Perspective

Adenovirus Overrides Cellular Checkpoints for Protein Translation

Clodagh C. O'Shea*

Serah Choi

Frank McCormick

David Stokoe

UCSF Cancer Center; San Francisco, California USA

*Correspondence to: Clodagh C. O'Shea; UCSF Cancer Center; 2340 Sutter Street; Box 0128; San Francisco, California 94115 USA; Tel.: 415.502.1868; Fax: 415.502.3179; Email: coshea@cc.ucsf.edu

ABSTRACT

mTOR is a critical regulator of protein translation, and plays an important role in controlling cellular replication. Recent studies indicate that nutrient and growth factor mediated activation of mTOR is deregulated in human cancer, and therefore represents an attractive tumor target. However, activation of mTOR is a complex process that is not yet fully understood. DNA viruses and tumor cells often perturb similar cellular pathways to facilitate their replication. In a recent study, we used adenovirus as a novel tool to probe the mechanisms underlying the inappropriate activation of mTOR upon virus infection of quiescent primary cells. These studies revealed that adenovirus encodes two viral proteins, E4-ORF1 and E4-ORF4, which activate mTOR, even in the absence of nutrient/growth factor signals, and which play a role in promoting viral replication. E4-ORF1 mimics growth factor signaling to mTOR by activating PI3-kinase, whereas E4-ORF4, which binds and relocalizes PP2A, can substitute for glucose mediated activation of mTOR. We discuss insights from this study, together with the similarities that may exist between viruses and tumor cells with respect to the mechanistic and functional requirements for mTOR activation in driving their aberrant DNA replication.

Many of the critical tumor targets, for example p53,[1,2] E2F[3] and PI3-kinase,[4] were first identified through the study of DNA tumor viruses. This is because there exists a profound functional overlap between DNA viruses and tumor cells with respect to the cellular checkpoints they each must perturb to facilitate their replication. In tumor cells, the Rb and p53 checkpoints are inactivated through mutations, while viruses encode proteins that bind and inactivate Rb and p53 directly, for example, adenovirus E1A and E1B-55K, respectively. This is no mere coincidence, but reflects, and indeed reveals, the obligate cellular players that must be perturbed in order to drive aberrant replication. In addition to E1A and E1B-55K, adenovirus encodes several other early viral proteins that are likely to impinge on cellular pathways critical for the onset of DNA replication and/or cell survival. By uncovering the cellular targets of these early viral proteins, we may identify critical cellular pathways that drive aberrant replication. This information can then be used to determine whether these same cellular targets are also deregulated in tumor cells, and/or could be exploited for novel cancer therapies. As such, DNA viruses are a powerful genetic system with which to identify and examine the integration of the pivotal cellular pathways that are necessary to drive aberrant cellular replication. In a recent study,[5] we used adenovirus to gain new insights into the regulation of protein translation and nutrient/growth factor signaling pathways to mTOR, an important tumor target.

ADENOVIRUS BYPASSES A NUTRIENT/GROWTH FACTOR CHECKPOINT FOR PROTEIN TRANSLATION

Together with DNA replication, protein synthesis is one of the most energy consuming processes within the cell, and plays a critical role in mediating cell growth. Thus, the initiation of both DNA replication and protein translation are strictly controlled and normally occur only under conditions conducive to cell growth, such as in the presence of nutrient/growth factor signals. However, both DNA viruses and tumor cells have evolved strategies to subvert such controls to replicate even in the harshest of cellular microenvironments. Tumor cell mechanisms that drive inappropriate S phase entry are now reasonably well understood and were greatly informed by the study of DNA viruses. Nutrients and growth factor signals are also required for the initiation of protein translation, a process that plays an important role in cellular growth. We reasoned that we could use adenovirus as a simple genetic tool to gain new insights into the regulation of protein translation in

aberrant replication. Therefore, we examined whether, in addition to usurping control of DNA synthesis, adenovirus also initiates protein translation in infected cells in the absence of extra-cellular growth factor/nutrient signals. Protein synthesis was monitored by ^{35}S-Methionine incorporation in primary quiescent human small airway epithelial cells (SAECs) incubated either in complete medium or in phosphate buffered saline (PBS), devoid of all components known to be required for translation (glucose, amino acids, growth factors). Predictably, the absence of growth factors and nutrients reduced normal cellular protein synthesis by 80% (Fig. 1). However, in cells infected with wild type adenovirus (at 24 hours post-infection and prior to DNA replication), the synthesis of both viral and cellular proteins was maintained, even in PBS (Fig. 1). These early effects on translation are entirely distinct from the global shutdown in cellular protein synthesis that is observed late in viral infection.[6] These data suggested to us that adenovirus may encode viral genes that function to bypass a nutrient/growth factor checkpoint for protein translation in infected cells.

ADENOVIRUS ENCODES EARLY PROTEINS THAT MIMIC NUTRIENT AND GROWTH FACTOR SIGNALING TO ACTIVATE MTOR AND PROTEIN TRANSLATION

The control of protein translation is poorly understood but is regulated in part by the mammalian target of rapamycin (mTOR), which plays an important role in regulating protein translation in response to nutrient and growth factor signals. mTOR is a kinase that was first isolated as the target of the macrolide drug rapamycin,[7] which has potent inhibitory effects on cell growth and replication. mTOR activation induces the phosphorylation of 4EBP1 and p70[S6K], which stimulates the translation of mRNAs with a high degree of secondary structure in their 5' untranslated regions, including that of many important growth regulatory messages (Fig. 2). For example, mRNAs regulated by mTOR include cyclin D1,[8] ornithine decarboxylase,[9] and c-myc.[10] In this way, mTOR activation is thought to serve as an important checkpoint for normal cell growth.[11]

mTOR is activated by the presence, and/or binding, of active GTP bound Rheb. The hamartin/tuberin complex (which we will refer to as the TSC1/TSC2 complex) inactivates Rheb-GTP through the GAP activity of TSC2 (Fig. 2).[12] Growth factors are thought to stimulate mTOR through activation of PI3-kinase and PKB/Akt, resulting in the phosphorylation of TSC2, inactivation of the TSC1/TSC2 complex, and ultimately increased Rheb-GTP loading.[13] In addition to growth factors, nutrients such as amino acids and glucose, which affect cellular ATP levels, are also important activators of mTOR. Low ATP levels stimulate the activity of AMP-dependent protein kinase (AMPK), which phosphorylates TSC2 and increases the activity of the TSC1/TSC2 complex.[14] However, the mechanisms underlying mTOR regulation by nutrients remain poorly understood.

We found that adenovirus bypasses a nutrient/growth factor checkpoint for protein translation in infected cells (Fig. 1). Therefore, we investigated whether adenovirus mediated these effects on protein translation through inappropriate activation of components of the PI3-kinase/mTOR signaling pathway. These studies revealed adenoviral proteins that activate PI3-kinase and mTOR, even under nutrient/growth factor deprived conditions, and which play a requisite role in viral replication. Using adenoviral mutants, we identified two early viral proteins, E4-ORF1 and E4-ORF4, which stimulate the activity of mTOR in distinct but
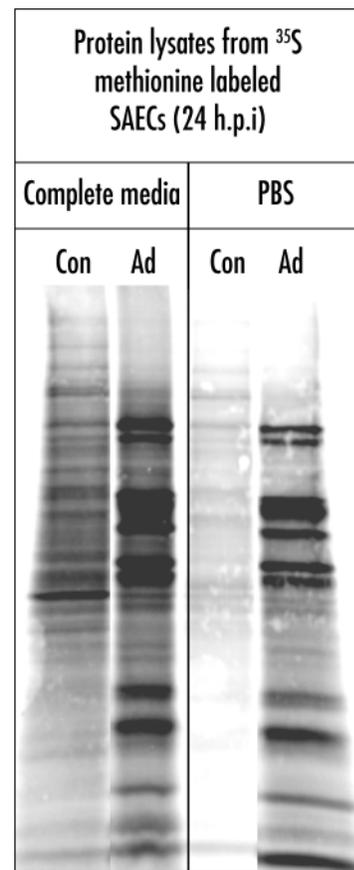

Figure 1. Adenovirus bypasses a nutrient/growth factor checkpoint for protein translation in infected primary cells. Primary quiescent small airway epithelial cells (SAECs) were infected with a nonreplicating control virus (Con) or with wild type adenovirus (Ad). At 24 hours post-infection *(h.p.i.)*, SAECs were incubated in either complete medium (lacking Cys/Met) or PBS for four hours, and then labeled with ^{35}S-Met for 1 hour. Normalized lysates were separated by gel electrophoresis and analyzed on a STORM phosphorimager.

complementary ways.[5] One of these viral proteins, E4-ORF1, uses a C terminal PDZ binding motif to activate PI3-kinase, a well known regulator of mTOR activity. However, an E4-ORF1 mutant virus that fails to activate PI3-kinase in infected primary cells is only partially compromised in its ability to activate p70[S6K]. This led us to find an additional adenoviral protein, E4-ORF4, which substitutes for glucose signaling and activates mTOR through a PI3-kinase/PKB-independent mechanism. Moreover, E4-ORF4 is the critical viral protein necessary for activation of the mTOR pathway in infected primary quiescent cells, while E4-ORF1/PI3-kinase plays a minor role.[5] A trivial explanation for the minor contribution of E4-ORF1/PI3-kinase, compared to E4-ORF4, in activating mTOR in infected SAECs may be that E4-ORF1 is not highly expressed in quiescent SAECs. However, a more intriguing explanation is that the hard-wiring of mTOR signaling pathways is different in specific tissue types or cellular states (such as G_0 versus G_1), with those activated by E4-ORF4 playing a particularly important role in quiescent SAECs. Thus, E4-ORF1 and E4-ORF4 expression may allow adenovirus to activate mTOR for its replication in pleiotropic cell-types. Distinguishing between these possibilities may have important implications for understanding mTOR regulation in normal and aberrant cell growth.
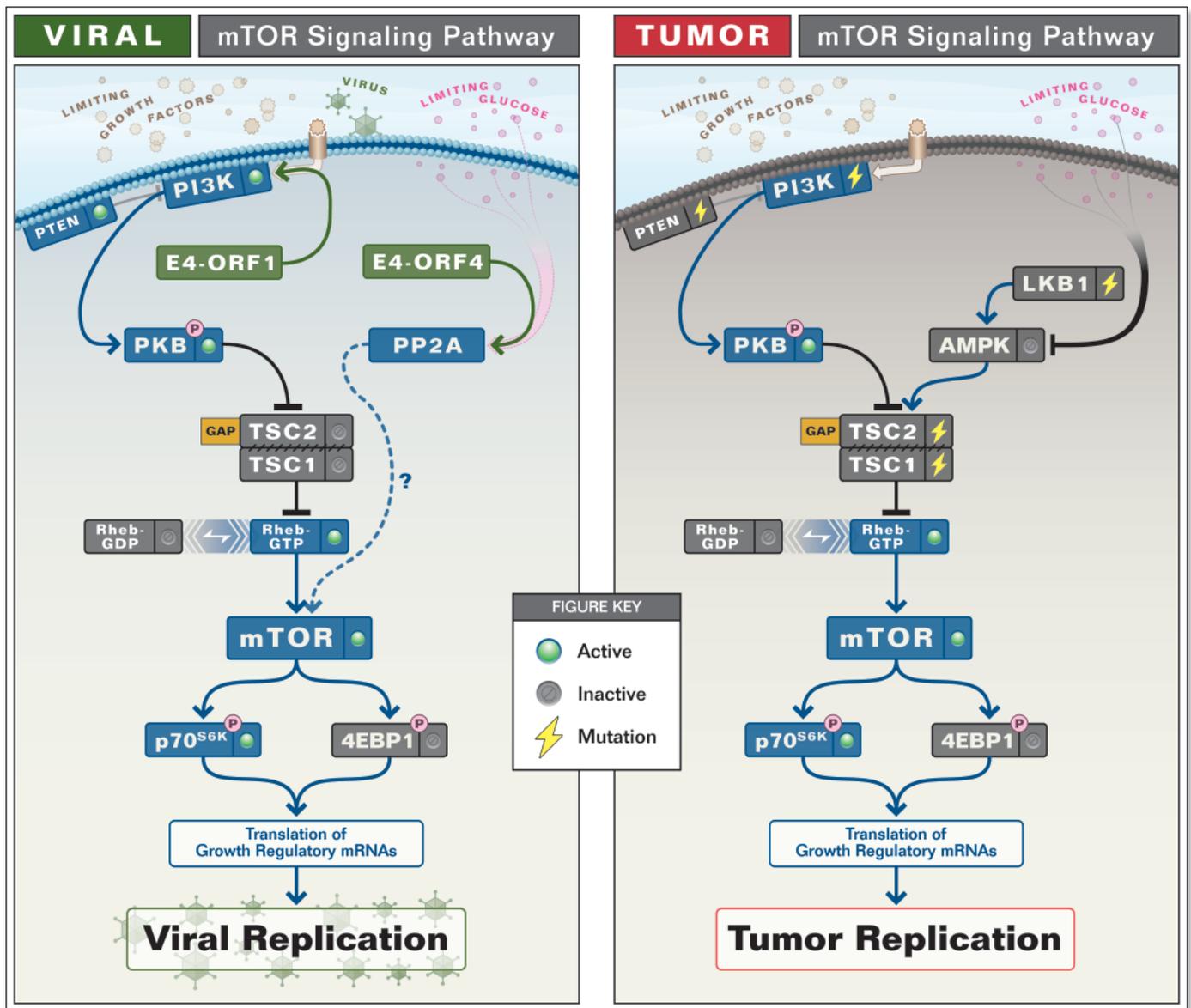
Figure 2. Deregulation of the mTOR signaling pathway in tumor cells versus adenoviral infected normal cells. The mTOR pathway integrates signals from growth factors and nutrients and regulates the translation of important growth regulatory mRNAs. Growth factors (insulin/EGF) stimulate mTOR through activation of PI3-kinase and PKB, resulting in the phosphorylation and inactivation of the TSC1/2 complex. The TSC1/2 complex acts as a RhebGAP, and converts active Rheb-GTP, which is necessary for mTOR activation, to inactive Rheb-GDP. mTOR activation induces the phosphorylation of key downstream effectors in the translation pathway, such as 4EBP1 and p70S6K. Nutrients, such as glucose, also activate mTOR, possibly through inhibition of AMPK, resulting in decreases in Rheb-GTP. In normal cells, the mTOR pathway is inactive in the absence of growth factors and nutrients. A) Viral mTOR signaling pathway: Adenovirus encodes two proteins, E4-ORF1 and E4-ORF4, that activate mTOR even under nutrient/growth factor deprived conditions. E4-ORF1 activates growth factor/PI3-kinase signaling, resulting in increased Rheb-GTP loading and mTOR activation. E4-ORF4 mediated activation of mTOR occurs in the absence of increases in Rheb-GTP loading, is inhibited by rapamycin, and substitutes for glucose signaling to mTOR. PP2A, a known binding partner of E4-ORF4, is a likely cellular target whereby E4-ORF4 activates mTOR. B) Tumor mTOR signaling pathway: Constitutive mTOR activation is associated with several inherited cancer predisposition syndromes. Examples include: Peutz-Jeghers disease, caused by inactivating mutations in *LKB1* (which is required for activation of AMPK); Cowden disease, caused by inactivating mutations in *PTEN*, the lipid phosphatase that antagonizes PI3-kinase activation of PKB; and tuberous sclerosis complex, caused by inactivating mutations in either *TSC1* or *TSC2*.

DEREGULATION OF THE MTOR SIGNALING PATHWAY IN TUMOR VERSUS VIRAL REPLICATION

At first, we were surprised to find that adenovirus encodes two proteins to activate mTOR. However, many of the pivotal growth regulating checkpoints in the cell are regulated by multiple signals that exert integrated or additive effects upon critical downstream effectors. Consistent with this, E4-ORF1 and E4-ORF4 have distinct and nonoverlapping functions in activating mTOR in viral infection. E4-ORF1 activates a growth factor/PI3-kinase signaling pathway, resulting in increased Rheb-GTP loading and mTOR activation (Fig. 2). In contrast, E4-ORF4 does not affect Rheb-GTP loading, and is redundant with both ectopic Rheb overexpression and glucose signaling in activating mTOR. The expression of both E4-ORF1

and E4-ORF4 together, but not individually, is sufficient to maintain p70^{S6K} phosphorylation even in cells incubated in the absence of growth factors and nutrients. Therefore, it is tempting to speculate that E4-ORF1 and E4-ORF4 functions may have evolved to ensure mTOR activation and viral replication even in an unfavorable cellular microenvironment, devoid of nutrient and growth factor signals. It will be interesting to determine whether tumor cells have had to evolve similar strategies and select for genetic lesions that deregulate both nutrient and growth factor signaling pathways to mTOR.

Indeed, recent observations from inherited cancer predisposition syndromes suggest that this may well be the case. Constitutive mTOR activation underlies the pathology associated with the inherited cancer syndrome tuberous sclerosis complex, a genetic disorder caused by inactivating mutations in either TSC1 or TSC2. In addition, the genetic cancer syndrome Peutz-Jeghers disease is caused by inactivating mutations in LKB1, a protein kinase that activates AMPK in response to increased cellular AMP/ATP ratios, such as occurs on nutrient withdrawal (Fig. 2). Thus, loss of LKB1 activity results in inactivation of the TSC1/TSC2 complex and constitutive mTOR activation even upon nutrient withdrawal.[15] Finally, germ-line mutations in PTEN, the lipid phosphatase that antagonizes PI3-kinase activity, results in Cowden disease, which is associated with increased frequency of benign and malignant tumors. Thus, inappropriate activation of the mTOR pathway appears to be a recurring theme in both tumor and viral replication.

Viral proteins-novel tools to probe mechanisms of inappropriate mTOR activation. Of the known pathways that stimulate mTOR, E4-ORF4 appears to most closely resemble, and indeed can substitute for, glucose signaling.[5] The nutrient signaling pathway to mTOR is relatively poorly understood. To date, AMPK phosphorylation, which is unaffected by E4-ORF4, is one of the few described targets in glucose signaling to mTOR.[14] Therefore, E4-ORF4 is a unique tool with which to reveal critical cellular targets in the glucose signaling pathway to mTOR, and whether they are also targeted in tumorigenesis. The best characterized cellular targets of E4-ORF4 include SR proteins,[16] which play a role in splicing; the tyrosine kinase and proto-oncogene c-Src; and the serine/threonine protein phosphatase 2A (PP2A). Of the known cellular targets of E4-ORF4, c-Src and PP2A seem the most likely to mediate E4-ORF4's effects on mTOR activation, given their well established roles in cellular signaling cascades.

E4-ORF4 has been shown to bind to the c-Src kinase domain in vitro,[17] and to affect the tyrosine phosphorylation of c-Src substrates in transfected cells.[18] c-Src has previously been reported to activate mTOR,[19] although these effects are thought to be mediated by c-Src induced activation of the growth factor/PI3-kinase signaling pathway. Whether c-Src also activates a nutrient signaling pathway to mTOR has not been reported. Therefore, it is possible that E4-ORF4 may activate mTOR via its interaction with c-Src. However, in contrast to transfected v-Src, E4-ORF4 does not affect PI3-kinase/PKB activation (our unpublished data). These data suggest that either c-Src is not the relevant cellular target whereby E4-ORF4 mediates its effects on mTOR activation, or if it is, it does so independently of c-Src mediated PI3-kinase activation.

E4-ORF4 also binds to the regulatory subunit of PP2A and is thought to modulate its activity.[20,21] PP2A is a heterotrimeric complex comprising of A, B and C subunits, of which there are at least 60 possible combinations. The large number of B subunits is thought to allow for different substrate specificities. Therefore, the ability of E4-ORF4 to activate mTOR may be through its binding

and modulation of the regulatory B subunit of PP2A (Fig. 2). Consistent with this, we demonstrated that E4-ORF4 point mutants that fail to bind to PP2A are defective for p70^{S6K} activation.[5] In addition, we have since demonstrated that wild type E4-ORF4 binds to both endogenous PP2A B and C subunits, unlike E4-ORF4 mutants that fail to activate p70^{S6K} (unpublished data). In contrast, we have been unable to detect any binding at all, of either wild type or mutant E4-ORF4, to endogenous c-Src. These data suggest that the ability of E4-ORF4 to activate mTOR is most likely through its binding and modulation of the regulatory B subunit of PP2A. Thus, E4-ORF4 may reveal a novel role for PP2A in the mTOR signaling pathway. Previously, in both yeast mutants, and mammalian cells treated with okadaic acid (a broad PP1/PP2A inhibitor), PP2A has been implicated in p70^{S6K} activation, but downstream of mTOR, as these effects were not inhibited by rapamycin.[22,23] In contrast, our results demonstrate that E4-ORF4's effects in stimulating p70^{S6K} and 4EBP1 phosphorylation are completely inhibited in the presence of rapamycin, indicating that PP2A may also play a role in signaling upstream of mTOR. Therefore, PP2A may act at multiple points in the mTOR pathway which could be modulated by different B subunit substrate specificities or signaling inputs.

It is intriguing that disparate DNA viruses have functionally converged to encode proteins that bind and modulate PP2A, for example Polyomavirus small T (PyST) and middle T (MT), and also SV40 small T (ST).[24] This suggests that the modulation of PP2A plays a critical role in driving aberrant DNA replication, including perhaps in tumorigenesis. Indeed the modulation of PP2A by SV40 ST, together with the catalytic subunit of telomerase, Ras and SV40 Large T mediated inactivation of Rb and p53, is critical for the in vitro transformation of human cells.[25-27] Moreover, PP2A A subunit mutations have been found in a subset of human tumors.[28,29] Understanding how different viral proteins or tumor mutations modulate PP2A activity, and whether this contributes to mTOR activation, may yield important insights into the role of PP2A in normal and tumor cell growth.

The role of PI3-kinase/mTOR activation in viral replication. The evolution of adenoviral proteins that function in the activation of PI3-kinase/mTOR, suggest that this signaling pathway may play an important role in facilitating viral replication. Moreover, adenovirus is not unique in encoding viral proteins that activate the PI3-kinase/mTOR pathway. Indeed, PI3-kinase was itself first isolated as a protein associated with polyomavirus MT.[4] Intriguingly, Human Papillomavirus 16 E6 protein has recently been shown to degrade TSC2 resulting in the activation of mTOR.[30] In addition, cytomegalovirus stimulates 4EBP1 phosphorylation in a rapamycin-independent manner. Whether viral proteins also target the rapamycin-independent functions of mTOR remains an area for future research.

To address the question of the requirement for PI3-kinase/mTOR activation in the viral growth deregulation program, we used pharmacological inhibitors of PI3-kinase/mTOR, and in parallel took the genetic approach of using E4-ORF1 and E4-ORF4 viral mutants. We found that the inhibition of mTOR activation in adenoviral infected quiescent primary cells significantly impaired DNA replication initiation, despite E1A mediated activation of E2F.[5] A requirement for p70^{S6K} activation in S phase entry has previously been demonstrated,[31] and rapamycin is known to arrest cells in the G$_1$ phase of the cell cycle.[32] Our data suggest that mTOR and the Rb family of proteins (Rb, p107, p130) have nonredundant roles in regulating S phase entry, at least in quiescent cells, and that mTOR acts downstream of, or in parallel to, E2F in initiating cell cycle entry.

We favor a hypothesis whereby mTOR regulates the translation, much like E2F regulates the transcription, of a panel of mRNAs important for the onset of DNA synthesis.[33] In many ways, the potentially sequential requirement for E2F and mTOR activation in S phase entry is reminiscent of the early work by Pardee and colleagues which showed that isoleucine deprivation caused a cell cycle arrest in late G_1, whereas growth factor deprivation caused a cell cycle arrest in mid G_1.[34] Interestingly, isoleucine deprivation is known to inhibit mTOR activity.[35] Some of the chemotherapeutic agents commonly used in the clinic target E2F transcriptional targets, for example, 5-Fluorouracil and thymidylate synthetase. Therefore, identifying the critical mRNAs that are translationally regulated at the beginning of S phase will not only further our understanding of normal cell cycle progression but potentially uncover novel cellular targets for cancer drug development. Microarray analysis of polysomes from primary cells infected with mutant viruses, such as ΔE4-ORF4, which readily distinguishes between E2F and mTOR activation in the onset of aberrant DNA replication, may be a novel strategy to elucidate such targets.

NOVEL ONCOLYTIC VIRAL STRATEGIES FOR TUMOR CELLS WITH DEREGULATED PI-3 KINASE/MTOR SIGNALING

In addition to their utility as tools with which to discover new tumor targets, viral mutants that fail to perturb critical growth regulatory pathways may undergo selective lytic replication in tumor cells in which these pathways are already deregulated through mutations. For example, ONYX-411 is a novel oncolytic virus that takes advantage of deregulated E2F activity in tumor cells that have inactivating mutations in RB/p16.[36] ΔE4-ORF1 and ΔE4-ORF4 replicate to wild type virus levels in HeLa cells[37] in which mTOR is activated through E6 mediated degradation of TSC2.[30] However in primary cells the replication of both ΔE4-ORF1 and ΔE4-ORF4 viruses was partially compromised, although not so severely as to suggest that either of these viruses would be restricted to tumor cells for their replication.[5] Nevertheless, LY294004, which inhibits both PI3-kinase and mTOR activity, results in a 15-fold defect in wild type viral replication in infected primary cells. This suggests that a virus defective for both E4-ORF1 and E4-ORF4 functions may be similarly inhibited and therefore, a novel and potentially efficacious oncolytic virus for the treatment of tumors that have deregulated the growth factor/PP2A/mTOR pathway.

PROSPECTIVE/OUTLOOK

Our discovery of viral proteins that impact mTOR signaling at multiple levels were prompted by our initial observation that adenovirus infected cells maintain global translation, as evidenced by ^{35}S-Methionine labeling, even in the absence of nutrient/growth factors (Fig. 1). A major question then is whether tumor cells also select for genetic lesions that deregulate not only growth factor, but also nutrient, signaling to mTOR. Therefore, we are using E4-ORF4 as a unique tool to identify novel cellular targets in the mTOR signaling pathway and whether they are deregulated in cancer. Nevertheless, mRNAs that depend on mTOR activity for their translation are thought to comprise a relatively small pool of 8–15% of the mRNAs within the cell.[38] Consistent with this, an E4-ORF4 viral mutant is defective in stimulating the assembly of eIF4E/eIF4G translation initiation complexes in infected cells,[5] but is effective in overriding a nutrient/growth factor checkpoint for global translation

(our unpublished observations). In contrast, an E4-ORF1 viral mutant that fails to activate PI3-kinase is partially, but by no means completely, defective in maintaining global protein synthesis. These data imply that adenovirus has yet more secrets to reveal with respect to viral strategies that usurp control of normal protein translation checkpoints. This not only underscores the importance of translation in driving inappropriate replication, but also the utility of adenovirus as a genetic tool with which to explore and target aberrant protein synthesis within the cell.

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