Usp18 Regulates Epidermal Growth Factor (EGF) Receptor Expression and Cancer Cell Survival via MicroRNA-7*§

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Jason E. Duey‡, Laurey Comeau§, Alexander Sorkin#1,2, Benjamin Purow§1,3, and Benjamin Kefas§1,4

From the§Department of Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261 and the§Division of Neuro-Oncology, Neurology Department, University of Virginia Health System, Charlottesville, Virginia 22908

Epidermal growth factor receptor (EGFR) is involved in development and progression of many human cancers. We have previously demonstrated that the ubiquitin-specific peptidase Usp18 (Ubp43) is a potent regulator of EGFR protein expression. Here we report that the 3′-untranslated region (3′-UTR) of the EGFR message modulates RNA translation following cell treatment with Usp18 siRNA, suggesting microRNA as a possible mediator. Given earlier evidence of EGFR regulation by the microRNA miR-7, we assessed whether miR-7 mediates Usp18 siRNA effects. We found that Usp18 depletion elevates miR-7 levels in several cancer cell lines because of a transcriptional activation and/or mRNA stabilization of miR-7 host genes and that miR-7 acts downstream of Usp18 to regulate EGFR mRNA translation via the 3′-UTR. Also, depletion of Usp18 led to a decrease in protein levels of other known oncogenic targets of miR-7, reduced cell proliferation and soft agar colony formation, and increased apoptosis. Notably, all of these phenotypes were reversed by a specific inhibitor of miR-7. Thus, our findings support a model in which Usp18 inhibition promotes upregulation of miR-7, which in turn inhibits EGFR expression and the tumorigenic activity of cancer cells.

Despite advances in cancer treatments, improvement of overall patient survival remains poor. One of the primary reasons for this low success is the inherent complexity of oncogenic pathways such as those driven by the epidermal growth factor (EGF)* receptor (EGFR). EGFR is a member of the receptor tyrosine kinase (RTK) family. Upon binding of ligand, EGFR autophosphorylates at tyrosine residues and triggers an intracellular signal transduction cascade, which ultimately promotes cellular survival and division (1, 2). Dysregulation of EGFR and downstream signaling events is found in a multitude of tumor types (3–6). Therefore, targeting the tyrosine kinase activity of EGFR with small molecule inhibitors or targeting EGFR with antibodies has been a focus in the treatment of several tumors, including brain (glioblastoma), cervical, lung, and head and neck (squamous cell carcinoma). However, this strategy has resulted in minimal success. A major limitation of these approaches is that tumor cells eventually develop resistance to the current therapeutics. The resistance develops through increased ligand expression, additional somatic mutations in the EGFR tyrosine kinase domain, and increased heterodimerization with other RTKs (3, 7–9).

As an alternative to developing approaches to directly inhibit EGFR signaling, our recent efforts focused on identifying allosteric modulators of EGFR protein levels. Inhibition of these modulators has the potential to significantly decrease EGFR protein levels irrespective of ligand levels or EGFR mutational status. Using a library of small interfering RNAs (siRNAs) that target deubiquitinating enzymes (DUBs), a class of proteins known to regulate receptor trafficking and expression (10–12), we identified a number of candidate proteins which regulate EGFR protein levels. One of these candidates is Usp18 (Ubp43) (13). Ubiquitin specific peptidase 18 (Usp18) is a cytosine protease which has been shown to remove ubiquitin and the ubiquitin-like molecule interferon stimulated gene 15 (ISG15) from substrates (14, 15). siRNA knockdown of Usp18 resulted in a 50–90% reduction in EGFR protein levels in a variety of cancer cell lines (13). Interestingly, this decreased synthesis occurs despite no change to EGFR mRNA levels (13). Such an observation hints that Usp18 regulation of EGFR protein occurs through EGFR 3′- and/or 5′-untranslated regions, suggesting the involvement of microRNAs (miRNAs) (16–18). In fact, miRNAs miR-128a,b (19) and miR-7 (20) have been shown to regulate EGFR.

miRNAs are a class of noncoding RNAs that regulate protein expression by binding to the 3′-UTR of mRNA targets (17, 18). They play critical roles in controlling cellular processes such as proliferation, apoptosis, development, and differentiation (16, 17, 20, 21). miRNAs are first transcribed in the cell nucleus as long primary transcripts (pri-miRNAs), typically several hundred nucleotides long, and then capped, spliced, and polyaden-
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nylated (22). These transcripts are processed in the nucleus by the ribonuclease enzyme Drosha into a precursor miRNA which is about 70 nucleotides in length (16–18, 22). The pre-
miRNA is exported to the cell cytosol and further processed by the enzyme Dicer to 19–23 nucleotide miRNA. The resultant
siRNA-like mature miRNA molecule is incorporated into the RISC complex, where it directs mRNA translational inhibition
and/or degradation (16–18, 22).

In the present study, we have identified the mechanism by which Usp18 controls EGFR down-regulation. We found that
Usp18 knockdown leads to increased miR-7 levels as a result of increased transcriptional activation and/or mRNA stabilization
of miR-7 host genes, mediating the effect on EGFR expression and other known oncogenic targets of miR-7. This is the first
study which demonstrates a role for a deubiquitinase enzyme in the regulation of a miRNA. Furthermore, we determined that
tumor cells depleted of Usp18 undergo apoptosis through the activation of miR-7. These data suggest that inhibiting Usp18
may serve as a means of activating miR-7 and ultimately as a therapy for tumors with dysregulated EGFR.

MATERIALS AND METHODS

Cell Culture—Glioma cell lines U87MG and T98G, and the cervical cell line, HeLa, were acquired from American Type Culture
Collection (ATCC). Head-and-neck squamous cell carcinoma UMSCC2 (referred to in this study as SCC2) cells origi-
nated from Dr. T. Carey (University of Michigan). All cell lines
were grown under previously described conditions (13, 23).

Materials—Pre-miR-7 and control-pre-miR were obtained
from Applied Biosystems/Ambion (Austin, TX). Usp18 siRNA
#5 and control siRNA were obtained from Qiagen (Valencia,
CA) (13) while Usp18 siRNA #6 was obtained from Dharmaco
(Lafayette, CO).

MicroRNA-7 and Control Inhibitor—Control and miR-7 spe-
cific inhibitor were obtained from Dharmaco (001005-01 and
300546-08, respectively). Briefly, the miR-7 inhibitor is a chemi-
cally engineered hairpin small RNA that includes a sequence that is perfectly complementary to the miR-7 sequence (24-
UUGAAGACUAGUGAU UUUGUUGU-46) and is modified
by the addition of a 2′-O-methyl group. The perfect comple-
mentarity allows it to bind specifically to and inhibit only miR-7. The control miR hairpin inhibitor is designed similarly to
the miR-7 inhibitor, but its sequence is based on two Caenorhabditis elegans miRNA sequences that have been con-
firmed to have minimal sequence identity with miRNAs in rats,
mice and humans (cel-miR-67 and cel-miR-239b: UCACAAC-
CUCCUAGAAAGAGUAGAU and UUGUACUAGACAAA-
GUACUG) (24, 25).

Transfection of Cells—Usp18 siRNAs and control siRNA
were transfected into HeLa, U87, T98, and SCC2 cells using
oligofectamine (Invitrogen, Carlsbad, CA) or Dharmafect 2
lipid reagents as previously described (13, 23, 26). For luciferase
assay of miR-7-reporter and EGFR-3′-UTR-reporter outputs,
HeLa and T98 cells were used. These cells were first transfected
with control-siRNA or Usp18 siRNAs and then with either 1.0
μg of EGFR 3′-UTR-reporter plasmid or miR-7-reporter plasmid
plus 0.05 μg of CMV-β-galactosidase or plus 0.05 μg of
Renilla luciferase reporter using Fugene HD (Roche).

Plasmid Construction—Wild-type Usp18 plasmid used in
this study encoded mouse Usp18 fused to an HA epitope tag
and cloned into a pcDNA3.1 background (Addgene plasmid
12454). The mutant, enzymatically inactive C61S mutant (15)
plasmid was generated from the wild-type construct using site-directed mutagenesis with the forward caacatcggaca-
gagctgttccttaacctcgttc and reverse gaagcaaggtaggaagaa-
gagctgttcctgagttt primers. miR-7-reporter plasmid and EGFR
3′-UTR reporter plasmid were constructed as previously described (20). The native EGFR-3′-UTR contains three sites
for miR-7 with 1–7 and 1–8 seed matches (20). Mutations in these
regions render the 3′-UTR unresponsive to treatment with
miR-7 (20, 27).

Usp18 Rescue of Usp18 siRNA—HeLa cells were transfected
with 1 μg of pcDNA3.1, pHA-Usp18W7, or pHA-Usp18C61S
plasmids using TransIT-LT1 lipid reagent as per manufacturer
protocol (Mirus Bio, Madison, WI). After 24 h they were then
transfected with 30 nm control or Usp18 siRNA using Lipo-
fectamine 2000 (Invitrogen). Total time of exposure to DNA
was 72 h while exposure to siRNA was 55 h. T98 cells were
transfected with control or Usp18 siRNA #5 together with either 0.5 μg of pcDNA3.1, pHA-Usp18W7t, or pHA-
Usp18C61S using Fugene HD (21, 28). Cell extracts were per-
formed as detailed previously (13) and subjected to SDS-PAGE
and immunoblot analysis.

Real-time PCR—The mRNA levels of Usp18 and EGFR were
determined as previously described (13). Briefly, T98, HeLa,
and SCC2 cells were treated with control or Usp18 siRNA
(50–75 nm) using Dharmafect 2 transfection reagent. At 72 h
post-transfection, total RNA was isolated using Qiagen RNeasy
Mini protocol. Samples were analyzed using an Applied Biosys-
tems (Foster City, CA) 7900HT and Taqman Expression Assays
(EGFR: Hs00193306_m1; Usp18: Hs00276441_m1; 18 S ribo-
somal RNA: 4308329). Usp18, luciferase, and EGFR mRNA
qPCR values were normalized to 18 S ribosomal RNA qPCR
values in each sample. For the determination of the levels of
miR-7 and its host genes, cells were transfected with either con-
trol or Usp18 siRNA and lysed using Qiazol and QiAquick
columns (Qiagen). RNA was isolated using the miRNeasy kit
and RT–PCR performed using miScript (Qiagen). cDNA was
generated and quantitative real-time PCR analysis for miR-7
and U6B were performed using miR7 and U6B specific forward
primers and a universal reverse primer (Qiagen). For Usp18,
pituitary gland specific factor 1a (PGSF1a), pri-miR-7–2 and
heterogeneous nuclear ribonucleoprotein K (hnRNPK), spe-
cific primers were used. All samples were analyzed with an
Applied Biosystems (StepOnePlus) real-time PCR system and
normalization to U6B small RNA (28). Data analysis was carried
out using StepOne Software v2.1 (Applied Biosystems).

Primer sequences used are: hnrRNPK-F: 5′-TGCGAGTG-GACGTGTGTAT-3′; hnrRNPK-R: 5′-TTAGCACTTGTAGAGTTG-CCCAATAATT-3′; PGSF1a-F: 5′-CCCAGAGCTCCCTCCACA-
ACT-3′; PGSF1a-R: 5′-TTCCCTGTGATAAATGGACGT-3′; Pri-miR-7–2-F: 5′-CACCAGGAGACATGCTTGGAA-3′;
Pri-miR-7–2-R: 5′-TGTAGTGCACTGGTGCAATC-3′; Luciferase-F: 5′-GGCGGTATTATTTATCGGAGTT-3′; Luci-
ferase-R: 5′-CCATACTGTTGACAAATTCAGTT-3′; 18S-F:
**RESULTS**

Usp18-dependent Regulation of EGFR Protein Occurs through the 3′-UTR of EGFR mRNA—We previously showed that Usp18 siRNA-mediated depletion of Usp18 in SCC2 and COS-1 cells leads to a decrease in EGFR protein synthesis despite eliciting no changes to EGFR mRNA (13). These observations strongly suggest that depletion of Usp18 in these cells leads to suppression of EGFR mRNA translation. To investigate this effect in additional cell types and further study the mechanism we employed several other cancer cell lines with various levels of EGFR expression, including the established glioma line T98 and the human cervical cancer cell line HeLa (3–5). Transfection of T98 and HeLa cells with two different siRNA oligonucleotides effectively decreased Usp18 mRNA (supplemental Fig. S1, A, B, and D), as previously observed in SCC2 cells (13). Furthermore, we confirmed that Usp18 siRNA treatment of T98 cells led to depletion of the Usp18 protein (Fig. 1A and supplemental Fig. S1C). We then compared EGFR protein levels in T98 and HeLa cells depleted of Usp18 using the above tested siRNAs. Immunoblot analysis demonstrated a dramatic decrease in EGFR protein levels in Usp18 siRNA-transfected T98 (Fig. 1, A, D, and E) and HeLa (Fig. 1, B and C) cells as compared to control siRNA-transfected cells.

The decrease in EGFR protein levels as a result of Usp18 siRNA treatment of Hela and T98 cells could be rescued with the simultaneous transfection of a wild-type mouse Usp18 construct that is insensitive to siRNA targeting human Usp18 (Fig. 1, B–E). Such an observation confirms the specificity of the siRNA effects. To test whether the catalytic activity of Usp18 is necessary for this rescue effect, enzymatically inactive mouse Usp18 was co-transfected with Usp18 siRNA. As shown in Fig. 1, B–E this Usp18 mutant did not restore EGFR levels in either Hela or T98 cells. These data suggest that the effect of Usp18 on EGFR protein expression is dependent on the ability of Usp18 to remove ISG15 or ubiquitin from substrates.

Despite EGFR protein levels decreasing following Usp18 depletion there was little to no change in EGFR mRNA levels (supplemental Fig. S1, A and B). This finding in T98 and Hela cells is consistent between two different Usp18 siRNA oligonucleotides and with observations made previously in SCC2 and COS-1 cells (13). This strongly suggests that Usp18 regulates EGFR protein levels by controlling the process of EGFR mRNA translation. Interestingly, Usp18 siRNA #5 elicited a 25% decrease in EGFR mRNA, but only in HeLa cells (supplemental Fig. S1B). This oligo did not elicit the same phenotype in T98 (supplemental Fig. S1A) and SCC2 (13) cell lines used in this study nor in U87 and COS-1 cell lines (data not shown). Therefore, an additional mechanism to down-regulate EGFR translation may occur in HeLa cells that involves mRNA cleavage (20, 27).

To test the prediction that the regulation of EGFR translation occurs through the EGFR 3′-UTR, we measured the luciferase output of a pGL3-luciferase vector bearing the EGFR 3′-UTR (20). T98 and HeLa cells transfected with Usp18 siRNA exhibited a decreased luciferase output as compared with control siRNA (Fig. 1, F and G). Measurements of luciferase mRNA levels revealed no changes when we compared Usp18 siRNA to
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control siRNA-transfected cells (supplemental Fig. S1D). These data confirm that EGFR down-regulation by Usp18 depletion occurs through the EGFR 3′-UTR.

Usp18 Depletion Leads to miR-7 Up-regulation—miRNAs have been shown to regulate protein levels by binding to the 3′-UTR regions of their mRNA targets (18). Based on the above observation and our previous data showing that forced expression of miR-7 in cells decreased EGFR levels (20), we hypothesized that the effects of Usp18 depletion occurred through the up-regulation of miR-7. Quantitative RT-PCR analysis of miR-7 levels in T98, HeLa, and SCC2 cells transfected with Usp18 siRNA revealed a 1.5–2-fold increase when compared with control siRNA (p < 0.05 and p < 0.01) (Fig. 2A and supplemental Fig. S2A). Such increases are within the range of miR
changes that are considered to be capable of eliciting potent effects on mRNA translation (31).

miR-7 activity was also analyzed using a miR-7 luciferase reporter to assess whether the changes in miR-7 levels correlated with changes in miR-7 activity (24, 32). Treatment of T98 and HeLa cells with Usp18 siRNA resulted in significantly reduced miR-7 reporter luciferase output (Fig. 2B). This reduced luciferase output was completely reversed in the presence of a miR-7 specific inhibitor in both T98 and HeLa cells (Fig. 2B). Also, the miR-7 inhibitor reduced the basal activity of miR-7 (at 72 h \( p < 0.05 \)), as indicated by an increased reporter output (supplemental Fig. S2B). Thus, HeLa and T98 cells express biologically active miR-7 under steady state conditions, and Usp18 depletion promotes an increase in both miR-7 expression and activity.

To further assess the involvement of Usp18 in the regulation of miR-7 expression, we compared the steady state levels of Usp18 and miR-7 in the cell lines used in this study. Our observations reveal that T98 and HeLa cells normally express high levels of Usp18 mRNA and corresponding low levels of miR-7 (Fig. 2C). In contrast, SCC2 cells express low levels of Usp18 mRNA and high levels of miR-7 (Fig. 2C). Thus, under steady state conditions in these cell lines lower Usp18 mRNA levels correlate with higher miR-7 levels, and vice versa. We did not observe this same correlation between Usp18 and EGFR mRNA (data not shown). However, this is consistent with the model that changes in Usp18 are affecting translation from EGFR mRNA and not changes in EGFR mRNA levels themselves. While analysis of a larger set of cell lines is necessary to definitively establish correlation between steady-state levels of Usp18 and miR-7, the observations made in these three cancer cell lines are consistent with the data above showing that depletion of Usp18 leads to increased levels of miR-7. Together the data strongly suggest that endogenous Usp18 is an important regulator of miR-7 expression and activity.

miR-7 Mediates the Effects of Usp18 Depletion on EGFR Expression—Based on our previous work (13, 20) and data above (Fig. 2 and supplemental Fig. S2A), we hypothesized that the reduced levels of EGFR protein observed in cells lacking Usp18 is due to increased miR-7 expression (Figs. 1, 2 and supplemental Fig. S2A). We tested this hypothesis using miR-7-specific inhibitors and analyzing EGFR expression in T98, HeLa, and SCC2 cells. Transfection of a miR-7-specific inhibitor in T98, HeLa, or SCC2 cells slightly increased the basal levels of the EGFR protein (Fig. 3, A and B). Importantly, a specific miR-7 inhibitor was able to completely rescue the decrease in EGFR protein expression associated with Usp18 depletion (Fig. 3, A and B).

To further confirm the increased miR-7 activity we analyzed the protein levels of other known targets of miR-7 such as IRS-1 and c-Raf-1 ((20, 27) and supplemental Fig. S3, A and B). Following treatment with Usp18 siRNA we observed a significant decrease in c-Raf-1 protein levels in T98 (supplemental Fig. S3C), and a significant decrease in IRS-1 protein levels in SCC2...
and HeLa cells (supplemental Fig. S3, D–F). Additionally, the decrease in IRS-1 was rescued with addition of the miR-7-specific inhibitor (supplemental Fig. S3, E and F). Thus, Usp18 controls the protein levels of EGFR, Raf-1, and IRS-1 in a miR-7-dependent manner.

Knowing that the EGFR 3′/5′UTR is necessary for Usp18-dependent regulation of EGFR (Fig. 1, F and G and Ref. 13) and that this regulation is also dependent on activation of miR-7 (Fig. 2 and supplemental Fig. S2A), we assessed the necessity of the EGFR 3′-UTR in the ability of the inhibitor to rescue Usp18 depletion phenotypes. We found that the Usp18 siRNA-dependent decrease in EGFR 3′-UTR-luciferase output was completely abrogated by miR-7 inhibitor in both T98 and HeLa cells (Fig. 3C). These data implicate miR-7 and the EGFR 3′-UTR as mediators of the Usp18-dependent regulation of EGFR protein expression.

Usp18 Regulation of miR-7 Likely Occurs at the Transcriptional Level—The three primary transcripts of mature miR-7 in the human genome originate from an intergenic region and the intronic regions of two different genes. In general, parallel transcription of host genes and miR-7 expression is likely to occur and thus similar expression patterns would be expected. Such a phenomenon has been observed for several other microRNAs and their host genes (33–35). Our studies here compared the mRNA levels of miR-7 host genes, hnRNPK and PGSF1a, as well as the abundance of intergenic pri-miR-7-2 (a long non-protein coding transcript). Quantitative RT-PCR analysis of T98, HeLa, and SCC2 cells depleted of Usp18 revealed significant increases in hnRNPK, PGSF1a, and pri-miR-7–2 mRNA levels relative to control siRNA cells (Fig. 4, A–C). For hnRNPK and pri-miR-7-2, the fold inductions were more pronounced in HeLa and T98 compared with SCC2 cells (Fig. 4A and C); for PGSF1a the increase was greater in SCC2 and HeLa when compared with T98 cells (Fig. 4B). This variation suggests that Usp18 depletion increases the expression of miR-7 from all three loci and possibly through different mechanisms in the different cell lines.

Usp18 Depletion Significantly Reduces Cellular Proliferation—As seen above, Usp18 depletion activates miR-7 in a number of different tumor cell lines (Figs. 2 and 3 and supplemental Fig. S2A). Previous studies from our laboratory showed that forced expression of miR-7 reduced the proliferation and viability of established glioblastoma cell lines including T98 cells (20). Therefore, we hypothesized that Usp18 depletion would pro-
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Usp18 Depletion Induces Apoptosis of Cancer Cells through miR-7

FIGURE 4. Usp18 regulates miR-7 expression at the transcriptional level and from multiple genomic sources. Plots of mRNA expression of (A) hnrNPK and (B) PGSF-1a (which encode miR-7-1 and -3 transcripts, respectively) and (C) pri-miR-7-2 (which originates from an intergenic source) in T98, Hela, and SCC2 cells transfected with either control or Usp18 siRNA (#6) for 48 h, measured by quantitative RT-PCR and normalized with U6B small RNA (**, \( p < 0.01 \) and *, \( p < 0.05 \)).

miR-7 activity. Additionally, tumor cells depleted of Usp18 undergo apoptosis as a direct result of miR-7 activation and also exhibit a decreased ability to form colonies in soft agar, suggesting that Usp18 may have oncogenic properties. This is the first study to date demonstrating a role for Usp18 in the regulation of microRNAs and cancer cell apoptosis. Such observations not only provide additional insight into the complex mechanisms of EGFR and miR-7 regulation, but also suggest that Usp18 inhibition has potential for cancer therapeutics.

Determination that enzymatically inactive Usp18 could not rescue the EGFR down-regulation phenotype associated with Usp18 knockdown strongly suggests that the activity of Usp18 is critical to this function. In contrast, studies linking Usp18 to interferon signaling and viral response have shown that the activity of the enzyme is irrelevant (37). Thus, Usp18 may have varied roles in distinct cellular processes but it appears that the inhibition of its activity will affect only some of these processes, such as EGFR expression. Such a point is critical when evaluating any enzyme for therapeutic inhibition.

Usp18 is generally considered an enzyme which removes ubiquitin-like ISG15 from substrates (15, 38), although it has also been shown to remove ubiquitin from substrates in vitro (14, 39). Currently, the association between cancer and ISG15 is still quite uncertain. Some studies have observed altered levels of ISG15 in cancer cells (40–42). However, it remains unclear as to whether increased ISG15 levels are a cause or effect of tumorigenesis. Clearly, future work investigating substrates of Usp18 and the ISG15 conjugation system will be helpful to the field of cancer.

We also show in this study that Usp18 depletion elicits a dramatic increase in miR-7 levels, which in turn affects cell viability and tumorigenicity. Earlier studies also provided in vivo and in vitro evidence for the roles of Usp18 in cellular death. These studies showed that mice lacking Usp18 suffered brain injury (44), and Usp18 knockdown sensitized cells to bortezomib, interferon \( \gamma \), and TRAIL-induced apoptosis (43). Similarly, overexpression of miR-7 has previously been shown to reduce cellular invasiveness and the viability of cancer cells, implying reduced tumorigenicity in response to increased annexin V staining (Fig. 5C) while simultaneous inhibition of miR-7 almost completely negated this increase in apoptosis. These observations demonstrate that Usp18 depletion induces apoptosis in T98 and HeLa cells in a miR-7-dependent manner.

Effects of Usp18 Depletion on Cell Growth in Soft Agar—Because Usp18 depletion increases apoptosis and decreases the number of T98 and HeLa cells, we tested whether it also suppresses the tumorigenic activity of these cell lines. To investigate this possibility we quantified the ability of these cell lines to form colonies in soft agar, a well-established in vitro tumorigenicity assay. Usp18 knockdown significantly reduced the number of colonies formed by both HeLa and T98 cells (Fig. 6). These data suggest that Usp18 may function as an oncogene and its inhibition may suppress proliferative and metastatic properties of tumor cells.

DISCUSSION

Usp18 and the ISG15 conjugation system will be helpful to the field of cancer. Clearly, future work investigating substrates of Usp18 and the ISG15 conjugation system will be helpful to the field of cancer.
miR-7 (20). Additionally, increased miR-7 expression was also shown to reduce the levels of pro-survival proteins such as IRS-1, IGF-1R, PAK1, and Raf-1 (20, 27, 45, 46). This suggests that Usp18 and miR-7 share similar pro-survival targets and thus, demonstrates that a decrease in Usp18 leads to increased miR-7, and in turn to reduced cell viability and tumorigenicity.

It was previously reported that increases in the levels of specific microRNAs may arise through increased transcriptional activation, mRNA stabilization of host genes, and/or increased processing of primary microRNA transcripts (46–48). The observations made in this study strongly suggest that the Usp18 knockdown-dependent induction of miR-7 levels occurs through transcriptional activation and/or mRNA stabilization of miR-7 host genes. We observed that knockdown of Usp18 results in a significant increase in the expression of miR-7 host genes hnRNPK and PGSF1a, as well as the intergenic pri-miR-7-2. The fold increase in miR-7 host genes and intergenic pri-miR-7-2 was different for the different cancer cells. This suggests that the degree to which each genomic source contributes to mature miR-7 differs in each cell line. This intriguing observation suggests that the regulation of transcription and/or mRNA stabilization of miR-7 host genes and the subsequent increase in miR-7 by Usp18 depletion is a process that may involve several mechanisms. One possible mechanism is based on a recent study which suggested that the level of mature miR-7 is regulated at the transcriptional level by the transcription factor HoxD10 (46). However, we did not find changes in the protein levels of HoxD10 following Usp18 depletion.
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This study has also shown that depletion of Usp18, and subsequent miR-7 up-regulation, leads to reduced cellular proliferation in two different cancer cell lines. It is important to point out that Usp18 and/or miR-7 are likely inhibiting multiple cellular processes and, in particular, the activity of multiple cell transformation proteins. For example, our studies and previous studies have shown that miR-7 has the ability to inhibit several pro-growth signaling proteins including EGFR, IRS-1, IGF-1R, PAK1, and Raf-1 (20, 27, 45, 46). Furthermore, Usp18, and its substrate ISG15, have also been shown to control the stability of pro-growth proteins (49, 50). Thus, it is likely that Usp18 activity is regulating cellular growth via a mechanism(s) that is additional to the Usp18/miR-7 pathway regulation of growth promoting proteins. This would explain the observation that miR-7 inhibition completely rescues Usp18 depletion-induced apoptosis but can only partially rescue Usp18 depletion-induced decreases in cell number.

In summary, the data presented here show that Usp18 is a potent regulator of miR-7 levels and activity. Depletion of Usp18 in numerous cancer cell lines leads to a dramatic increase in miR-7 activity, which in turn reduces the levels of EGFR and other oncogenic proteins. These changes ultimately lead to increased apoptosis and reduced cell numbers. Usp18 has now been identified, and miR-7 confirmed, as critical elements responsible for controlling cell growth and tumorigenicity of cancer cells.

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REFERENCES

1. Lemmon, M. A., and Schlessinger, J. Cell 141, 1117–1134
2. Wells, A. (1999) Int. J. Biochem. Cell Biol. 31, 637–643
3. Huang, S., Armstrong, E. A., Benavente, S., Chinnaiyan, P., and Harari, P. M. (2004) Cancer Res. 64, 5355–5362
4. Shen, L., Shui, Y., Wang, X., Sheng, L., Yang, Z., Xue, D., and Wei, Q. (2008) BMC. Cancer 8, 232
5. Ziegler, D. S., Wright, R. D., Kesari, S., Lemieux, M. E., Tran, M. A., Jain, M., Zawel, L., and Kung, A. L. (2008) J. Clin. Invest. 118, 3109–3122
6. Broniscer, A., and Gajjar, A. (2004) Oncologist 9, 197–206
7. Kobayashi, S., Boggon, T. J., Dayaram, T., Jänne, P. A., Kocher, O., Moyer, M., Johnson, B. E., Eck, M. J., Tenen, D. G., and Halmos, B. (2005) N. Engl. J. Med. 352, 786–792
8. Kruser, T. J., and Wheeler, D. L. (2010) Exp. Cell Res. 316, 1083–1100
9. Pao, W., Miller, V. A., Politi, K. A., Riely, G. J., Somwar, R., Zakowski, M. F., Kris, M. G., and Varmus, H. (2005) PLoS. Med. 2, e73
10. Bowers, K., Piper, S. C., Edeling, M. A., Gray, S. R., Owen, D. J., Lehner, P. J., and Luzio, J. P. (2006) J. Biol. Chem. 281, 5094–5105
11. McCullough, J., Clague, M. J., and Urban, S. (2004) J. Cell Biol. 166, 487–492
12. Mizuno, E., Iura, T., Mukai, A., Yoshimori, T., Kitamura, N., and Komada, M. (2005) Mol. Biol. Cell. 16, 5163–5174
13. Duez, J. E., and Sorkin, A. (2009) Mol. Biol. Cell. 20, 1833–1844
14. Liu, L. Q., Ilaria, R., Jr., Kingsley, P. D., Iwama, A., van Etten, R. A., Palis, J., and Zhang, D. E. (1999) Mol. Cell. Biol. 19, 3029–3038
15. Malakhov, M. P., Malakhova, O. A., Kim, K. I., Ritchie, K. J., and Zhang, D. E. (2002) J. Biol. Chem. 277, 9976–9981
16. Ambros, V. (2004) Nature 431, 350–355
17. Bartel, D. P. (2004) Cell 116, 281–297
18. Murchison, E. P., and Hannon, G. J. (2004) Curr. Opin. Cell Biol. 16, 223–229
19. Weiss, G. J., Bemis, L. T., Nakajima, E., Sugita, M., Birks, D. K., Robinson, W. A., Varella-Garcia, M., Bunn, P. A., Jr., Haney, J., Helfrich, B. A., Kato,
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H., Hirsch, F. R., and Franklin, W. A. (2008) Ann. Oncol. 19, 1053–1059
20. Kefas, B., Godlewski, J., Comeau, L., Li, Y., Abounader, R., Hawkinsion, M., Lee, J., Fine, H., Chiocca, E. A., Lawler, S., and Purow, B. (2008) Cancer Res. 68, 3566–3572
21. Kefas, B., Comeau, L., Floyd, D. H., Seleverstov, O., Godlewski, J., Schmit- tgen, T., Jiang, I., diPierro, C. G., Li, Y., Chiocca, E. A., Lee, J., Fine, H., Abounader, R., Lawler, S., and Purow, B. (2009) J. Neurosci. 29, 15161–15168
22. Kim, V. N. (2005) Nat. Rev. Mol. Cell Biol. 6, 376–385
23. Purow, B. W., Haque, R. M., Noel, M. W., Su, Q., Burdick, M. J., Lee, J., Sundaresan, T., Pastorino, S., Park, J. K., Mikolaenko, I., Maric, D., Eberhart, C. G., and Fine, H. A. (2005) Cancer Res. 65, 2353–2363
24. Meister, G., Landthaler, M., Dorsett, Y., and Tuschl, T. (2004) RNA 10, 544–550
25. Vermeulen, A., Robertson, B., Dalby, A. B., Marshall, W. S., Karpilow, J., Leake, D., Khvorova, A., and Baskerville, S. (2007) RNA 13, 723–730
26. Duex, J. E., Mullins, M. R., and Sorkin, A. (2010) Exp. Cell Res. 316, 2136–2151
27. Webster, R. J., Giles, K. M., Price, K. J., Zhang, P. M., Mattick, J. S., and Leedman, P. J. (2009) J. Biol. Chem. 284, 5731–5741
28. Kefas, B., Comeau, L., Erdle, N., Montgomery, E., Amos, S., and Purow, B. (2010) Neuro. Oncol. 12, 1102–1112
29. Kefas, B. A., Heimberg, H., H., Hogrefe, C., Erb, S., Bobach, C., Faubas, S., Wess, L., and Seiler, B. (2009) Oncogene 28, 2606–2620
30. Potter, H., Sgorbissa, A., and Brancolini, C. (2010) Cancer Res. 70, 655–665
31. Ritchie, K. J., Malakhov, M. P., Hetherington, C. J., Zhou, L., Little, M. T., Malakhova, O. A., Sipe, J. C., Orkin, S. H., and Zhang, D. E. (2002) Genes Dev. 16, 2207–2212
32. Jiang, L., Liu, X., Chen, Z., Jin, Y., Heidbreder, C. E., Kolokythas, A., Wang, A., Dai, Y., and Zhou, X. (2010) Biochem. J. 432, 199–205
33. Reddy, S. D., Ohshiro, K., Rayala, S. K., and Kumar, R. (2008) Cancer Res. 68, 8195–8200
34. Chatterjee, S., and Grosshans, H. (2009) Nature 461, 546–549
35. Viswanathan, S. R., Bale, D. G. Q., and Gregory, R. I. (2008) Science 320, 97–100
36. Feng, Q., Sekula, D., Guo, Y., Liu, X., Black, C. C., Galimberti, F., Shah, S. J., Sempere, L. F., Memoli, V., Andersen, J. B., Hassel, B. A., Dragnev, K., and Dmitrovsky, E. (2008) Mol. Cancer Ther. 7, 3780–3788
37. Malakhova, O. A., Yan, M., Malakhov, M. P., Yuan, Y., Ritchie, K. J., Kim, K. L., Peterson, L. F., Shuai, K., and Zhang, D. E. (2003) Genes Dev. 17, 455–460

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