Introduction

Mammalian target of rapamycin, mTOR, is a highly conserved serine/threonine kinase, which is ubiquitously expressed in cells to control growth and metabolism [1–3]. This protein is essential for normal development and viability [4] as knockout of mTOR results in embryonic lethality [5], and its ablation in some somatic cells leads to increased apoptosis [6]. As a key intermediate in the transmembrane signaling pathway, mTOR integrates various intracellular and extracellular stimuli to regulate many vital cellular processes. Thus, the dysregulation of mTOR pathway is implicated in an increasing number of diseases, including cancer, type 2 diabetes, and neurodegeneration (reviewed in [7]). Hyperactivation of mTOR signaling has been associated with aggressive tumor growth in many cancers [8], including breast cancer [9]. The mTOR pathway is implicated not only in tumorigenesis of breast cancer but also in tumor sensitivity to chemotherapy and hormonal treatment [10]. Activated mTOR pathway is known to promote numerous cellular functions consistent with tumor invasiveness such as proliferation, migration, and survival [11].

mTOR is activated in response to nutrients, growth factors, and cellular energy (reviewed in [2, 12]). Active mTOR exists in two complexes, mTORC1 and mTORC2, which consist of distinct sets of binding proteins [13]. Active mTOR phosphorylates different substrates to regulate distinct cellular functions [14] including protein synthesis, organization of the actin cytoskeleton, membrane traffic, and protein degradation (reviewed in [15]). Protein synthesis is a key feature of cancer cells [16] and mTOR regulates protein synthesis through its downstream targets.
p70 S6 kinase and eIF4E-BP [17, 18]. Another essential cellular function regulated by mTOR is autophagy [19], which is an intracellular degradation system that delivers cytoplasmic proteins to lysosomes [20].

Involvement of active mTOR pathway in the progression of breast cancer is well established [21, 22]. Its inhibition has been shown to sensitize breast cancer cells to the cytotoxic effects of chemotherapy in vitro [23]. Rapamycin and its analogs (rapalogs) are highly specific inhibitors of mTOR, and currently, they are being evaluated as anticancer agents in clinical trials. However, toxicity is a limiting factor that precludes the use of high doses of mTOR inhibitors, particularly rapalogs, in combinatorial treatment for breast cancer [24]. Moreover, evidence indicates that many human cancers have intrinsic resistance to treatment and the tumors initially sensitive to rapamycin demonstrate acquired resistance and become refractory to the treatment [25]. One of the potential mechanisms of the ensuing drug resistance in breast cancer is the upregulation of mTOR pathway, which may involve increased activity or increased levels of total proteins in the mTOR pathway. An earlier report showed that metformin, an antidiabetic agent, exerts antitumor effects via inhibition of mTOR activity [26]. Nonetheless, the molecular basis of the beneficial effects of metformin in breast cancer is far from being fully unraveled. Although metformin action on peripheral tissues requires high concentrations, its use is generally tolerable if avoided in patients with contraindications [27]. The relatively safe profile of metformin makes it a promising agent for mTOR inhibition in breast cancer, particularly that mTOR inhibitors are usually required in high doses to achieve better antitumor effects.

Total mTOR protein level is high in some cancers, such as colorectal cancer, and it correlates positively with the tumor stage [28], but the status of total mTOR protein and its impact in breast cancer cells are not well delineated. Although several mTOR inhibitors have shown promising antitumor effects [21], there is risk of emergence of drug resistance [29]. Notably, feedback upregulation of the mTOR pathway is one of the potential mechanisms of drug resistance in breast cancer. One of the possible mechanisms underlying the upregulation of mTOR pathway is the increased level of total mTOR protein itself. The mechanisms controlling mTOR protein expression and degradation in breast cancer cells are still poorly understood. Autophagy and the ubiquitin-proteasome system (UPS) are the main intracellular protein degradation pathways in eukaryotes [30]. In the UPS, proteins are degraded by the 26S proteasome complex [31]. In autophagy, protein degradation is induced by a specific autophagy inducer [32] such as starvation, oxidative stress [33], or proteasome inhibition. In normal cells, constitutive autophagy and the UPS pathways act in parallel to prevent the accumulation of proteins to prevent cells damage, however, the effect of these events in cancer cells are less understood [34]. In rapidly proliferating tumor cells, the endoplasmic reticulum sustains stress exceeding the degradative capacity of the proteasome and autophagy systems. As a result, misfolded proteins accumulate in perinuclear aggresomes, which are associated with induction of nonapoptotic cell death [35].

To understand possible mechanisms underlying elevated level of mTOR protein accumulation in breast cancer, we have undertaken this work. Our finding of defective proteolysis of mTOR protein could be potentially exploited for improving the efficacy of breast cancer treatment regimens and mitigating drug resistance as well as providing a basis for potential novel therapeutic modalities for breast cancer.

Materials and Methods

Cell lines and reagents

MCF-10A, MCF-7, and MDA-MB-231 breast cell lines obtained from the ATCC cultured and stored following ATCC protocol of authentication by short terminal repeat analysis. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing high glucose (4.5 g/L) (Gibco/Life Technologies, Carlsbad, CA, USA) supplemented with 7% fetal bovine serum (Harlan Bioproducts for Science, Inc., Indianapolis, IN, USA). Insulin, verapamil, MG132, chymostatin, leupeptin, pepstatin A, metformin, rapamycin, and PP242 purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Western blot analysis

Cell lysates from both control and treated breast cells were prepared by three rounds of freeze-thawing and vortexing of cell suspensions in a lysis buffer containing 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 1.5 mmol/L MgCl2, 10 mmol/L KC1, 0.5 mmol/L dithiothreitol (DTT), 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.5 mg/mL each of leupeptin, antipain, and pepstatin, 0.1 μg/mL chymostatin, 0.3 TU/mL aprotinin, and 0.5 mg/mL benzamidine. Equal protein amount was fractionated by electrophoresis in sodiumdodecyl sulfate-polyacrylamide gel (SDS-PAGE), transferred to PVDF transfer membrane (PerkinElmer, Waltham, MA, USA), stained with Ponceau S solution (Sigma Chemical Co., St. Louis, MO, USA), destained, and immunoblotted with the designated antibodies including β-actin to ensure equal loading. Anti-mTOR, anti-pmTOR (Ser-2448), anti-pP70S6K (T-389), anti-LC3B I, anti-LC3B II, anti-β-Actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-P70S6K antibody was purchased from EMD Millipore Corporation (Billerica, MA, USA). Blots were developed with enhanced chemiluminescece reagent (Pierce ECL, Thermo
Protein stability assays

Cycloheximide (CHX) assay was performed by treating the cells with CHX (Sigma Chemical Co.) at 200 μg/mL concentration for various time points, as indicated in Figure legend and the stability of mTOR protein was assessed by western blot (WB).

Immunocytochemistry

MCF-7 cells were plated on tissue culture chambers (Lab-Tek Chamber Slide; Nunc, Inc., St. Louis, MO, USA). Cells were stained with 1 μg/mL Acidine Orange (AO, Hartman-Leddon Co., Philadelphia, PA, USA), followed by formalin fixation, methanol antigen retrieval, 2% (W/V) fetal bovine serum (FBS) blocking, and anti-mTOR immunostaining.

MTT cell proliferation assay

Cells were grown in 24-well plates to 50–70% confluence and proliferation rate of the cells were determined using a live cell assay kit (CellTiter 96 Non-Radioactive Cell Proliferation Assay) and following the manufacturer’s protocol (Promega Corp., Madison, WI, USA). The cells were stained with 3-(4,5-dimethylthiazol-2-1)-2,5-diphenyltetrazolium bromide (MTT, 0.05 mg/mL). Absorbance was recorded at 562 nm using a NanoDrop spectrophotometer (Wilmington, DE, USA).

Wound healing migration assay

Breast cells were seeded on a flat bottom 24-well plate, incubated overnight to allow the cells to resume growth. The medium was changed with fresh growth media in 70–80% confluent monolayers and supplemented with insulin and mTOR inhibitors, as described in the Figure legend. Wound was initiated by scratching with a sterile 20-μL plastic pipette tip. Cell migration, indicated by wound closure, was evaluated by comparing the width of the clear line of cell-free zone with that of the initial wound using a bright field microscopy. The size of wound was measured at various time points 0, 6, 12, 24, and 48 h.

Autophagy assay

Cell lysates were fractionated in 4%/8% SDS-PAGE and immunoblotted for microtubule associated protein 1 light chain 3 isoforms LC3B I and II using antibodies obtained from Cell Signaling Technology. Autophagy was assessed by the relative ratio of LC3BII to LC3BI proteins.

Statistical analysis

Differences between study groups were analyzed by an one-way analysis of variance (ANOVA) with a post-hoc Holm–Sidak method. Results represent the average of three independent experiments (n = 3; mean ± SD, and *P < 0.05 was considered statistically significant), analyzed by Sigmplot software program 12.3 (Systat Software, Inc., San Jose, CA, www.sigmaplot.com).

Results

Level of total mTOR protein is higher in breast cancer cells compared to the noncancerous cells

To assess the status of total mTOR protein in breast cells, the WB analysis was performed on cancerous and noncancerous breast cell lines. As seen in Figure 1A, total mTOR protein is significantly higher in MCF-7 and MDA-MB-231 breast cancer cells compared to the noncancerous MCF-10A breast cells. The WB analysis also revealed high levels of phosphorylated mTOR (pmTOR) as well as phosphorylated P70-S6K (pP70-S6K), a downstream target of mTOR, in MCF-7 cells (Fig. 1B). Immunoblotting for pP70-S6K in the MCF-7 cells revealed an increase in another band consistent in molecular weight with the total nonphosphorylated form of P70-S6K protein as shown by the large dark arrow (Fig. 1B). Treatment of MCF-7 cells with mTOR inhibitor PP242 (Fig. 1C) resulted in an inhibition of mTOR phosphorylation activity in a dose-dependent manner as evident by the presence of lower levels of pmTOR and pP70-S6K. However, it also resulted in a concomitant increase in the levels of both mTOR and P70-S6K, as indicated by the small and large dark arrows, respectively (Fig. 1C). The dose-dependent effect of PP242 is also represented as line graph (bottom panel, Fig. 1C), which further elucidates above findings. Together, our data suggest that total mTOR protein level is high in breast cancer cells, particularly in the MCF-7 cells, which correlates with mTOR activity in these cells.

mTOR protein is more stable in breast cancer cells compared to noncancerous breast cells

The high level of total mTOR protein in the breast cancer cells could be attributed to increased expression and/or reduced degradation of mTOR protein. To investigate the possibility of reduced degradation of mTOR protein in the breast cancer cells, we compared the stability of mTOR


protein using CHX) treatment and immunoblotted for mTOR protein (Fig. 2). Our data show that mTOR protein is more stable in MCF-7 and MD-MB-231 breast cancer cells compared to the noncancerous MCF-10A cells. In MCF-10A cells, total mTOR protein level declined progressively following CHX treatment (Fig. 2A lanes 2 through 6). However, the level of this protein in both MCF-7 and MDA-MB-231 cells remained relatively unchanged (Fig. 2B and C). These findings (Fig. 2D) suggest that proteolysis of mTOR protein most likely contributes to the lowering the level of this protein in the noncancerous breast cells, but this degradation process is, most likely, less effective in the breast cancer cells.

To assess the nature of proteolysis of mTOR in these cells, we treated the cells with proteasome inhibitor MG132. In MCF-10A cells, proteasome inhibition resulted in a decrease in the total mTOR protein in a time-dependent manner (Fig. 2E, lanes 1–3). In contrast, proteasome inhibition caused no significant change in mTOR protein level in both MCF-7 and MDA-MB-231 cells (Fig. 2F and G, respectively). This finding suggested a possibility of proteasome-dependent mTOR degradation in normal breast epithelial cell, MCF-10A, but not in the breast carcinoma cells, MCF-7 and MDA-MB-231. Since ubiquitin–proteasome system (UPS) and autophagy are two main proteolytic pathways in eukaryotic cells and these two pathways work in a coordinated and complementary manner so that inhibition of proteasome induces autophagy [36], we examined this possibility. To test, we analyzed the cellular level of LC3B proteins, a family of well-known autophagy markers [36]. As shown in Figure 2H, proteasome inhibition in MCF-10A cells was associated with increased LC3B II (Fig. 2H, lane 3), which is consistent with activation of autophagy [37]. However, in MCF-7 and MDA-MB-231 cells, proteasome inhibition increased both LC3B isoforms with more increase in LC3B I than LC3B II isoform in a time-dependent manner (Fig. 2I and J, lanes 2 and 3). The accumulation of early intermediates of autophagy, such as LC3B I, likely represents a block in the later stages of autophagy [38]. Induction of autophagy marker LC 3BII [39] in MCF-10A cells following MG132 treatment suggests that proteasome inhibition may have caused induction of autophagy in MCF-10A cells (Fig. 2H). This event most likely leads to the degradation of mTOR protein in these cells. However, proteasome inhibition in the breast cancer cells did not induce autophagy pathway in breast cancer cells, which had resulted in an increased level of mTOR protein in the cancer cells.

**Metformin treatment of MCF-7 breast cancer cells decreases the level of total mTOR protein**

We next assessed whether inhibition of mTOR activity in breast cancer cells impacts mTOR degradation. Treatment of breast cancer cells with metformin and rapamycin, two known mTOR inhibitors, resulted in a significant decrease in the total level of mTOR protein in MCF-7 cells (Fig. 3A).
To assess whether the reduction in mTOR protein after metformin treatment could be due to protein degradation, we measured the mTOR half-life by CHX experiments in metformin-treated cells. Our data show higher rate of reduction in mTOR protein in the metformin-treated cells (Fig. 3B). To test if proteasomal activity is involved in the metformin-induced mTOR degradation, we treated the MCF-7 cells with proteasome inhibitor, MG132, with or without metformin treatment. Results, shown in Figure 3C, indicate that MG132 treatment is unable to rescue metformin-induced mTOR degradation. This finding suggests that mTOR reduction in MCF-7 is not proteasome-dependent. Assessment of the effect of metformin and rapamycin treatment on the status of mTOR downstream targets revealed a notable decrease in phospho P70-S6K (pP70-S6K). The level of P70-S6K protein, however, remains mostly unchanged (Fig. 3C).

**Metformin induces a perinuclear sequestration of mTOR protein in breast cancer cells**

To further verify the involvement of protein degradation in the metformin-mediated mTOR reduction in MCF-7...
cells, immunocytochemistry for the subcellular localization of mTOR protein was performed. MCF-7 cells were treated with metformin or verapamil, an autophagy inducer that is known to induce autophagy in vascular smooth muscle cells as well as adenocarcinoma cells [40, 41]. The cells were stained with AO for localization of acidic vacuoles in the cytoplasm. Verapamil treatment induced extensive vacuole formation in the cytoplasm of MCF-7 cells as shown by the arrow heads in Figure 4B, bottom panel, with no apparent effect on mTOR staining in the rim of condensed cytoplasm surrounding the vacuoles. In contrast to verapamil treatment, metformin treatment of
MCF-7 cells did not induce a noticeable vacuolization of the cytoplasm, but it induced accumulation of mTOR protein in the vicinity of the nucleus as shown by the arrows in Figure 4C, bottom two panels, with no focal increase in autophagic activity of AO staining. These findings indicate that metformin treatment induced aggregation of mTOR protein in a perinuclear region consistent with aggresome formation which is known to allow sequestration of misfolded abundant protein molecules and facilitates their clearance by degradation [42].

**The metformin-induced decrease in mTOR protein level correlates positively with a decrease in the proliferation and migration potentials of MCF-7 breast cancer cells**

To examine the impact of metformin-induced mTOR degradation on the phenotype of breast cancer cells, we compared the effect of various mTOR inhibitors on the proliferation and migration potentials of different breast cells. Metformin treatment of MCF-7 cells was associated with a marked decrease in the cells proliferation compared to the other mTOR inhibitors (Fig. 5A). These findings were further corroborated by the effect of mTOR inhibitors on the migration of breast cell lines. As shown in the wound healing assay (Fig. 5B), MCF-7 cells migration decreased profoundly with mTOR inhibition. The migration of MCF-7 cells was assessed by changes in the wound size in the treatment groups at the designated time points compared to the control groups. The wound size is indicated by the length of thick white line across the wound region (Fig. 5C), which is inversely proportional to the migration potential of the cells. The results show that metformin treatment dramatically inhibited MCF-7 cells migration (Fig. 5C, track iv compared to the other mTOR inhibitors PP242 and rapamycin (Fig. 5C, tracks iii and v, respectively). The line graph shows that metformin treatment in particular dramatically inhibited MCF-7 cells migration.
Discussion

The findings of this work emphasized results of previous research about beneficial role of mTOR inhibition in breast cancer. This study showed an evidence that metformin and rapamycin resulted in a decrease in the overall level of mTOR protein in MCF-7 breast cancer cells in addition to the inhibition of mTOR activation. Compared to other mTOR inhibitors, such as rapamycin and PP242, metformin treatment exerted more inhibitory effect on proliferation and migration of breast cancer cells.
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Furthermore, metformin elicited less rebound upregulation of total proteins in the mTOR pathway (namely, P70 S6K, as seen in Fig. 3C) in breast cancer cells, which potentially imposes a lesser risk of emergence of drug resistance to mTOR inhibition in breast cancer treatment regimens.

This study revealed that total mTOR protein is higher in the breast cancer cells compared to the noncancerous cells, which correlated positively with the level of mTOR activity (Fig. 1). Therefore, high mTOR protein could be potentially involved in promoting the cancerous phenotype of breast cancer cells. This hypothesis is substantiated by the relatively strong correlation between the decreased total mTOR protein induced by metformin (Fig. 3) and the resultant inhibition of proliferation and migration of breast cancer cells (Fig. 5).

The decreased mTOR protein degradation is one of the potential causes underlying the high mTOR protein level in breast cancer cells. Our findings revealed that mTOR protein is degraded more rapidly in the noncancerous breast cells compared to the breast cancer cells (Fig. 2). These findings suggest that the rate of mTOR degradation in breast cancer cells is, most likely, lower compared to that in the noncancerous cells. Such a difference between normal and cancer breast cells could be exploited to open a new avenue for novel antitumor agents by targeting these mechanisms preferentially in the breast cancer cells. Our data in Figures 3 and 4 provided evidence that metformin may be able to induce mTOR degradation in breast cancer cells by triggering aggresome formation.

This study revealed an increase in the LC3B I more than LC3B II isoform in breast cancer cells upon proteasome inhibition (Fig. 2), which suggests that these cells are likely to initiate autophagy, yet unable to finish the conversion process. These findings together could, at least in part, explain the high mTOR level in the breast cancer cells and low mTOR level in the noncancerous cells.

Treatment of MCF-7 cells with a known autophagy inducer, verapamil [40, 41], induced vacuolization of the cytoplasm consistent with autophagosome formation, but metformin treatment; however, did not induce such vacuolization. Instead, metformin treatment induced accumulation of mTOR protein in a perinuclear aggresome. Accumulating proteins in cells are generally transported toward the microtubule organizing center, where they are sequestered into a single large perinuclear aggresome [43]. Aggresome formation allows accumulated proteins to be sequestered in aggresome and facilitates their clearance by autophagy [42] Our results show that metformin induced sequestration of mTOR in perinuclear aggregation (Fig. 4). Metformin treatment also resulted in increased degradation of cytoskeletal proteins, which could explain decreased viability and proliferation of MCF-7 cells after metformin treatment. Our finding of growth regulation of metformin-treated breast cancer cells (Fig. 5) is consistent with a previous finding which showed metformin-induced inhibition of MCF-7 cell proliferation in an AMPK-dependent manner [44]. Since activation of AMPK causes inhibition of mTOR [45–47], our finding raises the possibility that AMPK–mTOR signaling event might also be involved in breast cancer cell growth inhibition. Furthermore, metformin induced degradation of mTOR (Fig. 3) plays an important role in triggering cell growth inhibition. These findings provide a novel mechanism involving the mode of action of metformin in breast cancer cells, could be utilized in improving the efficacy of breast cancer treatment, and counteracting emergence of resistance in breast cancer cells to the treatment modalities.

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Conflicts of Interest

No potential conflicts of interest were disclosed by the authors.

References

1. Raught, B., A. C. Gingras, and N. Sonenberg. 2001. The target of rapamycin (TOR) proteins. Proc. Natl. Acad. Sci. U. S. A. 98:7037–7044.
2. Dazert, E., and M. N. Hall. 2011. mTOR signaling in disease. Curr. Opin. Cell Biol. 23:744–755.
3. Gomez-Pinillos, A., and A. C. Ferrari. 2012. mTOR Signaling Pathway and mTOR Inhibitors in Cancer Therapy. Hematol. Oncol. Clin. North Am. 26:483–505.
4. Takei, N., and H. Nawa. 2014. mTOR signaling and its roles in normal and abnormal brain development. Front. Mol. Neurosci. 7:1–12.
5. Murakami, M., T. Ichisaka, M. Maeda, N. Oshiro, K. Hara, F. Edenhofer, et al. 2004. mTOR Is Essential for Growth and Proliferation in Early Mouse Embryos and Embryonic Stem Cells. Mol. Cell. Biol. 24:6710–6718.
6. Guo, F., S. Zhang, M. Grogg, J. A. Cancelas, M. E. Varney, D. T. Starczynowski, et al. 2013. Mouse gene targeting reveals an essential role of mTOR in hematopoietic stem cell engraftment and hematopoiesis. Haematologica 98:1353–1358.
7. Laplante, M., and D. M. Sabatini. 2012. mTOR signaling in growth control and disease. Cell 149:274–293.
8. Hansel, D. E., E. Platt, M. Orloff, J. Harwalker, S. Sethu, J. L. Hicks, et al. Mammalian Target of Rapamycin (mTOR) Regulates Cellular Proliferation and Tumor Growth in Urothelial Carcinoma. Am. J. Pathol. 176:3062–3072.

9. McAuliffe, P. F., F. Meric-Bernstam, G. B. Mills, and A. M. Gonzalez-Angulo. 2010. Deciphering the role of PI3K/Akt/mTOR pathway in breast cancer biology and pathogenesis. Clin. Breast Cancer 10(Suppl 3):S59–S65.

10. Margariti, N., S. Fox, A. Bottini, and D. Generali. 2011. “Overcoming breast cancer drug resistance with mTOR inhibitors”. Could it be a myth or a real possibility in the short-term future? Breast Cancer Res. Treat. 128:599–606.

11. Karar, J., and A. Maity. 2011. PI3K/AKT/mTOR pathway in Angiogenesis. Front. Mol. Neurosci. 4:1–8.

12. Ray, A., M. Alalem, and B. K. Ray. 2014. Insulin signaling network in cancer. Indian J. Biochem. Biophys. 51:493–498.

13. Kalender, A., A. Selvaraj, S. Y. Kim, P. Gulati, S. Brulé, B. Violett, et al. 2010. Metformin, Independent of AMPK, Inhibits mTORC1 in a Rag GTPase-Dependent Manner. Cell Metab. 11:390–401.

14. Dowling, R. J. O., I. Topisirovic, B. D. Fonseca, and N. Sonenberg. 2010. Dissecting the role of mTOR: lessons from mTOR inhibitors. Biochem. Biophys. Acta 1804:433–439.

15. Schmelzle, T., and M. N. Hall. 2000. TOR, a Central Controller of Cell Growth. Cell 103:253–262.

16. White-Gilbertson, S., D. T. Kurtz, and C. Voelkel-Johnson. 2009. The role of protein synthesis in cell cycling and cancer. Mol. Oncol. 3:402–408.

17. Vogt, P. K. 2001. PI 3-kinase, mTOR, protein synthesis and cancer. Trends Mol. Med. 7:482–484.

18. Gingras, A. C., B. Raught, and N. Sonenberg. 2001. Regulation of translation initiation by FRAP/mTOR. Genes Dev. 15:807–826.

19. Ma, T., C. A. Hoeffer, E. Capetillo-Zarate, F. Yu, H. Wong, M. T. Lin, et al. 2010. Dysregulation of the mTOR Pathway Mediates Impairment of Synaptic Plasticity in a Mouse Model of Alzheimer’s Disease. PLoS ONE 5:e12845.

20. Zhou, X., T. Ikenoue, X. Chen, L. Li, K. Inoki, and K.-L. Guan. 2009. Rheb controls misfolded protein metabolism by inhibiting aggresome formation and autophagy. Proc. Natl Acad. Sci. 106:8923–8928.

21. Yardley, D. A. 2013. Combining mTOR inhibitors with chemotherapy and other targeted therapies in advanced breast cancer: rationale, clinical experience, and future directions. Breast Cancer 7:7–22.

22. Noh, W., Y. Kim, M. Kim, J. Koh, H.-A. Kim, N. Moon, et al. 2008. Activation of the mTOR signaling pathway in breast cancer and its correlation with the clinico-pathologic variables. Breast Cancer Res. Treat. 110:477–483.

23. Liu, H., C. Scholz, C. Zang, J. H. Schefe, P. Habbel, A.-C. Regierer, et al. 2012. Metformin and the mTOR Inhibitor Everolimus (RAD001) Sensitize Breast Cancer Cells to the Cytotoxic Effect of Chemotherapeutic Drugs In Vitro. Anticancer Res. 32:1627–1637.

24. Chumsri, S., G. Sabnis, K. Tkaczuk, and A. Brodie. 2014. mTOR inhibitors: changing landscape of endocrine-resistant breast cancer. Future Oncol. 10:443–456.

25. Kurmasheva, R. T., S. Huang, and P. J. Houghton. 2006. Predicted mechanisms of resistance to mTOR inhibitors. Br. J. Cancer 95:955–960.

26. Ben Sahra, I., C. Regazzetti, G. Robert, K. Laurent, Y. Le Marchand-Brustel, P. Aubergé, et al. 2011. Metformin, Independent of AMPK, Induces mTOR Inhibition and Cell-Cycle Arrest through REDD1. Cancer Res. 71:4366–4372.

27. Bailey, C. J., and R. C. Turner. 1996. Metformin. N. Engl. J. Med. 334:574–579.

28. AlQurashi, N., V. Gopalan, R. A. Smith, and A. K. Y. Lam. 2013. Clinical impacts of mammalian target of rapamycin expression in human colorectal cancers. Hum. Pathol. 44:2089–2096.

29. Chen, X., M. Zhao, M. Hao, X. Sun, J. Wang, Y. Mao, et al. 2013. Dual Inhibition of PI3K and mTOR Mitigates Compensatory AKT Activation and Improves Tamoxifen Response in Breast Cancer. Mol. Cancer Res. 11:1269–1278.

30. Wang, X. J., J. Yu, S. H. Wong, A. S. L. Cheng, F. K. L. Chan, S. S. M. Ng, et al. 2013. A novel crosstalk between two major protein degradation systems. Autophagy 9:1500–1508.

31. Ding, W.-X., H.-M. Ni, W. Gao, T. Yoshimori, D. B. Stolz, D. Ron, et al. 2007. Linking of Autophagy to Ubiquitin-Proteasome System Is Important for the Regulation of Endoplasmic Reticulum Stress and Cell Viability. Am. J. Pathol. 171:513–524.

32. Heras-Sandoval, D., J. M. Pérez-Rojas, J. Hernández-Damián, and J. Pedraza-Chaverri. 2014. The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. Cell. Signal. 26:2694–2701.

33. Vessoni, A. T., E. C. Filippi-Chiela, C. F. Menck, and G. Lenz. 2013. Autophagy and genomic integrity. Cell Death Differ. 20:1444–1454.

34. Mathew, R., V. Karantza-Wadsworth, and E. White. 2007. Role of autophagy in cancer. Nat. Rev. Cancer 7:961–967.

35. Suh, D. H., M.-K. Kim, H. S. Kim, H. H. Chung, and Y. S. Song. 2012. Unfolded protein response to death. Death Differ. 20:1444–1454.

36. Kuma, A., M. Matsui, and N. Mizushima. 2007. LC3, an Autophagosome Marker, Can be Incorporated into
Protein Aggregates Independent of Autophagy: caution in the Interpretation of LC3 Localization. Autophagy 3:323–328.
37. Mizushima, N., and T. Yoshimori. 2007. How to Interpret LC3 Immunoblotting. Autophagy 3:542–545.
38. Mizushima, N., T. Yoshimori, and B. Levine. 2010. Methods in Mammalian Autophagy Research. Cell 140:313–326.
39. Klionsky, D. J., F. C. Abdalla, H. Abeliovich, R. T. Abraham, A. Acevedo-Arozena, K. Adeli, et al. 2012. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 8:445–544.
40. Salabei, J. K., A. Balakumaran, J. C. Frey, P. J. Boor, M. Treinen-Moslen, and D. J. Conklin. 2012. Verapamil Stereoisomers Induce Antiproliferative Effects In Vascular Smooth Muscle Cells Via Autophagy. Toxicol. Appl. Pharmacol. 262:265–272.
41. Pająk, B., E. Kania, B. Gajkowska, and A. Orzechowski. 2012. Verapamil-induced autophagy-like process in colon adenocarcinoma COLO 205 cells; the ultrastructural studies. Pharmacol. Rep. 64:991–996.
42. Olzmann, J. A., L. Li, and L. S. Chin. 2008. Aggresome Formation and Neurodegenerative Diseases: therapeutic Implications. Curr. Med. Chem. 15:1–14.
43. Rodriguez-Gonzalez, A., T. Lin, A. K. Ikeda, T. Simms-Waldrip, C. Fu, and K. M. Sakamoto. 2008. Role of the Aggresome Pathway in Cancer: targeting Histone Deacetylase 6–Dependent Protein Degradation. Cancer Res. 68:2557–2560.
44. Zakikhani, M., R. Dowling, I. G. Fantus, N. Sonenberg, and M. Pollak. 2006. Metformin Is an AMP Kinase-Dependent Growth Inhibitor for Breast Cancer Cells. Cancer Res. 66:10269–10273.
45. Laplante, M., and D. M. Sabatini. 2009. mTOR signaling at a glance. J. Cell Sci. 122:3589–3594.
46. Steinberg, G. R., and B. E. Kemp. 2009. AMPK in Health and Disease. Physiol. Rev. 89:1025–1078.
47. Kudchodkar, S. B., G. Q. Del Prete, T. G. Maguire, and J. C. Alwine. 2007. AMPK-Mediated Inhibition of mTOR Kinase is Circumvented during Immediate-Early Times of Human Cytomegalovirus Infection. J. Virol. 81:3649–3651.