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ORIGINAL ARTICLE

Oncofetal protein IGF2BP3 facilitates the activity of proto-oncogene protein eIF4E through the destabilization of EIF4E-BP2 mRNA

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RNA-binding proteins (RBPs) have important roles in tumorigenesis. Although IGF2BP3, an evolutionally conserved RBP, has been reported as a useful diagnostic marker for various cancers and has been considered a regulator of tumorigenesis, little is known of the function of IGF2BP3 because of lack of information regarding IGF2BP3 target mRNAs. Here, we report the identification of IGF2BP3 target mRNAs and IGF2BP3 function in cancer proliferation. We identified mRNAs with altered expression in IGF2BP3-depleted cells by massive sequencing analysis and IGF2BP3-binding RNAs by immunoprecipitation of IGF2BP3 followed by massive sequencing analysis, resulting in the identification of 110 candidates that are negatively regulated by IGF2BP3. We found that IGF2BP3 destabilized EIF4E-BP2 and MEIS3 mRNAs. Co-immunoprecipitation analysis revealed the interaction between IGF2BP3 and ribonucleases such as XRN2 and exosome component. The retarded proliferation of IGF2BP3-depleted cells was partially rescued by the depletion of EIF4E-BP2, which negatively regulates eukaryotic translation initiation factor 4E (eIF4E), an activator of translation and a well-known proto-oncogene. Consistent with this observation, IGF2BP3 depletion reduced phosphorylated eIF4E, the active form, and translational efficiency of eIF4E target transcripts. Reduction of phosphorylated eIF4E by IGF2BP3 depletion was rescued by EIF4E-BP2 depletion. At last, we found an inverse correlation between the expression level of *IGF2BP3* and *EIF4E-BP2* in human lung adenocarcinoma tissues. Together, these results suggest that IGF2BP3 promotes eIF4E-mediated translational activation through the reduction of EIF4E-BP2 via mRNA degradation, leading to enhanced cell proliferation. This is the first report demonstrating that IGF2BP3 is an RNA-destabilizing factor. Notably, here we provide the first evidence for the functional linkage between two previously well-known cancer biomarkers, IGF2BP3 and eIF4E.

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INTRODUCTION

Gene expression is a complicated process controlled by diverse biochemical reactions mediated by the cooperative function of various proteins. Among these factors, RNA-binding proteins (RBPs) bind to specific motifs in RNA sequences or specific RNA structures and have a central role in diverse processes, such as transcription, RNA processing, localization, translation and degradation.[1] Eukaryotic cells encode ~500 RBPs, which typically contain RNA-binding domains such as the RNA recognition motif and the K homology domain, with unique RNA-binding specificity and specific protein–protein interaction activity.[2] Because RBPs have important roles in various biological processes, dysfunction of RBPs causes diseases, such as neural diseases, metabolic disease and cancers.[3,4] For instance, upregulation of eukaryotic translation initiation factor 4E (eIF4E), which promotes translation by binding to mRNAs through the recognition of the cap structure of mRNAs under the regulation of the phosphatidylinositol 3′-kinase-Akt-mammalian target of rapamycin (PI3K-AKT-mTOR) pathway, facilitates tumorigenesis.[3,5] Therefore, knowledge of RBP functions is critical for understanding RNA-mediated biological networks and numerous diseases including cancer.

To elucidate the biological functions of RBPs, the identification of the target RNAs regulated by RBPs is crucial.[6] Various *in vivo* and *in vitro* methods have been developed to identify the RNAs that bind to RBPs. In particular, reliable and robust high-throughput methods with massive RNA-sequencing analysis (RNA-seq) to detect protein–RNA interactions have been recently developed.[7] For instance, ribonucleoprotein complex immunoprecipitation followed by massive RNA sequencing (RIP-seq) has been developed to capture genome-wide RNA transcripts that physically interact with certain proteins or protein complexes.[8] RIP-seq has successfully identified RNAs that associate with various RBPs involved in tumorigenesis, such as Polycomb repressive complex 2, TDP-43, HuR and Argonaute proteins.[8–11]

Insulin-like growth factor II messenger RBPs (IGF2BPs), also known as IMPs, are evolutionally conserved RBPs in metazoan genomes. The human genome encodes three IGF2BPs, IGF2BP1, -2 and -3, and these proteins contain evolutionally conserved RNA recognition motif 1 domains and K homology domains.[12] IGF2BP1 is the best studied IGF2BP. IGF2BP1 acts in various important biological pathways, such as RNA localization, translation and RNA stability and is involved in various important cellular functions, such as cell polarization, migration, proliferation and differentiation. IGF2BP1 facilitates the localization of beta-actin mRNA to lamellae of embryonic fibroblasts,[13] and enhances the production of IGF-2 and beta-actin through

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translational activation.[14,15] IGF2BP1 also stabilizes several mRNAs such as c-myc mRNA.[16]

IGF2BP3 was first identified in pancreatic carcinoma in a large-scale screening for differentially expressed genes in pancreatic cancer.[17] Because IGF2BP3 and IGF2BP1 show a high identity (73%) with each other, it has been speculated that these proteins share similar biological functions.[12] However, IGF2BP3 function has not been well examined. IGF2BP3 has been proposed as a clinically meaningful oncofetal protein because it is expressed in a variety of cancers, such as cervical cancer and lung adenocarcinoma.[12,18,19] Cancers expressing high levels of IGF2BP3 show poor prognosis with metastasis.[20] Hence, IGF2BP3 has been widely used as a clinically important biomarker to determine cancer stage and tumor aggressiveness in many organ systems. This clinically useful cancer-associated property is an attractive characteristic of IGF2BP3 among IGF2BPs.

Transgenic mice highly expressing IGF2BP3 showed metaplasia of pancreatic acinar cells with enhanced cell proliferation, supporting a causal role of IGF2BP3 in transformation.[21] Previous reports suggested that IGF2BP3 functions to increase tumor cell proliferation by upregulation of IGF-II through the stabilization of IGF-II mRNA. However, it has conversely been suggested that the functionality of IGF2BP3 in tumorigenesis is not mediated by IGF-II function.[22] Thus, although it has been demonstrated that IGF2BP3 has an important role in cancer proliferation, the exact mechanism by which IGF2BP3 promotes cancer proliferation has not been completely elucidated.

To elucidate the mechanism by which IGF2BP3 promotes tumorigenesis, here we used high-throughput approaches with RNA-seq analysis to identify the target transcripts of IGF2BP3 and examine the function of IGF2BP3.

RESULTS

Identification of mRNAs regulated by IGF2BP3

We first identified the RNAs associated with IGF2BP3 by RIP-seq analysis. Cell lysates prepared from HeLa TO cells that transiently express FLAG-tagged IGF2BP3 (FLAG-IGF2BP3) approximately threefold higher than endogenous IGF2BP3 (Supplementary Figure S1a) were subjected to immunoprecipitation using anti-FLAG antibody. RNA was isolated from immunoprecipitants, followed by massive sequencing analysis. By calculating fold enrichment of immunoprecipitated RNAs compared with input RNAs, we identified 2201 RNAs with over twofold enrichment in IGF2BP3 immunoprecipitations as IGF2BP3-associated RNAs (white circles in Figure 1a). We confirmed the interaction between endogenous IGF2BP3 and IGF2BP3-associated RNA, which was identified by using RIP-seq (Supplementary Figure S1b).

Next we prepared IGF2BP3-depleted HeLa TO cells using two different small interfering RNA (siRNAs) (siIGF2BP3_1 or _2) (Figure 1b). We performed gene expression profiling analysis using RNA-seq of total RNAs isolated from control and IGF2BP3 knockdown cells. We identified RNAs whose expression levels were increased or decreased >1.5-fold in IGF2BP3-depleted cells compared with control cells, and identified 128 decreased and 419 increased transcripts in IGF2BP3 knockdown cells (black circles in Figure 1a). By integrating RIP-seq data and RNA-seq data,

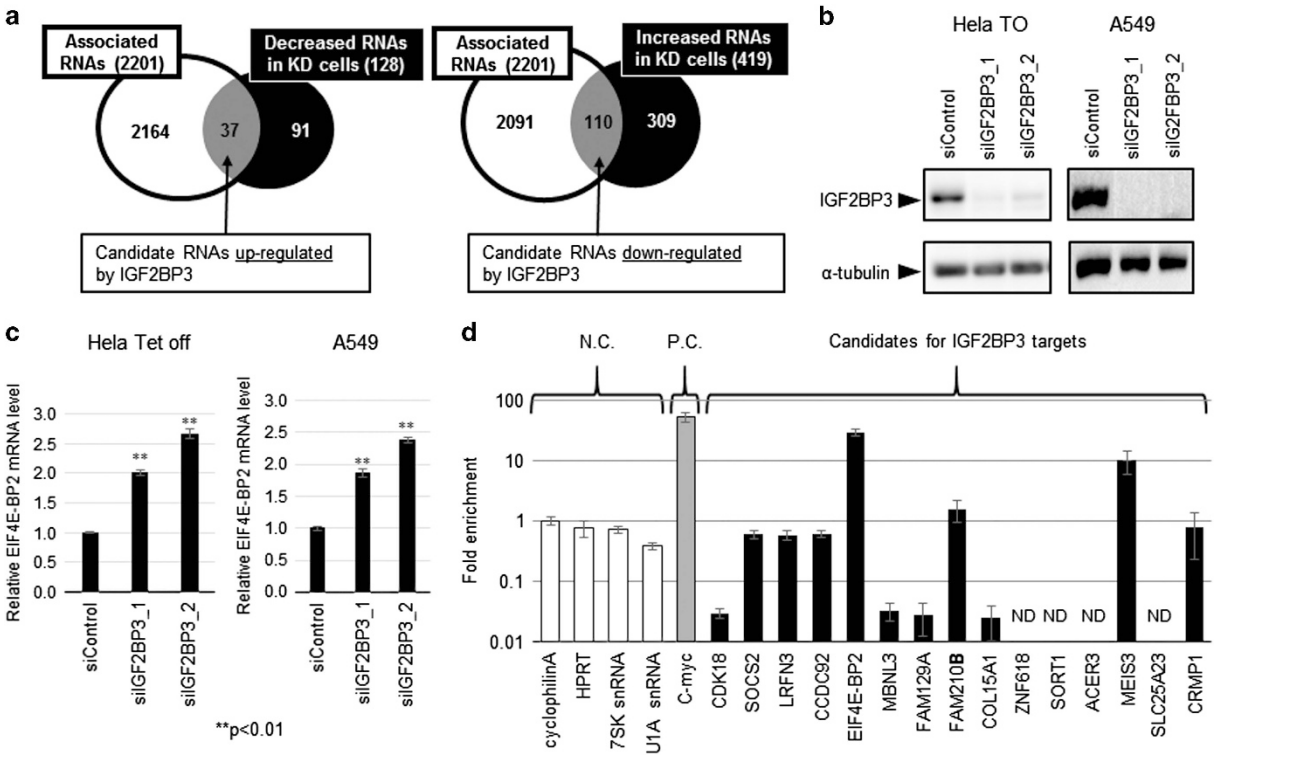

Figure 1. Determination of IGF2BP3 target mRNAs. (**a**) IGF2BP3 target mRNAs were determined by genome-wide analyses. IGF2BP3-associated mRNAs were identified by RIP-seq (white circle). Decreased mRNAs (black circle, left) and increased mRNAs (black circle, right) in IGF2BP3 knockdown cells were identified by RNA-seq. (**b**) Western blot analysis of IGF2BP3 in HeLa TO cells and A549 cells transfected with IGF2BP3 siRNAs. Alpha-tubulin was used as a loading control. (**c**) Increased expression of EIF4E-BP2, one of the IGF2BP3 target mRNAs, in IGF2BP3 knockdown cells was determined by RT-qPCR. *P*-values were obtained by Student's *t*-test ($n=4$). (**d**) Determination of direct binding mRNAs to IGF2BP3 *in vivo*. HeLa TO cells transfected either with pcDNA3-FLAG-IGF2BP3 or control pcDNA3-FLAG vector were irradiated with 360 nm UV light to induce cross-linking between FLAG-IGF2BP3 and its binding mRNAs for *in vivo* cross-linking analysis. Cell lysates were prepared under strong denaturing conditions, followed by immunoprecipitation with anti-FLAG antibody. RT-qPCR was performed to determine fold-enrichment in *in vivo* cross-linking analysis. Negative controls (NC) and positive controls (PC) indicate previously reported mRNAs that do not bind or bind to IGF2BP3, respectively.

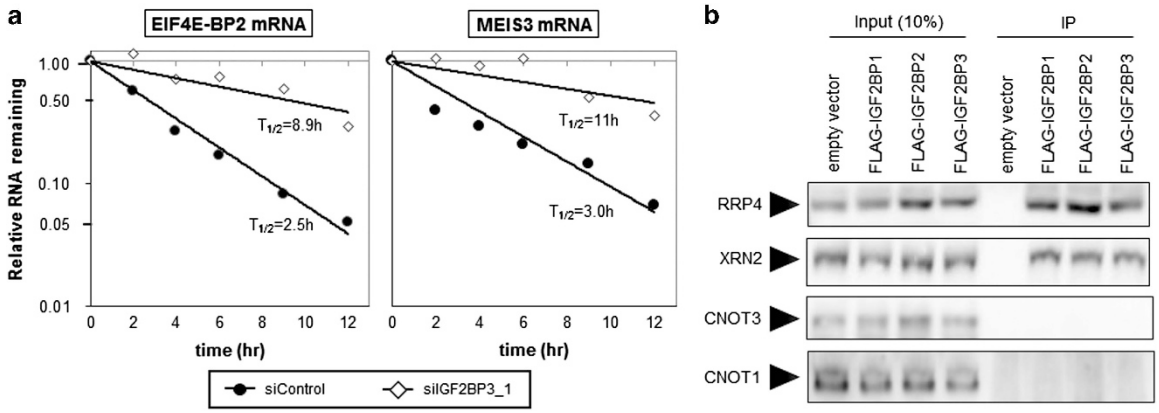

Figure 2. Determination of RNA stabilities of IGF2BP3 targets. (**a**) HeLa TO cells were treated with control or IGF2BP3 siRNA, and stabilities of the IGF2BP3 target mRNAs (*EIF4E-BP2* and *MEIS3*) were determined using BRIC. BrU-labeled total RNAs were collected at 0, 2, 4 and 6 h after BrU-pulse labeling. RT-qPCR was performed to determine the relative RNA remaining at each time point compared with time 0 h in control cells (black circles) and in IGF2BP3-depleted cells (open squares). The half-life of each RNA was calculated by drawing the best-fitted curve based on the least-squares analysis. (**b**) Co-immunoprecipitation (IP) of FLAG-tagged IGF2BP3 and endogenous ribonucleases in HeLa TO cells. FLAG-tagged IGF2BP3 was immunoprecipitated with anti-FLAG antibody, and western blot was performed using indicated antibodies.

we identified 37 transcripts upregulated by IGF2BP3; these transcripts were associated with IGF2BP3 and were decreased in IGF2BP3 knockdown cells (gray color intersection of left Venn diagram in Figure 1a). We also identified 110 transcripts downregulated by IGF2BP3; these transcripts associated with IGF2BP3 and were increased in IGF2BP3 knockdown cells (gray color intersection of right Venn diagram in Figure 1a). Data of massive sequencing analysis were confirmed by reverse transcriptase (RT)-qPCR (Figure 1c, Supplementary Figure S1c). We also confirmed IGF2BP3-depletion-mediated altered expression level of RNAs in A549 cells, a human lung adenocarcinoma cell line (Figure 1c, Supplementary Figure S1d).

We further investigated whether IGF2BP3-associated transcripts directly interact with IGF2BP3 by cross-linking and immunoprecipitation analysis that enables confirmation of direct protein–RNA interaction based on UV-mediated cross-linking reactions between RNA and protein. We determined the fold-enrichments of representative IGF2BP3 targets, and selected the top 15 transcripts that were highly upregulated mRNAs in IGF2BP3 knockdown cells and were immunoprecipitated with FLAG-IGF2BP3. Among these, EIF4E-BP2 and MEIS3 mRNAs were highly enriched as much as c-myc (a positive control for binding to IGF2BP3), supporting the notion that these RNAs directly bind to IGF2BP3 *in vivo* (Figure 1d).

IGF2BP3 regulation of RNA degradation

Previous studies showed that IGF2BP3 stabilizes CD44 mRNA and HMGA2 mRNA,[23,24] and thus IGF2BP3 has been considered as a RNA stabilizing factor. We initially expected that there would be more target RNAs upregulated by IGF2BP3 than those downregulated by IGF2BP3. However, our genome-wide integrated analysis showed more target RNAs downregulated by IGF2BP3 than those upregulated by IGF2BP3 (Figure 1a). This result suggests a novel function for IGF2BP3 as a RNA-destabilizing factor. We then tested the hypothesis that IGF2BP3 is involved in RNA degradation.

We monitored the stability of the RNA targets of IGF2BP3 in IGF2BP3-depleted cells using the 5′-bromo-uridine (BrU) immunoprecipitation chase (BRIC) method,[25] in which the nascent RNAs are labeled with BrU during transcription, followed by isolation of total RNAs containing BrU-labeled RNAs in a time-lapse manner. BrU-labeled RNAs are then isolated by a specific antibody and subjected to RT-qPCR analysis to determine the half-life of the specific transcript. We determined the half-lives of transcripts regulated by IGF2BP3 and found that IGF2BP3 depletion stabilized EIF4E-BP2 mRNA and MEIS3 mRNA (Figure 2a). These data indicate that IGF2BP3 destabilizes these RNAs.

The results presented above indicate that IGF2BP3 appears to have a role in RNA degradation. However, prediction analysis of the amino-acid sequence of IGF2BP3 did not reveal any ribonuclease domain, suggesting that IGF2BP3 may not have ribonuclease activity. Thus, we hypothesized that IGF2BP3 destabilizes target RNAs through its interaction with ribonucleases. To test this idea, FLAG-tagged IGF2BP3 was immunoprecipitated with anti-FLAG antibody, and western blotting was performed with several anti-ribonuclease antibodies. We detected the co-immunoprecipitation of RRP4 (one component of the 3′–5′ exonuclease complex) and XRN2 (5′–3′ exonuclease) in HeLa TO cells (Figure 2b) and A549 cells (Supplementary Figure S2a). In contrast, co-immunoprecipitation between IGF2BP3 and components of the CCR4-NOT deadenylase complex was not detected. We confirmed interaction between endogenous IGF2BP3 and endogenous nucleases, RRP4 and XRN2 by co-immunoprecipitation assay (Supplementary Figure S2b). We also determined EIF4E-BP2 mRNA level upon depletion of ribonucleases to reveal the contribution of XRN2 and RRP4 in degradation of EIF4E-BP2 mRNA. Double knockdown of XRN2 and RRP4 significantly increased EIF4E-BP2 mRNA. By contrast, single knockdown of either XRN2 or RRP4 slightly affected the expression level of EIF4E-BP2 mRNA (Supplementary Figure S2c). Furthermore, we observed that no additional increment of EIF4E-BP2 mRNA level by simultaneous depletion of RRP4 or XRN2 in addition to IGF2BP3 (Supplementary Figure S2d), indicating that XRN2 and exosome degrade EIF4E-BP2 mRNA through the function of IGF2BP3. These results suggest that IGF2BP3 recruits the exosome and XRN2 onto target transcripts, resulting in degradation of the RNAs.

IGF2BP3 promotes cell proliferation through repression of EIF4E-BP2

It has been proposed that IGF2BP3 promotes cell proliferation, leading to oncogenic transformation. However, little is known about the molecular mechanism of IGF2BP3-mediated enhancement of cell proliferation. Because EIF4E-BP2, whose mRNA is degraded by IGF2BP3, functions in the suppression of cell proliferation,[26] we predicted that IGF2BP3 promotes cell proliferation through repression of EIF4E-BP2. We tested this hypothesis using a double-knockdown strategy for IGF2BP3 and EIF4E-BP2 in HeLa TO cells (Figure 3a) and A549 cells (Supplementary Figure S3a). Although the expression level of EIF4E-BP2 protein was increased in IGF2BP3 knockdown cells, that of EIF4E-BP2 in double-knockdown cells was comparable to control cells (Figure 3a). In addition, cell proliferation was retarded in IGF2BP3-depleted cells, and was partially but significantly rescued by EIF4E-BP2 depletion (Figures 3b

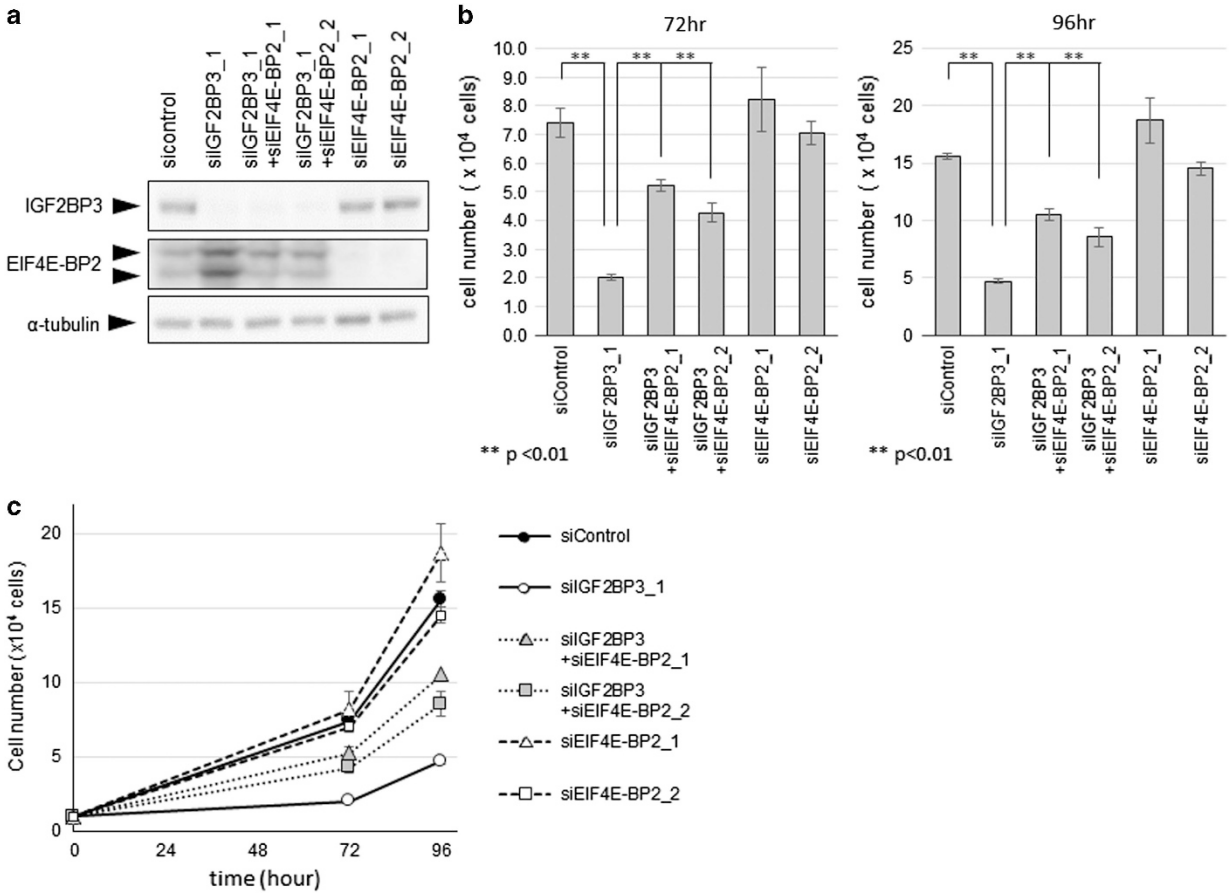

Figure 3. Retarded cell proliferation of IGF2BP3 knockdown cells was rescued by EIF4E-BP2 depletion. (**a**) Downregulation of IGF2BP3 (top) and EIF4E-BP2 (middle) in HeLa TO cells transfected with indicated siRNAs were confirmed by western blotting analysis. Alpha-tubulin was used as a loading control. (**b**) Viable HeLa TO cells were counted at 72 h or 96 h after first siRNA transfection using auto cell counter *P*-values were obtained by Student's *t*-test ($n = 4$). (**c**) Viable cell numbers were monitored after siRNA transfection by using auto cell counter (Millipore). Data at 72 h and 96 h are transferred from (**b**).

and c, Supplementary Figures S3b–d). These results suggest that IGF2BP3 promotes cell proliferation through downregulation of EIF4E-BP2 via enhancing mRNA degradation.

IGF2BP3 modulates eIF4E activity

Previous studies showed that EIF4E-BP2 downregulates cap-dependent translation through sequestration of eIF4E from the eukaryotic translation initiation factor complex.[27] eIF4E is a rate-limiting determinant of translation initiation and phosphorylation of eIF4E at S209 promotes tumorigenesis.[28] Several reports have reported that altered activity of EIF4E-BPs results in the altered phosphorylation status of eIF4E. For instance, downregulation of EIF4E-BP renders eIF4E phosphorylation.[29] Based on our data and previous reports, we hypothesized that downregulation of EIF4E-BP2 by IGF2BP3 activates eIF4E. To test this hypothesis, we evaluated the level of phosphorylated eIF4E (S209) in IGF2BP3-depleted cells. Western blotting analysis showed attenuation of phosphorylated eIF4E in IGF2BP3-depleted cells. Concomitant depletion of IGF2BP3 and EIF4E-BP2 restored the level of phosphorylated eIF4E (Figure 4a). Polysome profiling analysis showed that the distributions of VEGFA, FGF2 and ODC1 mRNAs, all target mRNAs of eIF4E,[30–32] were shifted from heavy fractions (the peak fractions of indicated mRNAs are indicated by black arrows in Figure 4b) to light fractions (the peak fractions of indicated mRNAs are indicated by white arrows in Figure 4b) by IGF2BP3 depletion, and their altered distributions were returned to heavy fractions by IGF2BP3 and EIF4E-BP2 double-knockdown (the peak fractions of indicated mRNAs are indicated by gray arrows in Figure 4b), indicating that IGF2BP3 depletion reduced translational activity of eIF4E target mRNAs via EIF4E-BP2. The distribution of alpha-tubulin mRNA, a non-eIF4E target, showed no difference among control, IGF2BP3 knockdown and IGF2BP3 and EIF4E-BP2 double-knockdown cells. Consistent with results of polysome profiling analysis, protein level of ODC1 was reduced by IGF2BP3 single knockdown and ODC1 reduction was rescued by IGF2BP3 and EIF4E-BP2 double-knockdown (lowest panel in Figure 4a). These results indicate that eIF4E activity is activated through the IGF2BP3-mediated repression of EIF4E-BP2.

EIF4E-BP2 mRNA is decreased in human lung adenocarcinoma tissues that highly express IGF2BP3

Finally, we investigated whether EIF4E-BP2 is repressed by IGF2BP3 not only in cultured cells but also in clinical specimens. We extracted total RNAs from human lung adenocarcinoma tissues ($n = 27$) and determined the expression level of IGF2BP3 mRNAs and EIF4E-BP2 mRNAs by RT-qPCR. Samples were grouped into high IGF2BP3 expressing and low IGF2BP3 expressing tissues based on their mRNA expression levels in comparison with the median expression of all tissues. Western blot analysis revealed high IGF2BP3 protein expression level in the high IGF2BP3 mRNA-expressing group tissues compared with low IGF2BP3 mRNA-expressing group tissues or their normal counterparts (Figure 5a, Supplementary Figure S5). We then examined EIF4E-BP2 mRNA expression levels in high and low IGF2BP3 expressing groups, and found that the expression level of EIF4E-BP2 mRNA was significantly inversely correlated with that of

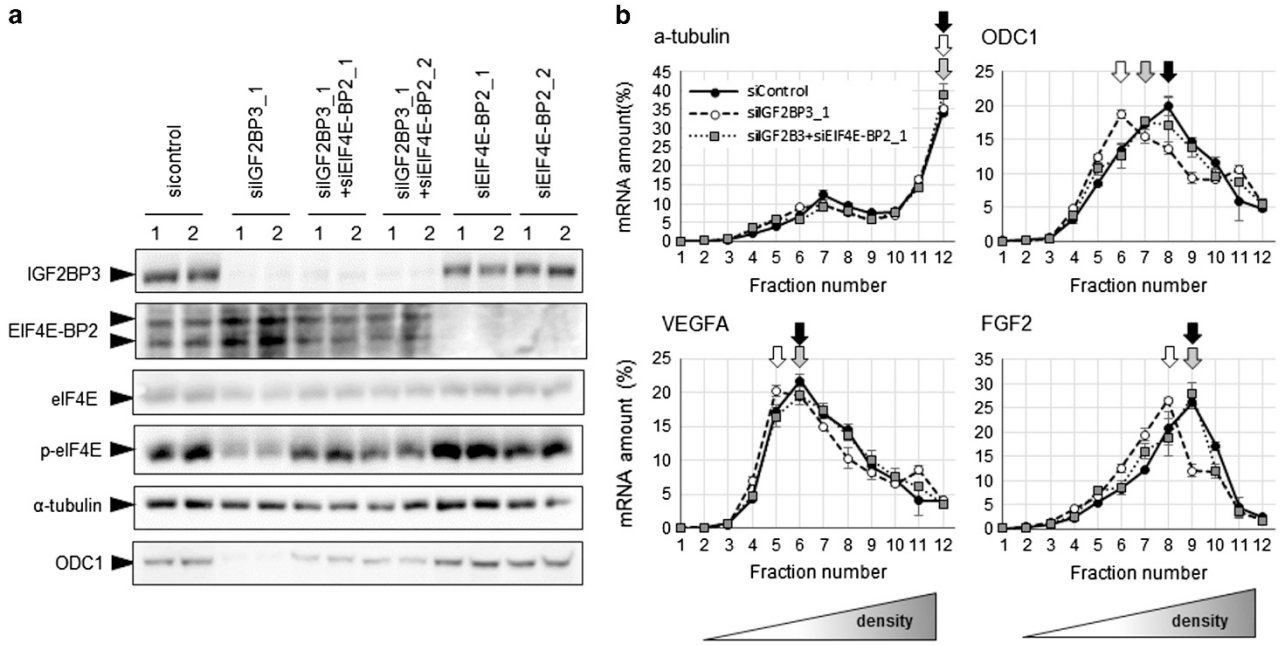

Figure 4. Reduction of eIF4E activity in IGF2BP3 knockdown cells. (**a**) Western blot analysis was performed in cells transfected with indicated siRNAs using indicated antibodies. (**b**) Polysomal distribution of eIF4E-targeted mRNAs in control cells (black circles), IGF2BP3 knockdown cells (open circles), or IGF2BP3 and EIF4E-BP2 double-knockdown cells (gray circles). Alpha-tubulin served as the negative control mRNA. Error bars show experimental error of two experiments. Black, white and gray arrows indicate the peak fractions of indicated mRNAs among polysomal distribution of cells transfected with siControl, siIGF2BP3 and siIGF2BP3+siEIF4E-BP2, respectively.

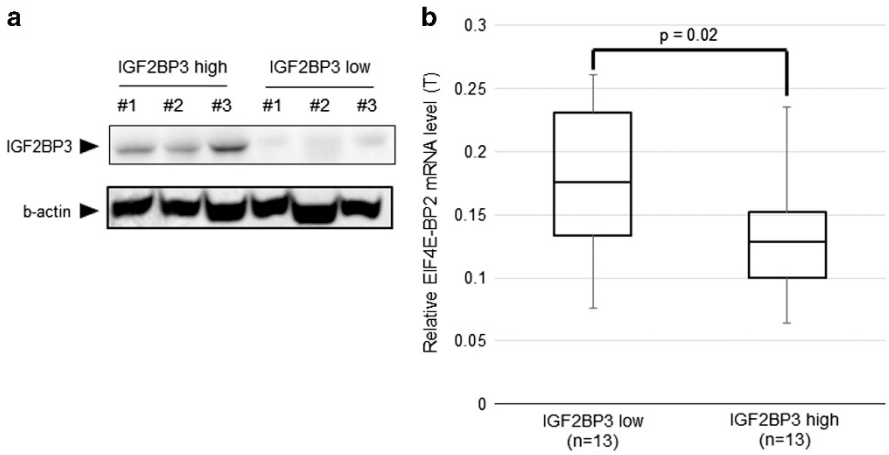

Figure 5. Inversely correlated expression of IGF2BP3 and EIF4E-BP2 in human lung adenocarcinoma tissues. (**a**) Western blot analysis of IGF2BP3 protein expression in human lung adenocarcinoma tissues with high or low IGF2BP3 mRNA expression tissues. (**b**) Box-plot of relative EIF4E-BP2 levels in human lung adenocarcinoma tissues. Total RNAs were extracted from human lung adenocarcinoma tissues (T), and RT-qPCR was performed to determine RNA levels of *IGF2BP3* and *EIF4E-BP2*. The expression levels of *b-actin* and *PGK1* in each tissues were used as reference mRNA for normalization. Samples were grouped into high IGF2BP3 expressing lung adenocarcinoma (IGF2BP3 high) and low IGF2BP3 expressing lung adenocarcinoma (IGF2BP3 low) based on gene expression in comparison with the median expression of IGF2BP3 mRNA of all tumors. The top bar is the maximum value, bottom bar is the minimum value, the top of the box is the upper or third quartile, the bottom of box is the lower or first quartile and the middle bar is the median value. *P*-value was obtained by a Wilcoxon rank sum test. ($n = 27$).

IGF2BP3 mRNA (Figure 5b). This result agrees well with the *in vitro* experiments and supports our hypothesis that IGF2BP3 suppresses EIF4E-BP2 mRNA in human lung adenocarcinoma tissues.

DISCUSSION

Several studies showed that high (Figure 6) expression of IGF2BP3 promotes tumor development. However, the mechanism underlying how IGF2BP3 promotes tumorigenesis has been under-investigated. Based on the knowledge that IGF2BP3 shares a high identity of 73% with IGF2BP1, previous studies focused on the functions of IGF2BP3 in translational activation and stabilization of RNA stability for target transcripts. Indeed, IGF2BP3 was shown to enhance translation of IGF-2 mRNA in U373 glioma cells[33] and K562 leukemia cells.[34] IGF2BP3 also stabilizes CD44 mRNA and PDPN mRNA through a yet-to-be-defined mechanism.[23,35] More recently, IGF2BP3 was reported to stabilize the transcript of HMGA2, an oncofetal protein associated with poor prognosis and low overall survival,[36] by preventing let-7 miRNA-directed mRNA degradation.[24] Although the elevated expression of IGF2BP3 is associated with upregulation of HMGA2 in several tumors, simultaneous elevated expression of IGF2BP3 and HMGA2 was

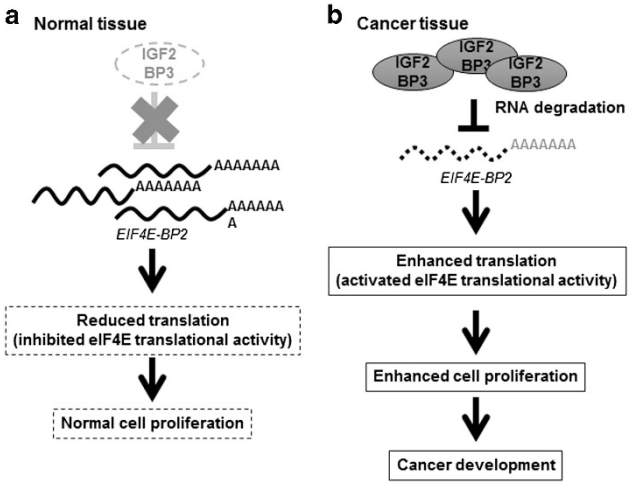

Figure 6. Model of IGF2BP3-mediated tumorigenesis. (**a**) In the normal tissues, low expression of IGF2BP3 enables the high expression of EIF4E-BP2 mRNA, resulting in reduced global translational activity that leads to normal cell proliferation. (**b**) High expression of IGF2BP3 facilitates the degradation of EIF4E-BP2 mRNA, resulting in enhancement of global translational activity that leads to accelerated cell proliferation and tumorigenesis.

not observed in several tumors such as breast cancers and colorectal cancers.[24] This suggests that another gene(s) or mechanism(s) should link IGF2BP3 function and tumorigenesis in addition to HMGA2.

To elucidate the molecular mechanism of how IGF2BP3 promotes tumorigenesis such as facilitated cell proliferation, we surveyed mRNAs with altered expression in IGF2BP3 knockdown cells that also associate with IGF2BP3 using RIP-seq and RNA-seq. Integration of RIP-seq data and RNA-seq data identified 37 transcripts upregulated by IGF2BP3 and 110 transcripts downregulated by IGF2BP3 (Figure 1). The identification of 37 transcripts upregulated by IGF2BP3 is consistent with the previously proposed function of IGF2BP3 in acting as an RNA stabilizing factor. The 110 transcripts downregulated by IGF2BP3 were unrelated to previously determined IGF2BP3 functions. Because the absence of RNA degradation factors causes an accumulation of direct targets, we hypothesized that IGF2BP3 degrades a set of RNAs and showed that IGF2BP3 depletion led to stabilization of mRNA targets such as EIF4E-BP2 mRNA or MEIS3 mRNA. In accordance with this *in vitro* observation, we observed the inverse correlation of the expressions of IGF2BP3 and EIF4E-BP2 mRNAs in clinical samples. To rule out the possibility of IGF2BP3-mediated regulation of EIF4E-BP2 at the protein level, we performed a cycloheximide chase experiment (Supplementary Figure S4g) and a polysome-profiling experiment (Supplementary Figure S4h) to assess protein stability of EIF4E-BP2 protein and translational efficiency of EIF4E-BP2 mRNA, respectively. We showed that IGF2BP3 depletion did not affect protein stability of EIF4E-BP2 and translational efficiency of EIF4E-BP2 mRNA, suggesting that mRNA destabilization is the sole mechanism of IGF2BP3-mediated EIF4E-BP2 regulation. In addition, we found that IGF2BP3 interacted with ribonucleases such as XRN2 and exosome components, suggesting that IGF2BP3 recruits ribonucleases onto target mRNAs, leading to mRNA degradation. A recent study reported that IGF2BP1, but not IGF2BP2 or IGF2BP3, destabilizes the long noncoding RNA HULC through recruitment of the CCR4-NOT deadenylase complex.[37] Importantly, IGF2BP3 did not interact with CCR4-NOT, suggesting that the mechanisms that facilitate RNA degradation between IGF2BP1 and IGF2BP3 are distinct. IGF2BP3-mediated RNA stability regulation (stabilization or destabilization) was apparently dependent on RNA species. The composition of the IGF2BP3-containing ribonucleoprotein complex to direct RNA stability likely depends on target mRNAs, leading to apparent incompatibility. Although this assumption remains largely speculative, a previous observation of transcript-specific IGF2BP1-containing ribonucleoprotein complexes[38] supports this idea. Another possibility is that different cellular localizations of IGF2BP3 might enable its binding different proteins and exhibiting different functions in the control of RNA stability. The observation of multiple IGF2BP3 localization patterns[39] supports this idea.

Enhanced translation facilitates cell proliferation, and elevated cell proliferation has an important role in tumorigenesis. Indeed, a previous report showed that eIF4E, a rate-limiting component of translation initiation, facilitates tumorigenesis by selectively stimulating the translation of a subset of tumor-promoting mRNAs, such as cyclins and c-myc.[40] Consistent with this function, eIF4E is frequently overexpressed in human cancers.[41] As eIF4E is negatively regulated by EIF4E-BP1 and EIF4E-BP2, EIF4E-BPs leads to reduced cell proliferation through the reduction of cap-dependent translational initiation.[42] It was reported that ectopic expression or activation of EIF4E-BP1 suppresses tumorigenicity[43–45] and that EIF4E-BP1/2-deficient mice showed reduced lung tumorigenesis induced by 4-(methylnitrosamino)-l-(3-pyridyl)-1-butanone.[46] In this regard, EIF4E-BPs is considered a tumor suppressor.[42] Consistent with this notion, inactivation of EIF4E-BP1 was found in aggressive breast carcinomas.[43] Importantly, EIF4E-BP2 was reported to exhibit a stronger influence on breast tumor behavior than EIF4E-BP1.[47] In this work, we found that impaired cell proliferation of IGF2BP3 knockdown cells was partially restored by EIF4E-BP2 depletion. We also showed that IGF2BP3 interacted with EIF4E-BP2 mRNA but not EIF4E-BP1 mRNA (Supplementary Figure S4e). These results suggest that IGF2BP3 promotes cell proliferation through the downregulation of EIF4E-BP2, a negative regulator of eIF4E. Moreover, we showed that phosphorylation status of eIF4E was regulated by the IGF2BP3-EIF4E-BP2 pathway. Soundness of phosphor-eIF4E by western blot analysis was confirmed by paramethoxyamphetamine-treatment, a well-known inducer of eIF4E phosphorylation via p38 phosphorylation (Supplementary Figure S4b). In addition, depletion of IGF2BP3, EIF4E-BP2 or IGF2BP3+EIF4E-BP2 did not alter phosphorylation status of p38, ruling out the possibility that the IGF2BP3-EIF4E-BP2 pathway regulates phosphorylation of eIF4E through alteration of p38 phosphorylation (Supplementary Figure S4a). Furthermore, no interaction of eIF4E mRNA with IGF2BP3 was observed (Supplementary Figure S4f), ruling out the possibility that IGF2BP3 directly regulates eIF4E mRNA. Taken together, these results suggest that IGF2BP3 activates eIF4E through the degradation of EIF4E-BP2 mRNA. As shown by results in Figure 3, EIF4E-BP2 pathway cannot completely describe a role of IGF2BP3 in cell proliferation. We found other IGF2BP3 target mRNAs from genome-wide analysis such as MEIS3, a cell survival regulator, and we also found that IGF2BP3 has a potential for regulating internal ribosome entry site-mediated translation similar to IGF2BP1 (Supplementary Figure S4c and d). Therefore, several pathways regulated by IGF2BP3 must be involved in the regulation of cell proliferation. Although we have some unresolved issues in understanding the function of IGF2BP3 so far, here we provide the first evidence for a functional connection between two well-known cancer biomarkers, the IGF2BP3 oncofetal gene and eIF4E proto-oncogene.

In this study, we also performed genome-wide analysis for IGF2BP1 and IGF2BP2 (Supplementary Figure S6a and b). One-hundred and thirty mRNA targets were upregulated by IGF2BP1, and 18 mRNA targets were downregulated by IGF2BP1. IGF2BP1 was demonstrated to function as a stabilizing factor for specific mRNAs.[16] We identified more mRNAs that were upregulated by IGF2BP1 than those upregulated by IGF2BP3. These data suggest that IGF2BP1 is more involved in RNA stabilization than in RNA degradation. In contrast, IGF2BP3 is more involved in RNA degradation than in RNA stabilization. Little has been known about the modulation of target mRNAs by IGF2BP2. Our genome-wide analysis identified 10 mRNAs upregulated by IGF2BP2 and 42 mRNAs downregulated by IGF2BP2.

IGF2BP2 might also regulate the stability of a set of target mRNAs. Moreover, our genome-wide analysis revealed that there was little overlap in IGF2BPs target mRNAs (Supplementary Figure S6c), and these results indicate the importance of an individual approach for the elucidation of each IGF2BP function. Although further investigation is required to uncover the specific functions of IGFBPs, this work provides a worthwhile starting point.

RBPs are thought to have a central role in tumorigenesis by regulating the expression of genes involved in cell cycle, cell motility and apoptosis.[48] However, technical limitations in identifying the bona fide target RNAs regulated by RBPs have prevented uncovering the functions of a large number of RBPs in tumorigenesis. In the current study, we performed genome-wide analyses to uncover the function of IGF2BP3. Our results demonstrate that IGF2BP3 acts as a RNA-destabilizing factor for a set of transcripts whose protein functions contribute to tumorigenesis. Moreover, we link two previously well-known cancer biomarkers, IGF2BP3 and eIF4E, through the IGF2BP3-mediated post-transcriptional regulation of EIF4E-BP2.

MATERIALS AND METHODS

Cell culture

HeLa Tet-off (HeLa TO) (Clontech, Palo Alto, CA, USA) and A549 cells (kindly obtained from Dr Nobukuni in FMI) were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and antibiotics at 37 °C in a humidified incubator with 5% CO_2.

siRNA treatment

The siRNA sequences are listed in Supplementary Table S2. To improve knockdown efficiency, siRNAs were transfected into cells sequentially using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. In brief, siRNA duplexes (final concentration 10 nM) were transfected into 6×10^4 cells (1st transfection), and then cells were incubated for 6 h at 37 °C/5% CO_2. After changing the medium, cells were incubated overnight. The following day, siRNA duplexes (final concentration 10 nM) were transfected into the cells again (2nd transfection) followed by incubation for 6 h at 37 °C/5% CO_2. After changing medium, cells were incubated for 24 h.

RNA immunoprecipitation

FLAG-tagged IGF2BP1, FLAG-tagged IGF2BP2 or FLAG-tagged IGF2BP3 plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The next day, cells were harvested and washed in cold phosphate-buffered saline. The cell pellet was resuspended in radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.1% DOC, 0.5 U μl^{-1} RNasin plus RNase inhibitor (Promega, Madison, WI, USA) and protease inhibitor cocktail (Sigma, St Louis, MO, USA)), and then sonicated by a Bioruptor UCD-250 (Cosmo Bio, Tokyo, Japan). The supernatants were obtained by centrifugation and used for immunoprecipitation using an anti-FLAG M2 mouse antibody (Sigma) or normal mouse immunoglobulin G (MBL, Nagoya, Japan). Antibodies were incubated with Dynabeads protein G (Invitrogen) for 1 h at 4 °C, and beads were washed three times with cold radioimmunoprecipitation assay buffer. ISOGEN LS (Nippon Gene, Osaka, Japan) was then added, followed by RNA isolation according to the manufacturer's instructions. The levels of transcripts were determined by RT-qPCR or massive sequencing.

Massive sequencing and data analysis

RNA-seq and RIP-seq complementary DNA libraries were prepared from 1 μg of total RNA using the mRNA Seq Sample Preparation Kit (Illumina, San Diego, CA, USA), according to the manufacturer's protocol. Thirty-six base-pair single-end read RNA-seq tags were generated using Illumina HiSeq 2000, according to the standard protocol. The fluorescent images were processed to sequences using the analysis Pipeline supplied by Illumina. Eland was used to map the reads to human reference genome sequences (hg19) with no mismatch errors. Splitting reads that originated from mRNA exon-exon junctions were also mapped to the human RefSeq transcriptome downloaded from the UCSC genome browser. Abundance of RefSeq transcripts was calculated from RNA-seq and RIP-seq sequence reads, based on reads per kilobase of exon model per million mapped reads (RPKM) as a means of normalizing for gene length and depth of sequencing. We used RefSeq transcripts that passed the cutoff of RPKM $\geqslant 1$ for data analysis. The accession numbers for the sequencing data produced in this study are (DDBJ:DRA002815).

In vivo cross-linking analysis

Cells treated for 4 h with 150 μM 4′-thio-uridine, which enhances the cross-linking between protein and RNA under 360 nm UV light, were harvested and washed in cold phosphate-buffered saline, followed by irradiation on ice with 360 nm UV light. Cells were collected in 1.5 ml microfuge tubes, and cell pellets were resuspended in 200 μl of lysis buffer (2% sodium dodecyl sulfate, 50 mM Tris-Cl (pH 8), 1 mM EDTA, 1 mM dithiothreito) and boiled at 95 °C for 5 min. After dilution with four volumes of dilution buffer (1.25% NP-40, 0.625% sodium deoxycholate, 62.5 mM Tris-Cl (pH 8), 1.75 mM EDTA, 187.5 mM NaCl), each sample was gently sonicated three times and centrifuged for 90 min at 4 °C. The supernatants were directly subjected to immunoprecipitation as described above.

BRIC

BRIC was performed as described previously.[49] In brief, cells were incubated at 37 °C in the presence of 150 μM BrU (Wako, Tokyo, Japan) for 24 h in a humidified incubator with 5% CO_2. At indicated time points after replacing BrU-containing medium with BrU-free medium, cells were harvested. Total RNA was isolated using RNAiso Plus (Takara, Shiga, Japan), followed by isolation of BrU-labeled RNA by anti-BrdU mouse antibody (clone 2B1, MBL). The isolated RNA was used for RT-qPCR.

Co-immunoprecipitation

For co-immunoprecipitation of FLAG-tagged IGF2BP3 and ribonuclease, we transfected FLAG empty plasmid (as a negative control) or FLAG-tagged IGF2BP expression vector into HeLa TO cells or A549 cells. Twenty-four hours after transfection, cells were lysed in 3-(3-cholamidepropyl) dimethylammonio-1-propanesulphonate (CHAPS) lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 0.3% CHAPS) followed by 0.01 μg RNaseA and 0.4 unit RNaseT1 treatment. The cell lysates were immunoprecipitated using anti-FLAG antibody. For co-immunoprecipitation of endogenous IGF2BP3 and ribonuclease, HeLa To cells were lysed in lysis buffer (50 mM Tris-HCl (pH 8), 250 mM NaCl, 5 mM EDTA, 0.5% NP-40), and immunoprecipitations were performed using normal rabbit immunoglobulin G (MBL) or anti IGF2BP3 rabbit antibody (Abcam, Cambridge, UK, #ab177942). Immunoprecipitants were examined by western blot analysis.

Research ethics

This study was conducted according to the principles expressed in the Declaration of Helsinki. The human tissue samples were prepared at the Asahi General Hospital. The Asahi General Hospital Institutional Review Board and The University of Tokyo Institutional Review Board approved the use of the human tissue in this study according to the Ethical Guidelines of the Ministry of Health, Labour and Welfare of Japan. Written informed consent for study participation was obtained from all participants and was recorded by the physician on a study participation sheet. The data were analyzed anonymously. This clinical trial registration number is 12–5(3).

Clinical specimens

Surgically resected specimens from 27 patients with a diagnosis of lung adenocarcinoma were obtained in Asahi General Hospital from 2011 to 2013. All specimens were trimmed to an approximate $5 \times 5 \times 5$ mm cube after surgical resection and immediately stored in RNA later solution Life Technologies, Carlsbad, CA, USA) at −80 °C. After homogenizing tissues with Tissue Ruptor (QIAGEN, Hilden, Germany), total RNA and protein were extracted with RNA iso Plus (Takara) and radioimmunoprecipitation assay buffer, respectively. Isolated RNA and extracted protein were subjected to RT-qPCR and western blotting analysis, respectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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