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PGE_2-induced colon cancer growth is mediated by mTORC1

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ABSTRACT

The inflammatory prostaglandin E_2 (PGE_2) cytokine plays a key role in the development of colon cancer. Several studies have shown that PGE_2 directly induces the growth of colon cancer cells and furthermore promotes tumor angiogenesis by increasing the production of the vascular endothelial growth factor (VEGF). The signaling intermediaries implicated in these processes have however not been fully characterized. In this report, we show that the mechanistic target of rapamycin complex 1 (mTORC1) plays an important role in PGE_2-induced colon cancer cell responses. Indeed, stimulation of LS174T cells with PGE_2 increased mTORC1 activity as observed by the augmentation of S6 ribosomal protein phosphorylation, a downstream effector of mTORC1. The PGE_2 EP_4 receptor was responsible for transducing the signal to mTORC1. Moreover, PGE_2 increased colon cancer cell proliferation as well as the growth of colon cancer cell colonies grown in matrigel and blocking mTORC1 by rapamycin or ATP-competitive inhibitors of mTOR abrogated these effects. Similarly, the inhibition of mTORC1 by downregulation of its component raptor using RNA interference blocked PGE_2-induced LS174T cell growth. Finally, stimulation of LS174T cells with PGE_2 increased VEGF production which was also prevented by mTORC1 inhibition. Taken together, these results show that mTORC1 is an important signaling intermediary in PGE_2 mediated colon cancer cell growth and VEGF production. They further support a role for mTORC1 in inflammation induced tumor growth.

1. Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer related death. Chronic inflammation has been identified as a major risk factor for developing CRC. In fact, clinical studies have demonstrated that the regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the risk of CRC by up to 50% [1,2]. NSAIDs primarily inhibit cyclooxygenase-1 and 2 which are enzymes that catalyze the production of prostaglandins and thromboxane A_2 from arachidonic acid. In the context of CRC, the production of the proinflammatory prostaglandin E_2 (PGE_2) plays a significant role in promoting tumor development [3]. High PGE_2 levels were detected in human samples of CRC and PGE_2 was shown to induce colon cancer formation in mice models [4–6]. At the cellular level, PGE_2 promotes cancer growth and spreading by directly inducing colon cancer cell proliferation, survival and migration. Furthermore, PGE_2 also influences cells in the tumor microenvironment and favors tumor growth by suppressing immunosurveillance and promoting tumor angiogenesis [7].

The mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that exerts its biological effects by being part of two distinct protein complexes named mTORC1 and mTORC2 [8,9]. mTORC1 plays a central role in cell growth by regulating key functions including protein and lipid synthesis as well as energy metabolism. It is activated by growth factors, amino acids and the cellular energy status. Following activation, mTORC1 phosphorylates several substrates including the ribosomal S6 kinase and 4E-BP1. mTORC1 activity is inhibited by the macrolide rapamycin or its analogs called rapalogs. mTORC2, which is primarily activated by growth factors, is involved in cell survival and cytoskeletal organization by phosphorylating members of the AGC kinases family including AKT. mTORC2 is classically resistant to the inhibitory effects of rapamycin. However, in certain cell types prolonged exposure to rapamycin might inhibit mTORC2, presumably by blocking the de novo assembly of the complex [10]. Several studies have demonstrated the importance of mTOR in cancer progression and targeting mTOR has been a successful approach in

Abbreviations: CRC, colorectal cancer; NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; PGE_2, prostaglandin E_2; mTOR, mechanistic target of rapamycin; VEGF, vascular endothelial growth factor; TNFα, tumor necrosis factor α.

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delaying cancer progression in patients [11]. In the context of CRC, clinical studies have shown that mTORC1 is activated in most CRC and the inhibition of mTOR by rapalogs reduces colon cancer cell growth in vitro and the formation of tumor in vivo [12–16].

Although PGE_2 and mTOR are involved in CRC growth, little is known about the effect of PGE_2 on mTOR signaling pathway and its relevance to CRC. In this study, we have found that PGE_2-induced colon cancer growth requires mTORC1 activity suggesting that mTORC1 plays a role in linking inflammation with cancer.

2. Materials and methods

2.1. Antibodies and reagents

The antibodies used included anti-phospho S6 ribosomal protein (M3500) from Spring Biosciences (Pleasanton, CA, USA), as well as anti-S6 ribosomal protein (2217), anti-raptor (2280) and anti-actin (4967) all form Cell Signaling Technology (Danvers, MA, USA). NVP-BEZ235 (CD0195) and PP-242 (CD0258) were purchased from Chemdea (Ridgewood, NJ, Switzerland). Rapamycin (R-5000) was from LC-laboratories (Woburn, MA, USA). Prostaglandin E_2 (14010), sulprostone (14765), butaprost (13741) and prostaglandin E_1 alcohol (13020) were from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Cell culture

The human colon cancer cell line (LS174T) was maintained in DMEM (D5796, Sigma–Aldrich, Buchs, Switzerland) containing 10% FBS (10270) and 1% penicillin G/streptomycin (15140-122) both from Life Technologies (Zug, Switzerland).

2.3. Western blot analysis

Cells were treated as indicated and subsequently harvested in RIPA lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptine, 1 mM sodium orthovanadate. Protein concentrations were measured using BCA Assay (Pierce, Rockford, IL, USA). Equal amounts of protein (20 μg) were separated on 4–12% polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Zug, Switzerland). Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) and immunoblotted with primary antibodies followed by infrared secondary antibodies. Bands from immunoreactive proteins were visualized by an Odyssey infrared imaging system.

2.4. Reverse transcription polymerase chain reaction

Total RNA was prepared from LS174T cells using the RNeasy system (Qiagen, Basel, Switzerland). One micrograms of total RNA was reversed transcribed (Superscript II, Invitrogen), and cDNA was subjected to PCR amplifications as previously described [17].

2.5. Vascular endothelial growth factor (VEGF) ELISA

LS174T cells were cultured in 24-well plates and serum starved for 24 h. Cells were subsequently treated with rapamycin for 1 h before being stimulated with PGE_2 (100 nM) for 24 h. Supernatants were collected and levels of VEGF in the culture media were quantified using an ELISA kit (R&D Systems Europe Ltd., Abingdon, United Kingdom).

2.6. Lentiviruses infection

Lentiviruses were produced by transfecting HEK-293T cells with pLKO1-raptor (plasmid 1857, Addgene, Cambridge MA, USA) or pLKO1-scramble (plasmid 1864, Addgene) as well as pMD2 (plasmid 12259, Addgene) and psPAX2 (plasmid 12260, Addgene) plasmids using FuGENE (Roche Diagnostics, Rotkreuz, Switzerland) and following the manufacturer's instruction.

Virus-containing supernatants were collected 48 and 72 h after transfection. Cells were infected in the presence of protamine sulfate and selected for puromycin resistance.

2.7. Cell count

Cells were plated in six-well plates at a density of 100,000 cells/well and cultured in DMEM 10% FBS. Twelve hours later, cells were treated with rapamycin (10 nM), NVP-BEZ235 (100 nM), or PP242 (100 nM) for four hours before being stimulated with PGE_2 (100 nM). Adherent cells were collected after 48 h and trypan-blue negative cells were counted using a Neubauer hemocytometer.

2.8. Cell growth in matrigel

Ten thousand LS174T cells were suspended in 0.5 ml of 1:2 diluted Matrigel®(Collaborative Biomedical Products, Bedford, MA, USA), and plated into 24-well plates. PGE_2 (100 nM) was added to the cell culture every other day and rapamycin (10 nM), PP242 (100 nM) or NVP-BEZ235 (100 nM) were added once a week. After 14 days of culture, colonies were photographed and the relative colony size was determined by measuring 10 random colonies in each slide (30 measurements/well). The mean for each treatment set was calculated.

3. Results

3.1. Activation of mTORC1 by PGE_2 in LS174T colon cancer cells

We tested the effect of PGE_2 on mTORC1 activity by analyzing the phosphorylation of S6 ribosomal protein, a downstream target of mTORC1 signaling pathway. We found by Western blot that PGE_2 increased the phosphorylation of S6 ribosomal protein (Fig. 1A). This effect was apparent after 10 min of stimulation and peaked after 1 h. A concentration of PGE_2 of 0.1 μM was sufficient to induce mTORC1 activity. Four different subtypes of membrane receptors (EP_1, EP_2, EP_3, and EP_4) mediate the physiological effects of PGE_2 [18]. We therefore next determined which receptor was responsible for the activation of mTORC1 by PGE_2 in LS174T cells. By PCR, we detected EP_2 and EP_4 mRNA in LS174T cells but not mRNA of EP_1 and EP_3 (Fig. 1B). To further demonstrate the ability of these receptors to activate mTORC1, we used selective chemical agonists. We found that PGE_1 alcohol, a selective EP_4 receptor agonist, but not butaprost, a selective EP_2 agonist increased S6 ribosomal protein phosphorylation (Fig. 1C). Consistent with the PCR results, sulprostone, an EP_3 agonist, did not influence mTORC1 activity. Taken together, these results show that PGE_2 activates mTORC1 in colon cancer cells mainly through the activation of EP_4.

3.2. Targeting mTORC1 blocks PGE_2 induced LS174T cell proliferation

PGE_2 promotes colon cancer progression in part by inducing colon cancer growth [7]. We therefore tested whether the inhibition of mTORC1 signaling could block the proliferative effect of PGE_2. We used three different mTORC1 inhibitors; rapamycin, an allosteric inhibitor of mTORC1, PP242, an ATP-competitive inhibitor of mTOR that blocks both mTORC1 and mTORC2 and

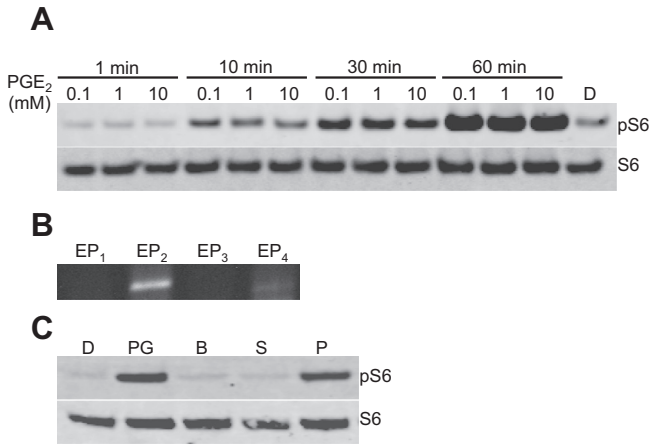

Fig. 1. PGE_2 activates mTORC1 in colon cancer cells through EP_4 receptor. (A) LS174T cells stimulated with PGE_2 for the indicated times and concentrations or treated with DMSO (D) as a control. Lysates were analyzed for pS6 ribosomal protein and S6 ribosomal protein expression by Western blot. The illustrated blots are representative of three independent experiments. (B) Expression of EP_1, EP_2, EP_3 and EP_4 receptors mRNA in LS174T were analyzed by reverse transcription PCR. (C) LS174T cells were exposed during 1 h to DMSO (D), PGE_2 (PG), butaprost (B, EP_2 agonist), sulprostone (S, EP_3 agonist) or PGE_1 OH (P, EP_4 agonist) at a concentration of 0.1 μM. Lysates were analyzed for pS6 ribosomal protein and S6 ribosomal protein expression by Western Blot. The illustrated blots are representative of three independent experiments.

NVP-BEZ235, a dual PI3K/mTOR inhibitor [19]. By Western blot, we observed that all three inhibitors were able to block PGE_2-induced mTORC1 activation (Fig. 2A). In addition, they also inhibited PGE_2-induced LS174T cell proliferation (Fig. 2B). To further demonstrate that blocking mTORC1 prevents the proliferative effects of PGE_2, we inhibited mTORC1 by downregulating raptor using RNA interference. We found that downregulation of raptor efficiently inhibited PGE_2-mediated mTORC1 activation (Fig. 2C). Moreover, PGE_2 did not induce proliferation in LS174T cells that did not express raptor (Fig. 2D). Taken together, these results show that targeting mTORC1 inhibits PGE_2-induced colon cancer cell proliferation.

3.3. Targeting mTORC1 blocks the growth of LS174T colonies induced by PGE_2

PGE_2 also promotes the growth of LS174T colonies when grown in matrigel [20]. We thus investigated whether mTOR inhibitors can prevent this effect and for this purpose cultured LS174T cells in matrigel in the presence or absence of mTOR inhibitors. As expected, PGE_2 had a growth stimulatory effect on LS174T colonies. We found however that mTOR inhibitors reduced the size of LS174T colonies and blocked the growth of LS174T colonies induced by PGE_2 (Fig. 3A and B).

3.4. Targeting mTORC1 blocks PGE_2-induced VEGF expression in LS174T cells

PGE_2 is also known to promote tumor growth by stimulating tumor angiogenesis. In particular, PGE_2 increases the production of VEGF which in turn contributes to tumor growth [21,22]. Since VEGF expression is also regulated by mTORC1 [23], we wanted to assess whether mTORC1 activity was necessary for PGE_2-induced VEGF production. To test this, LS174T cells were stimulated with PGE_2 in the presence or absence of rapamycin and VEGF production was assessed in cell culture supernatants by ELISA. As expected, we

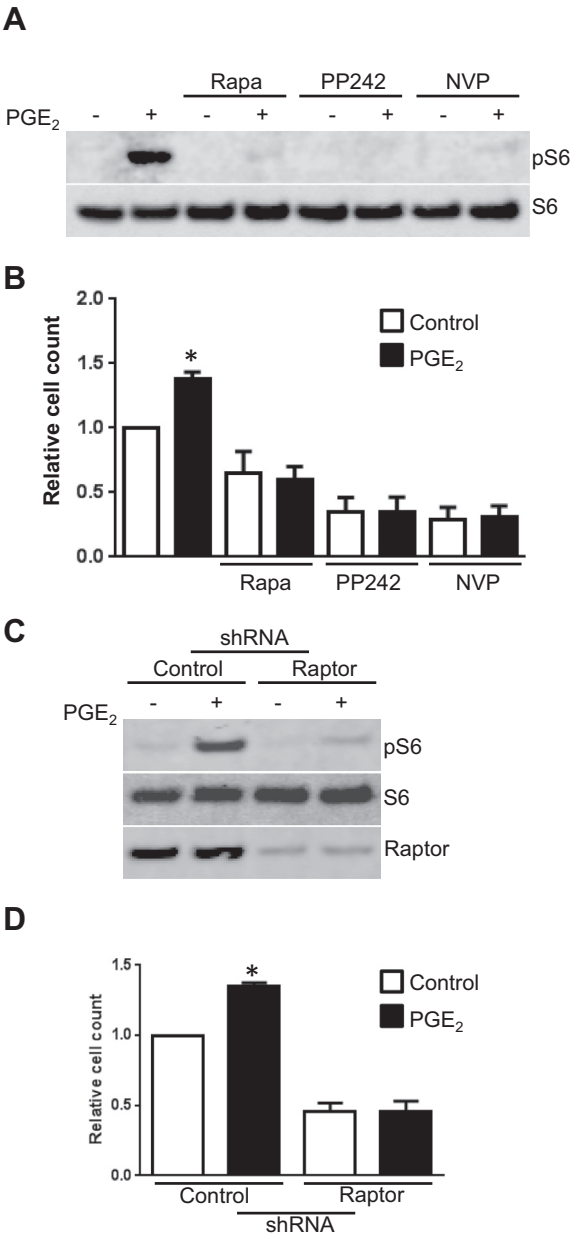

Fig. 2. Targeting mTORC1 blocks PGE_2-mediated cell proliferation. (A) LS174T cells were treated with rapamycin (R, 10 nM), PP242 (PP, 100 nM) or NVP-BEZ235 (NVP, 100 nM) for 4 h. Cells were subsequently stimulated with PGE_2 (100 nM, 1 h). Lysates were analyzed for pS6 ribosomal protein and S6 ribosomal protein expression by Western blot. The illustrated blots are representative of three independent experiments. (B) LS174T cells were treated with rapamycin (R, 10 nM), PP242 (PP, 100 nM) or NVP-BEZ235 (NVP, 100 nM) for 4 h. Cells were subsequently stimulated with PGE_2 (100 nM) for 48 h. Cells were then counted by light microscopy using a Neubauer hemocytometer. Columns, mean cell count of three independent experiments expressed as relative values; bars, SD; $^*p$-value = 0.0057 compared to untreated cells (Student's *t*-test). (C) LS174T cells were infected with lentiviruses expressing a raptor targeting shRNA or a control shRNA. Following selection with puromycin cells were stimulated with PGE_2 (100 nM) for 1 h. Lysates were analyzed for pS6 ribosomal protein and S6 ribosomal protein expression by Western blot. The illustrated blots are representative of three independent experiments. (D) LS174T cells were infected with lentiviruses expressing a raptor targeting shRNA or a control shRNA. Twenty-four hours later, cells were subsequently stimulated with PGE_2 (100 nM) for 72 h. Cells were collected and counted by light microscopy using a Neubauer hemocytometer. Columns, mean cell count of three independent experiments expressed as relative values; bars, SD; $^*p$-value = 0.0017 (Student's *t*-test).

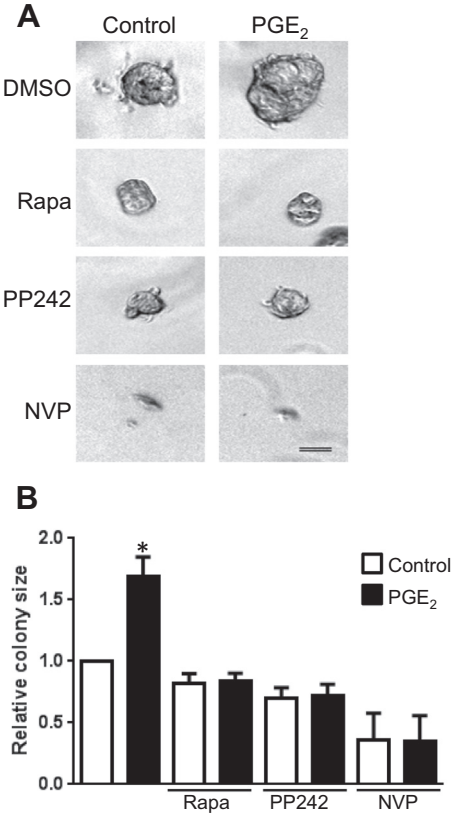

Fig. 3. mTOR inhibitors block the growth of colon cancer cell colonies induced by PGE_2. (A) LS174T cells were grown in Matrigel coated plates and treated with rapamycin (Rapa, 10 nM), PP242 (PP, 100 nM) or NVP-BEZ235 (NVP, 100 nM) once a week. In addition, cells were also stimulated with PGE_2 (100 nM) every other day for 2 weeks. Representative photographs of colonies after 2 weeks of culture (magnification, 40×; scale bar, 50 μm). (B) The size of the colonies was measured. Columns, mean colony size of three independent experiments expressed as relative values; bars, SD; *p-value = 0.0029 (Student's t-test).

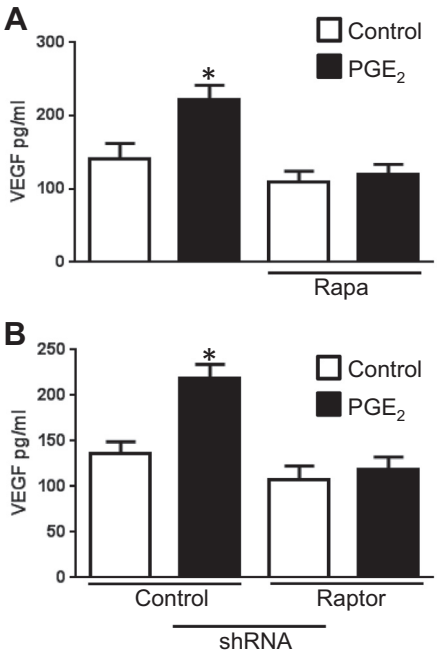

Fig. 4. Blocking mTORC1 reduces PGE_2-induced VEGF production. (A) LS174T cells were stimulated with PGE_2 (100 nM) in the presence or absence of rapamycin (Rapa, 10 nM) for 24 h. VEGF levels in the supernatants were quantified by ELISA. Plotted is the mean ± SD of VEGF (pg/ml) performed in duplicate (n = 3). *p-value = 0.0089 (Student's t-test). (B) LS174T cells were infected with a control or a raptor shRNA expressing lentivirus. Following puromycin selection, cells were stimulated with PGE_2 (100 nM) for 24 h. VEGF concentration was evaluated in the supernatants by ELISA. Plotted is the mean ± SD of VEGF (pg/ml) performed in duplicate (n = 3). *p-value = 0.0017 (Student's t-test).

found that PGE_2 increased the production of VEGF. This effect was however markedly decreased by rapamycin (Fig. 4A). Similarly, the downregulation of raptor by RNA interference inhibited PGE_2-mediated VEGF production (Fig. 4B).

4. Discussion

Targeting COX-2 by NSAIDs prevents the development of CRC [7]. It is therefore important to identify the molecular mechanisms involved in this process in order to better understand the pathophysiology of CRC. Over the past years, PGE_2 has emerged as the major cytokine produced by COX-2 and implicated in colon cancer growth. In particular, PGE_2 directly stimulates colon cancer growth and upregulates tumor angiogenesis [3]. Here, we report that mTORC1 plays a role in these processes. We have observed that PGE_2 increased mTORC1 activity in colon cancer cells. Furthermore, blocking mTORC1 prevented PGE_2-induced colon cancer cell growth and VEGF production. Several mechanisms can lead to mTORC1 activation by PGE_2. mTORC1 activity is classically regulated by a complex of two proteins called TSC1 and TSC2 that links extracellular stimuli to mTORC1. Both TSC1 and TSC2 are phosphorylated by multiple kinases including AKT, ERK and GSK3 [8]. Since PGE_2 has been shown to influence the activity of these three kinases, the activation of mTORC1 by PGE_2 might result from the coordination of multiple signals [20,22,24,25]. Using specific PGE_2 receptor agonists, we further showed that the EP_4 receptor was responsible for PGE_2-mediated mTORC1 activation. Consistent with this observation, it was previously reported that PGE_2-mediated colon cancer cell growth is mediated by EP_4-induced activation of the PI3K/AKT signaling pathway [20]. Therefore, these findings suggest that PGE_2 might activate mTORC1 mainly through AKT.

It is well established that chronic inflammation contributes to tumor progression [26]. In fact, inflammatory cells infiltrating tumors release multiple cytokines and chemokines that influence the behavior of cancer cells. For example, one of the most important inflammatory factors, the tumor necrosis factor α (TNFα) activates prosurvival signals and stimulates tumor angiogenesis thereby promoting tumor growth. Interestingly, it was observed that TNFα-induced tumor angiogenesis and tumor progression was dependent on mTORC1 suggesting that mTORC1 is an important link between inflammation and tumor growth [27]. Consistent with a role of mTORC1 in inflammation-driven tumor progression, we also found that mTORC1 is an important signaling intermediary in PGE_2-mediated colon cancer cell growth and VEGF production.

In addition to influencing the behavior of cancer cells, PGE_2 also modulates the cellular responses present in the tumor microenvironment. PGE_2 promotes the angiogenic reaction not only by increasing the production of VEGF but also by stimulating endothelial cell proliferation and migration [17]. Interestingly, rapamycin also inhibited PGE_2-induced endothelial cell responses suggesting that the effect of rapamycin is not restricted to cancer cells but also involves elements of the tumor microenvironment [28]. It is however worth noting that PGE_2 has also been shown to affect the immune response found in the tumor microenvironment by inducing a shift from an anti-tumor to an immunosuppressive tumor microenvironment [3]. Since rapamycin is primarily an immunosuppressive drug, the efficacy of rapamycin to block PGE_2-induced tumor growth might be reduced due to the inhibition of the

immune response [29]. Therefore, further studies are needed to fully characterize the effects of rapamycin on the immune reaction against tumors and in particular in the context of PGE_2-driven tumorigenesis.

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