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RagD regulates amino acid mediated-casein synthesis and cell proliferation *via* mTOR signalling in cow mammary epithelial cells

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This research paper addresses the hypothesis that RagD is a key signalling factor that regulates amino acid (AA) mediated-casein synthesis and cell proliferation in cow mammary epithelial cells (CMECs). The expression of RagD was analysed at different times during pregnancy and lactation in bovine mammary tissue from dairy cows. We showed that expression of RagD at lactation period was higher ($P < 0{\cdot}05$) than that at pregnancy period. When CMECs were treated with methionine (Met) or lysine (Lys), expression of RagD, β-casein (CSN2), mTOR and p-mTOR, and cell proliferation were increased. Further, when CMECs were treated to overexpress RagD, expression of CSN2, mTOR and p-mTOR, and cell proliferation were up-regulated. Furthermore, the increase in expression of CSN2, mTOR and p-mTOR, and cell proliferation in response to Met or Lys supply was inhibited by inhibiting RagD, and those effects were reversed in the overexpression model. When CMECs were treated with RagD overexpression together with mTOR inhibition or conversely with RagD inhibition together with mTOR overexpression, results showed that the increase in expression of CSN2 and cell proliferation in response to RagD overexpression was prevented by inhibiting mTOR, and those effects were reversed by overexpressing mTOR. The interaction of RagD with subunit proteins of mTORC1 was analysed, and the result showed that RagD interacted with Raptor. CMECs were treated with Raptor inhibition, and the result showed that the increase in expression of mTOR and p-mTOR in response to RagD overexpression was inhibited by inhibiting Raptor.

In conclusion, our study showed that RagD is an important activation factor of mTORC1 in CMECs, activating AA-mediated casein synthesis and cell proliferation, potentially acting *via* Raptor.

Keywords: RagD, casein synthesis, cell proliferation, mTOR signalling, cow mammary epithelial cells.

RagD is one of the small G proteins that belong to the Ras small G protein family, and has GTPase activity (Sasaki et al. 2012). In recent years, studies have found that RagD is an important signalling factor that regulates the mammalian target of rapamycin complex 1 (mTORC1) pathway (Kim et al. 2008; Sancak et al. 2008). In mammalian cells, the mTOR pathway is stimulated by hormones, nutrients and environmental stress. Amino acids (AA) are important nutrients that regulate mTOR pathway. After stimulation by AA, mTOR moves to the surface of lysosomes and is phosphorylated, then the mTOR pathway is activated (Sancak et al. 2010; Bar-Peled & Sabatini, 2014). Recent studies found that small G protein Rag family protein is the key activator for targeting mTORC1 to the lysosomal surface, and this process is necessary for the activation of mTOR pathway regulated by AA (Kogan et al. 2010; Bar-Peled et al. 2012).

The main signalling pathways of casein synthesis and cell proliferation regulated by AA in cow mammary epithelial cells (CMECs) are mTOR signalling pathways (Bionaz & Loor, 2011; Appuhamy et al. 2014). In CMECs, mTOR was activated by AA, and then the activated mTOR modulates the activity of two important translational regulators, the ribosomal S6 kinases 1 (S6K1) and the eukaryotic initiation factor 4E (eIF4E). These mTOR-regulated effectors control the casein synthesis and contribute to the cell proliferation of CMECs (Rhoads & Grudzien-Nogalska, 2007; Burgos et al. 2010). However, the mechanism of AA action on mTORC1 is poorly understood. While in some kinds of cells, such as HELA and HEK293 cell, several studies have investigated this mechanism in a variety of formats (Linares et al. 2015; Wolfson et al. 2016), it has not yet been studied in the regulation of dairy cow mammary lactation.

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To test the hypothesis that RagD is a key signalling factor that regulates AA mediated-casein synthesis and cell proliferation in CMECs, this study aimed to investigate the regulatory function of RagD on AA mediated-casein synthesis and cell proliferation and its mechanism in CMECs.

Material and methods

The experimental procedures followed The People's Republic of China Law on Animal Protection and were approved by the Animal Care Committee of the Northeast Agricultural University.

Animals and tissue samples

Holstein cows at lactation period ($n = 3$) and pregnancy period ($n = 3$) were used for the animal (in-vivo) experiment. Cows were slaughtered by exsanguination, and the mammary tissue was aseptically excised and stored at −80 °C for later experiments.

Cell preparation and treatments

The CMECs were cultured in DMEM/F12 medium containing 10% fetal bovine serum (FBS) according to previous report (Luo et al. 2013). CMECs were plated into 6 well plates, when they reached 80% confluence, cells were starved with DMEM/F12 medium devoid of all AA for 6 h, and then treated with DMEM/F12 media devoid of all AA (AA-), added 0·06 mM Met (Met+) or added 0·12 mM Lys (Lys+) for 24 h (Lu et al. 2012 and 2013). Then the cells were collected for later experiments.

Western blotting analysis

Western blotting (WB) analysis was performed according to previous report (Wang et al. 2014). The primary antibodies used in this study were shown as follow: RagD, Raptor, Deptor, PRAS40, GβL and β-actin were purchased from Santa Cruz (USA), mTOR and p-mTOR were purchased from Cell Signaling Technology (USA), Flag and Myc were purchased from Beyotime (China) and β-casein (CSN2) was purchased from Abbiotec (USA). The HRP-conjugated secondary antibodies were purchased from ZSGB-BIO (Beijing, China). Antibodies purchased from Santa Cruz and Abbiotec were used at 1 : 200 dilutions, purchased from Cell Signaling Technology and Beyotime were used at 1 : 1000 dilutions. The secondary antibodies were used at 1 : 2000 dilutions. Further details are given in online Supplementary File.

Cell proliferation assay

Cell proliferation (cell numbers and cell viability) assay was determined using a CASY model TT Analyser System

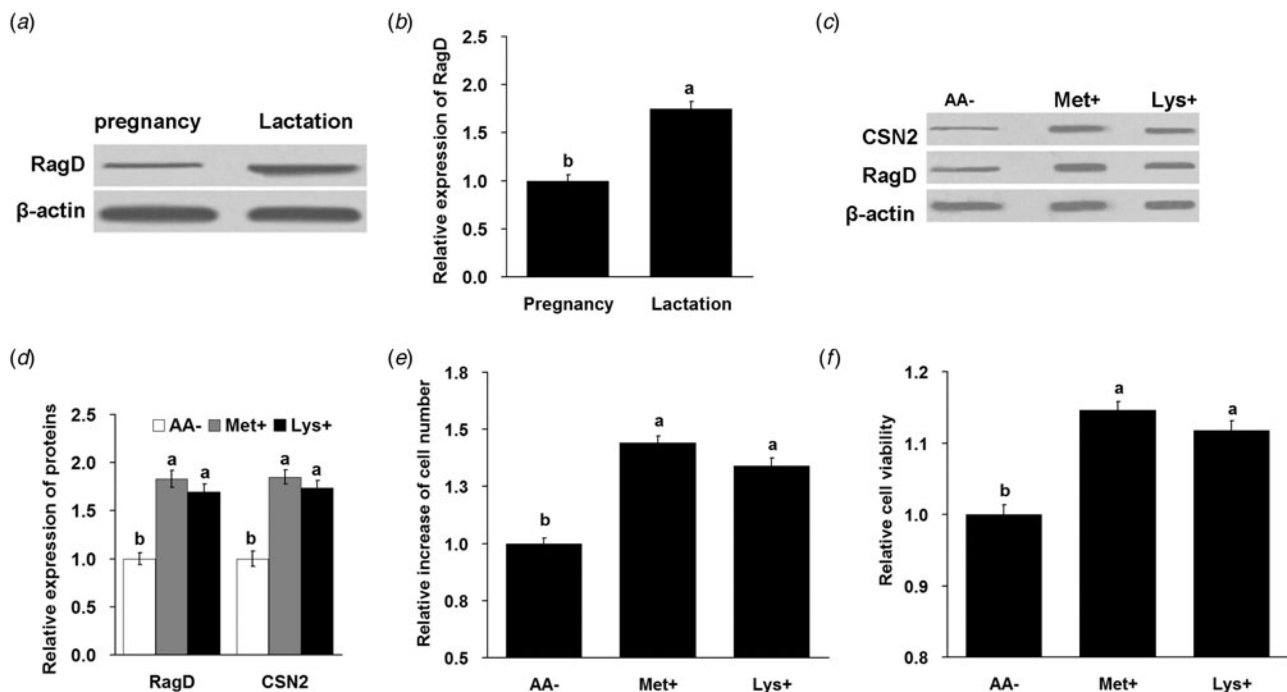

Fig. 1. Expression of RagD in cow mammary tissues and CMECs. Expression of RagD and CSN2 was determined by western blotting. (a, b) comparison of mammary tissue expression from pregnant and lactating cows. (c, d) Expression in CMEs treated with Met or Lys. (1e, f) Changes in cell number (1e) and cell viability in response to treatment with Met and Lys. AA-, Met+ and Lys+: CMECs cultured with DMEM/F12 medium devoid of all AA (AA-), or with additional 0·06 mM Met (Met+) or 0·12 mM Lys (Lys+) for 24 h. Data are presented as means ± SD. In the bar charts, different superscript lowercase letters indicate significant difference ($P < 0·05$).

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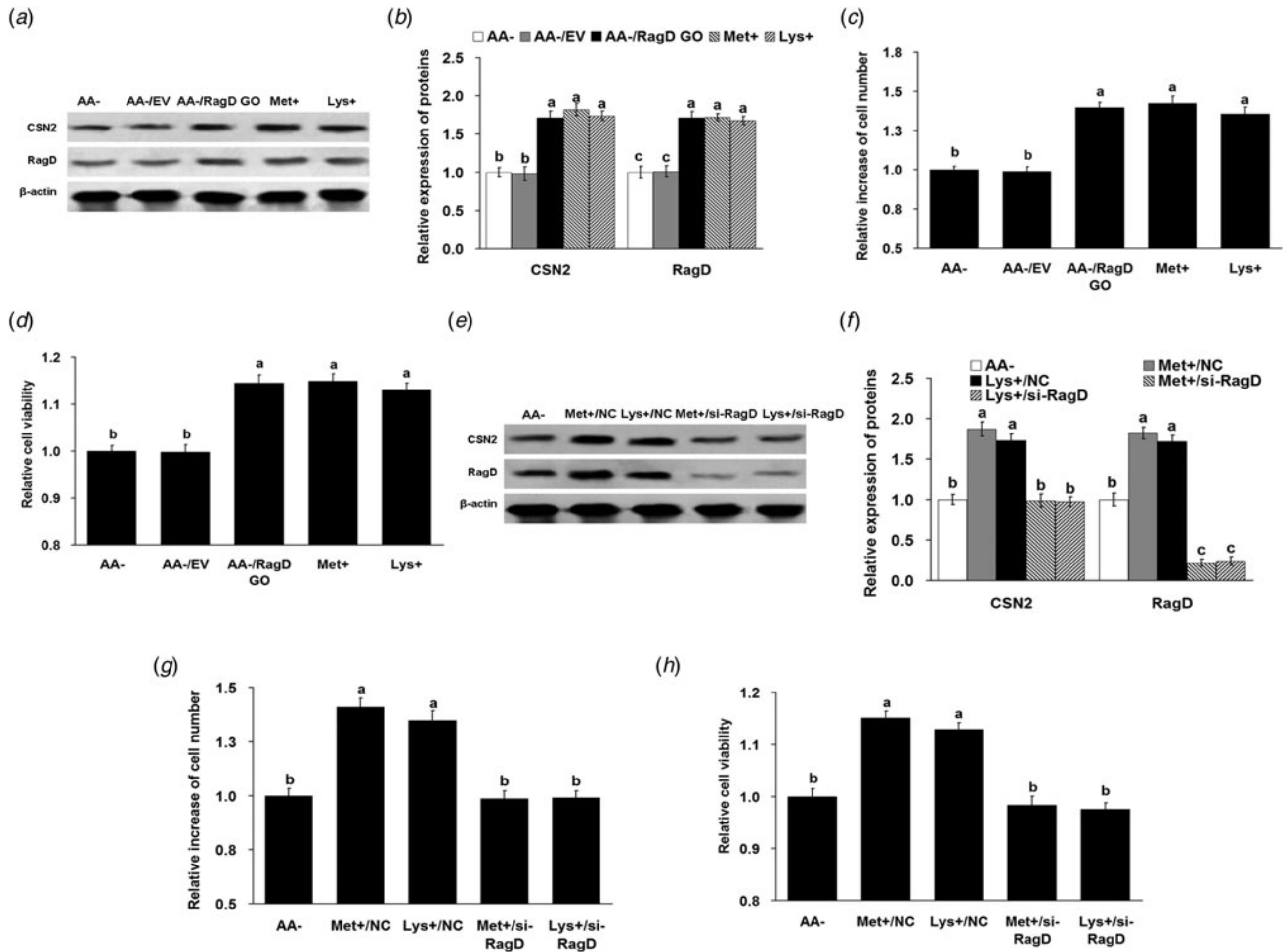

Fig. 2. RagD up-regulated AA-mediated casein synthesis and cell proliferation. (a, b) Stimulation of expression of CSN2 (and RagD) in response to overexpression of RagD in CMECs and its comparison with stimulation by AA addition. (c, d) stimulation of cell number and cell viability by the same treatments. (e, f) inhibition of AA-stimulated expression of CSN2 (and RagD) by RagD silencing. (g, h) inhibition of cell number and cell viability by the same treatments. EV: CMECs were transfected with empty vector; RagD GO: CMECs were transfected with RagD overexpression vector; NC: CMECs were transfected with negative control siRNA; si-RagD: CMECs were transfected with si-RagD. Other treatment codings as for Fig. 1. Data are presented as means ± SD. In the bar charts, different superscript lowercase letters indicate significant difference ($P < 0{\cdot}05$).

(Schärfe System GmbH, Reutlingen, Germany) according to the manufacturer's instructions.

Plasmid construction, small interfering RNA synthesis and transfection

The plasmid construction was performed using standard techniques as reported previously (Li et al. 2014). The primers used for plasmid construction are shown in online Supplementary File Table S1 and S2. The empty vectors used in this research were pCMV-C-Flag (Beyotime) and pCMV-C-Myc (Beyotime). The recombinant plasmids were as follow: Raptor-Flag and RagD-Myc. RagD-specific siRNA, mTOR-specific siRNA, Daptor-specific siRNA and negative control siRNA were synthesised by GenePharma Co., Ltd. (Shanghai, China). CMECs were transfected with plasmids or specific siRNAs using the Lipofectamine TM2000 transfection reagent (Invitrogen,) according to the manufacturer's instructions. The transfected CMECs were incubated at 37 °C in 5% CO2 for 24 h. Then the CMECs were collected for later experiments. Non-transfected CMECs were used as blank control (B), transfected with empty vectors were used as empty vectors control (EV) and transfected with negative siRNA were used as negative control (NC).

Co-immunopricipitation

The Co-immunoprecipitation (Co-IP) experiments were performed according to previous report (Yu et al. 2014) using the Pierce Co-immunoprecipitation Kit (Pierce, USA). About 200 μg of CMECs lysates were used for immunoprecipitation

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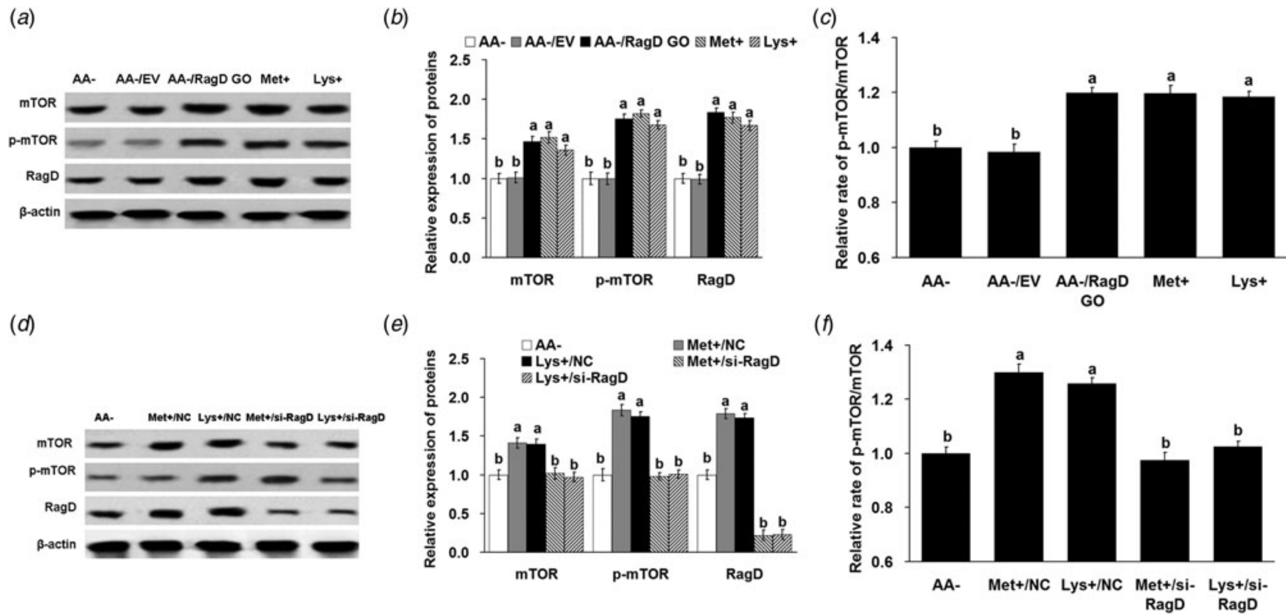

Fig. 3. RagD up-regulated AA-mediated mTORC1 pathway. (a, b) Expression of mTOR and p-mTOR (and RagD) in response to overexpression of RagD in CMECs and its comparison with stimulation by AA addition. (c): ratio of p-mTOR/mTOR in the same treatments. (d, e) inhibition of AA-stimulated expression of mTOR and p-mTOR (and RagD) by RagD silencing. (f) ratio of p-mTOR/mTOR in the same treatments. Treatment codings as for previous figures. Data are presented as means ± SD. In the bar charts, different superscript lowercase letters indicate significant difference ($P < 0{\cdot}05$).

and incubated with anti-RPL35 antibodies. The mouse IgG was used as negative controls. The equal volumes of lysates were incubated with control resin in the same way. The identity of the proteins in the immunoprecipitates was determined by WB with anti-mTOR, anti-p-mTOR, anti-Raptor, anti-Deptor, anti-PRAS40 and anti-PRAS40 GβL.

For the Co-IP used Flag antibody, CMECs were co-transfected with Raptor-Flag/RagD-Myc. Then, 200 μg of cell lysates were used for immunoprecipitation with anti-Flag antibody. The identity of proteins in the immunoprecipitates was determined by WB with anti-Myc antibody.

Statistical analysis

The data were analysed using the t-test by Sigma Plot 9·0 software and the data were presented as mean ± SD. The effects were considered significant at a probability value of $P < 0{\cdot}05$. Grey-scale scanning of WB results was analysed by ImageJ software. All data were obtained from at least three independent experiments.

Results

Expression of RagD in cow mammary gland tissues and CMECs in vitro

The expression of RagD in cow mammary tissues and CMECs cultured in vitro was tested (Fig. 1). In mammary tissue from lactating cows, the expression of RagD was higher ($P < 0{\cdot}05$) than that in mammary tissue from pregnant cows (Fig. 1a, b). In the cells cultured in vitro, the expression of CSN2 and RagD was increased ($P < 0{\cdot}05$) in Met+ or Lys+ group (Fig. 1c, d). In addition, cell number (Fig. 1e) and cell viability (Fig. 1f) were both increased ($P < 0{\cdot}05$) in Met+ or Lys+ group.

RagD up-regulated AA-mediated casein synthesis and cell proliferation

RagD overexpression and silencing were performed (Fig. 2). The expression of RagD, CSN2 (Fig. 2a, b), cell number (Fig. 2c) and cell viability (Fig. 2d) were higher ($P < 0{\cdot}05$) in RagD overexpressing CMECs than in either negative controls (AA-) or empty vector controls (AA-/EV). The stimulation of RadD and CSN2 expression (Fig. 2e, f) as well as cell number (Fig. 2g) and viability (Fig. 2h) by Lys + and Met+ was blocked by RagD silencing.

RagD up-regulated AA-mediated mTORC1 pathway

Effects of RagD overexpression and silencing on the mTORC1 pathway were studied (Fig. 3). Expression of mTOR and p-mTOR as well as the ratio of p-mTOR/mTOR (Fig. 3a–c) were increased ($P < 0{\cdot}05$) by RagD overexpression and also by Met+ and Lys+. The effect of amino acid addition was blocked by RagD silencing (Fig. 3e, f).

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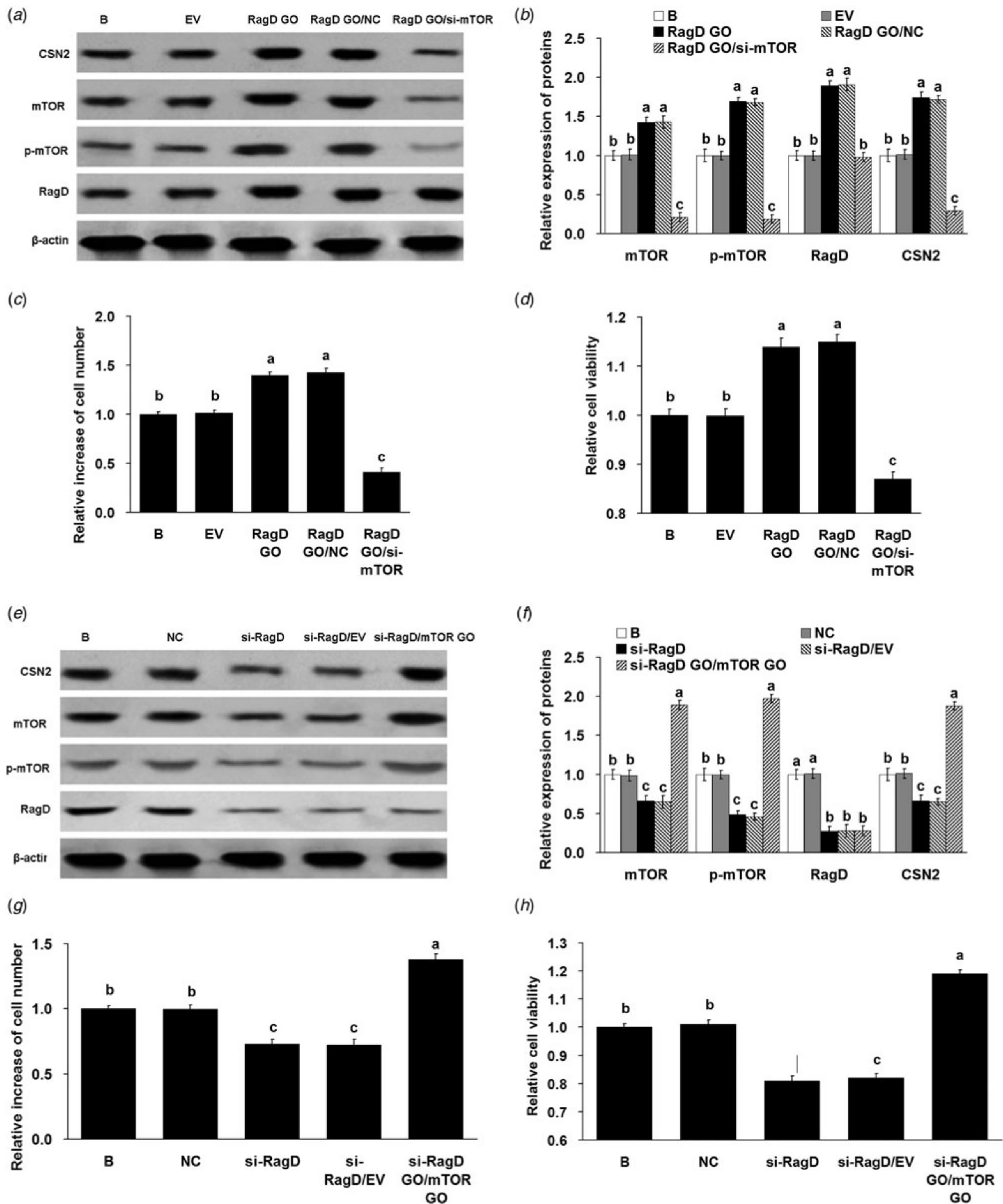

Fig. 4. RagD up-regulated casein synthesis and cell proliferation *via* mTORC1 pathway. (a, b) Stimulation of mTOR, p-mTOR, CSN2 (and RagD) by RagD overexpression and its inhibition by mTOR silencing. (c, d) Stimulation and inhibition of cell number and cell viability by the same treatments. (e, f) Inhibition of mTOR, p-mTOR, CSN2 (and RagD) by RagD silencing and its reversal by overexpression of mTOR. (g, h) Inhibition of cell number and cell viability and its reversal by the same treatments. NC: CMECs were transfected with negative control siRNA. Other treatment codings as for previous figures, or derivatives thereof. Data are presented as means ± SD. In the bar charts, different superscript lowercase letters indicate significant difference ($P < 0{\cdot}05$).

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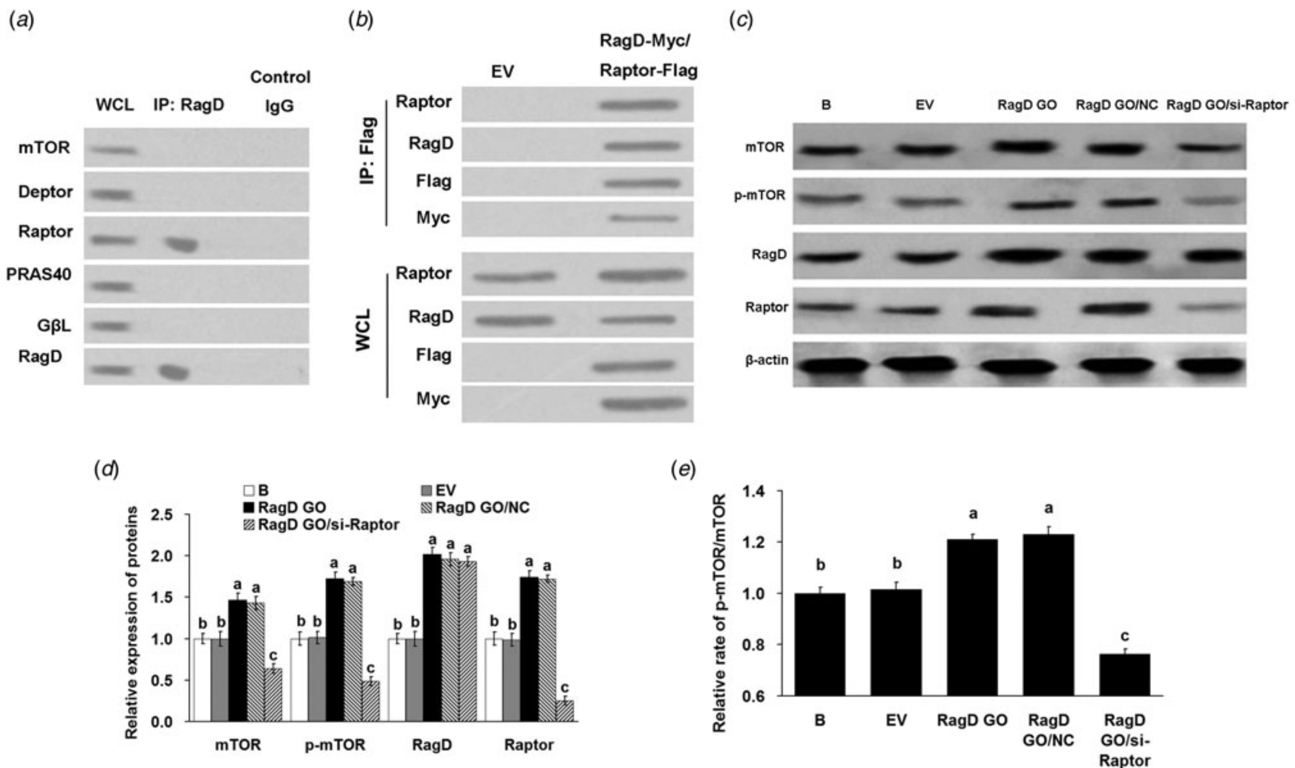

Fig. 5. Rag D interacts with Raptor and activates mTORC1 pathway *via* Raptor. (a) Interaction of RagD with individual subunit proteins showing positive co-immunoprecipitation with Raptor. (b) Co-immunoprecipitation of Raptor-Flag and RagD-Myc from CMECs co-transfected with Raptor-Flag and Rag-D-Myc. (c, d) Stimulation of mTOR, p-mTOR, Raptor (and Rag-D) expression by overexpression of RagD and its inhibition by Raptor silencing. (e) ratio of p-mTOR/mTOR in the same treatments. WCL: whole cell lysates. IP: immunoprecipitation. Control IgG: mouse IgG. Other treatment codings as for previous figures, or derivatives thereof. Data are presented as means ± SD. In the bar charts, different superscript lowercase letters indicate significant difference ($P < 0{\cdot}05$).

RagD up-regulated casein synthesis and cell proliferation via mTORC1 pathway

To investigate whether RagD up-regulated casein synthesis and cell proliferation *via* mTORC1 pathway, mTOR overexpression and silencing was performed (Fig. 4). The expression of CSN2 (Fig. 4a, b) and cell proliferation (Fig. 4c, d) were increased ($P < 0{\cdot}05$) in RagD overexpression group, but these increases were blocked by mTOR silencing. In addition, expression of CSN2 (Fig. 4e, f) as well as cell proliferation and viability (Fig. 4g, h) were decreased ($P < 0{\cdot}05$) in RagD silencing group, but these decreases were restored by mTOR overexpression.

Rag D interacts with Raptor and activates mTORC1 pathway via Raptor

To investigate how mTORC1 pathway was regulated by RagD, the interacting proteins of RagD were tested by co-immunoprecipitation, Co-IP (Fig. 5). Of five protein subunits (mTOR, Raptor, GβL, PRAS40 and Deptor) of mTORC1, only Raptor interacted with RagD (Fig. 5a). To examine the specific nature of this interaction, the CMECs were co-transfected with Raptor-Flag and RagD-Myc, and the Co-IP experiment was completed with Flag antibody. After Co-IP with anti-Flag antibody, the RagD-Myc was detected in immunoprecipitates against anti-Myc antibody or anti-RagD antibody (Fig. 5b). These results suggest that in the five proteins of mTORC1 complex, RagD interacts with Raptor but not other proteins. To investigate whether RagD activates mTOR *via* Raptor, the experiment of Raptor silencing was performed. The expression of mTOR and p-mTOR as well as the ratio of p-mTOR/mTOR (Fig. 5c, e) were increased ($P < 0{\cdot}05$) in RagD overexpression group, but these increases were blocked by Raptor silencing.

Discussion

RagD is a small G protein, and recent studies found that it was an activator of the mTOR signalling pathway, and plays an important role in the proliferation of cells (Efeyan et al. 2013). In this study, we found that the mammary expression of RagD was significantly higher during lactation compared to during pregnancy. This result suggests that RagD might have some functions in mammary gland development and lactation. However, since mammary cell proliferation is higher during pregnancy than during lactation, any in vivo role requires further confirmation and clarification.

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AA are important nutrient factors for the lactation and proliferation of CMECs (Arriola Apelo et al. 2014; Paz & Kononoff, 2014). Met and Lys are two essential AA for casein production in lactating dairy cows (Appuhamy et al. 2011; Wang et al. 2012; Awawdeh, 2016). In our study, Met and Lys added to the medium increased casein synthesis (CSN2 expression) and cell proliferation, and also stimulated RagD expression. These results suggest that RagD may be a regulatory factor involved in the AA-mediated regulation of casein synthesis and cell proliferation.

By the experiments of overexpression and inhibition of RagD, we demonstrated that RagD expression of CSN2 and mTOR as well as cell proliferation were promoted by RagD in CMECs. Recent studies have showed that RagD is a necessary regulatory factor for the activation of mTOR in human cells (Petit et al. 2013; Tsun et al. 2013). In our study, expression of CSN2 and cell proliferation in CMECs were increased in response to RagD overexpression, but when mTOR was inhibited, these increases were blocked, and vice versa. These results suggest that RagD is a positive regulatory factor for the activation of mTOR, and may have up-regulated the expression of CSN2 and cell proliferation *via* activation of mTOR in CMECs.

mTOR complex 1 (mTORC1) is composed of mTOR itself complexed with regulatory associated protein of mammalian target of rapamycin (Raptor), mammalian lethal with SEC13 protein 8 (mLST8; also known as GβL), proline-rich Akt substrate of 40 kDa (PRAS40, also known as AKT1S1) and DEP domain-containing mTORinteracting protein (Deptor). The mTORC1 complex is a master regulator that couples AA availability to cell proliferation and protein synthesis (Foster & Fingar, 2010; Jewell et al. 2013). Recently, Rag GTPases were shown to be AA-specific regulators of the mTORC1 pathway. Activation of the mTORC1 pathway by AA correlates with the movement of mTORC1 from an undefined location to a compartment containing Rab7, a marker of late endosomes and lysosomes. Between the processes of AA inducing the movement of mTORC1 to the lysosome, the Rag GTPases located in lysosome are essential (Sancak et al. 2010; Zoncu et al. 2011). Recently, studies in human cells showed that the Rag GTPase has four subunit proteins, RagA, RagB, RagC and RagD. The RagA/B and RagC/D form Rag heterodimers. AA induce the interaction between Rag heterodimers and mTORC1 by promoting the loading of RagB with GTP, after which mTORC1 is activated by Rag heterodimers (Sancak et al. 2008).

In our study, Raptor was identified by Co-IP as the interaction protein of RagD in mTORC1 In addition, the experiments of overexpression and inhibition of Raptor suggested that the stimulation of mTOR, p-mTOR, and cell proliferation by RagD overexpression may involve activation *via* Raptor, since it was blocked by Raptor silencing.

Conclusion

Our findings suggest that RagD is a key regulator in the promotion of AA-mediated casein synthesis and mammary cell proliferation, activating mTORC1 signalling *via* an interaction with Raptor. The physiological significance of these in vitro findings remains to be elucidated.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S0022029918000146.

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