression of mTOR, p70S6K, and S6 in GFb cells (Fig. 4). The expression of these proteins was inhibited, which suggests that the mTOR signaling pathway is associated with the expression of these genes, though the exact mechanisms involved remain unclear. Although more work is needed to comprehend these mechanisms, these new data suggest that the genes were expressed in an mTOR-dependent manner.

In summary, this current study shows for the first time that the mTOR signaling pathway plays a critical role in regulating the proliferation of GFb cells. Although more physiological studies are needed to elucidate the precise mechanisms of the mTOR signaling pathway in goat cells, we propose that mTOR may act as a key regulator in the development of goat with good meat and wool traits. This will form the focus of our future research.

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Disclosure Statement

No competing financial interests exist.

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