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Heterogeneous Nuclear Ribonucleoprotein K (hnRNP-K) Promotes Tumor Metastasis by Induction of Genes Involved in Extracellular Matrix, Cell Movement, and Angiogenesis

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Background: Cancer metastasis is a major hurdle in cancer therapy and needs identification of novel targets for drug designing.
Results: hnRNP-K is highly expressed in cancer cells and regulates extracellular matrix, cell motility, and angiogenesis pathways.
Conclusion: hnRNP-K is a potential target for metastasis therapy.
Significance: hnRNP-K expression level may serve as a marker of metastatic cancers, and hnRNP-K-inhibiting drugs could be candidate anti-cancer and anti-metastasis reagents.

Cancer is a leading cause of death and still awaits effective therapies. Rapid industrialization has contributed to increase in incidence of cancer. One of the reasons why most of the cancers fail therapy is due to their metastatic property. Hence identification of factors leading to metastasis is highly important to design effective and novel anti-cancer therapeutics. In our earlier study (Inoue, A., Sawata, S. Y., Taira, K., and Wadhwa, R. (2007) Loss-of-function screening by randomized intracellular antibodies: identification of hnRNP-K as a potential target for metastasis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 8983–8988), we had reported that the involvement of heterogeneous nuclear ribonucleoprotein K (hnRNP-K) in metastasis. Here, we established hnRNP-K-overexpressing and -underexpressing derivative cell lines and examined their proliferation and metastatic properties *in vitro* and *in vivo*. Whereas hnRNP-K compromised cells showed delayed tumor growth, its overexpression resulted in enhanced malignancy and metastasis. Molecular basis of the hnRNP-K induced malignant and metastatic phenotypes was dissected by cDNA microarray and pathway analyses. We found that the hnRNP-K regulates extracellular matrix, cell motility, and angiogenesis pathways. Involvement of the selected genes (*Cck*, *Mmp-3*, *Ptgs2*, and *Ctgf*) and pathways was validated by gene-specific expression analysis. Our results demonstrated that the hnRNP-K is a potential target for metastasis therapy.

Heterogeneous nuclear ribonucleoprotein K (hnRNP-K)[3] is a 66-kDa unique RNA- and DNA-binding component of the ribonucleoprotein particles. It is a multifunctional signaling protein localized in the nucleus, cytoplasm, and mitochondria and is involved in the regulation of structural organization of the chromatin, transcription, pre-mRNA processing, splicing, mature mRNA transport to the cytoplasm, translation, nuclear transport, signal transduction, and DNA repair (1–8). Several studies have demonstrated its role as a docking platform to integrate cross-talk between nucleic acids and kinases that mediate signal transduction (9). Several proteins, including hepatitis C virus (HCV) core protein C, herpes simplex virus-1 (HSV-1) immediate early protein IE63, casein kinase 2, and p32 Y-box-binding protein, N-WASP, RNA binding motif protein-42, and androgen receptor have been shown to interact with hnRNP-K (10–17). Dividing cells have a higher level of hnRNP-K expression, suggesting its proproliferation function (18–20). Consistently, a variety of cancers, including breast carcinoma (21), esophageal squamous cell carcinoma (22), head-and-neck/oral squamous cell carcinomas (23, 24), lung adenocarcinoma (25, 26), pancreatic carcinoma (27), melanoma (28), colorectal carcinoma (29), and chronic myeloid leukemia (30) were found to be enriched in hnRNP-K expression. It was shown to regulate the anti-apoptotic protein FLICE-like inhibitory protein (FLIP) and VEGF in cancer cells (31, 32). The functional significance and molecular mechanism of the role of hnRNP-K in cancer cells yet remain unclear.

Using the loss-of-function screening system achieved by intracellular expression of single domain antibodies in human metastatic cells, we had previously identified the involvement of hnRNP-K in *in vitro* migration (33) of human cancer cells. To dissect its functional significance in metastasis, we established hnRNP-K-overexpressing and compromised derivative cell lines of NIH 3T3 (mouse immortal cells) and HT1080 (human fibrosarcoma). *In vitro* and *in vivo* phenotype and molecular analyses demonstrated that the hnRNP-K promotes metastasis by induction of genes involved in extracellular matrix, cell movement, and angiogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Transfections—Mouse (NIH 3T3, immortal fibroblasts) and human (HT1080, fibrosarcoma)

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[3] The abbreviations used are: hnRNP-K, heterogeneous nuclear ribonucleoprotein K; iAb, intracellular antibody; MMP, matrix metallopeptidase; CCK, cholecystokinin; PTGS, prostaglandin-endoperoxide synthase; CTGF, connective tissue growth factor; sdpr, serum deprivation response.

cells were obtained from Japanese Collection of Research Bioresources and human osteosarcoma (U2OS) from the ATCC and cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C, in an atmosphere of 5% CO_2 and 95% air in a humidified incubator. hnRNP-K cDNA was cloned into the NotI and XhoI site of pCMV-Tag1 vector. An expression vector encoding intracellular antibody to hnRNP-K (iAb-hnRNP-K) was generated as described previously (33). hnRNP-K expression plasmids were transfected into the non-malignant model cell lines NIH 3T3 and U2OS. The malignant model cell line, HT1080, was transfected with iAb-hnRNP-K vector using FuGENE 6 reagent (Roche Applied Science). Typically, 6 μg of plasmid DNA was used to transfect cells in 10-cm dishes at ~70–80% confluency. Transfected cells were selected in a medium supplemented with G418 (500 μg/ml). hnRNP-K expression was examined in individual clones (10 clones for each cell line) by Western blotting and immunostaining with anti-hnRNP-K antibody. The clones with high level of expression were selected and maintained in the presence of G418 (300 μg/ml) for further analyses.

Gene Expression Analysis—Cells transfected with hnRNP-K and iAb-hnRNP-K expression plasmids were lysed in radioimmune precipitation assay buffer (Thermo Scientific). Aliquots of 20 μg of total protein were resolved on SDS-PAGE and examined for the expression of hnRNP-K and its downstream effectors by Western blotting as described previously (33), and antibodies such as anti-COX-2, anti-CCK, anti-CTGF, anti-VEGF, and anti-matrix metallopeptidase-3 (MMP) (Santa Cruz Biotechnology). Anti-actin antibody (Chemicon) was used as an internal control.

For immunostaining, cells ($\times 10^4$) were plated on a glass coverslip placed in a 12-well culture dish. After 24 h, when cells had attached to the surface and spread well, they were washed with cold PBS three times and then fixed with prechilled methanol/acetone (1:1) mixture for 5 min. Fixed cells were washed twice with PBS, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked with 0.2% BSA/PBS for 10 min. They were incubated with anti-hnRNP-K antibody (ImmuQuest) for 1 h at room temperature, washed three times with 0.2% Triton X-100 in PBS, and then incubated with Alexa Fluor 594-conjugated goat anti-mouse (Molecular Probes) secondary antibodies. After extensive washings with 0.2% Triton X-100 in PBS, cells were examined on a Carl Zeiss microscope (Axiovert 200 M).

In Vitro and in Vivo Proliferation and Malignant and Metastasis Assays—hnRNP-K overexpressing non-malignant (NIH 3T3) and hnRNP-K compromised malignant cells (HT1080) cells were analyzed for their proliferation rate, colony-forming efficiency, chemotaxis, and invasion assays.

For proliferation rate, equal number of control and transfected cells were plated in 24-well plate. After 48 h, cells were trypsinized, and an aliquot (20 μl) was mixed with an equal volume of 0.4% trypan blue solution. After 5 min of incubation, number of viable (unstained) and dead (stained) cells was counted either by hemocytometer in a quadrant or Vi-CELL viability analyzer (Beckman Coulter).

For colony-forming assays, 500 cells were plated in a six-well plate and left to form colonies for the next 10–15 days with a regular change of medium on every third day. Colonies were fixed in methanol, stained with 0.1% crystal violet solution, photographed, and counted.

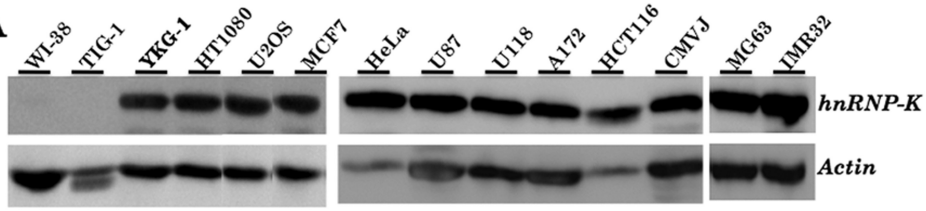
For chemotaxis assays, cells at 60–70% confluency were washed with cold PBS, trypsinized, and resuspended in DMEM supplemented with 0.5% bovine serum albumin (Sigma) at 5×10^4 cells/ml. 2.5×10^4 cells were plated in BioCoat™ Matrigel™ Invasion Chambers (8-mm pore, BD Biosciences), and the invasion assay was performed following the manufacturer's instructions. Cells that moved through the chamber were counted under a microscope.

For *in vitro* cell invasion assays, cells were grown in a monolayer. A wound was made in the monolayer of cells by scratching the cells in a line with a 200-μl pipette tip. Cells were washed a few times with PBS to remove cell debris, and a fresh medium was added. The time of scratching wound was designated as time 0. Cells were allowed to proliferate and migrate into the wound during the next 24 h and recorded under a phase contrast microscope with a 10× phase objective. Migration capacity was quantitated by measuring the percent of open area in 6–10 randomly captured images.

In vivo malignant and metastasis assays were performed using nude mice subcutaneous and tail vein xenograft models, respectively. Balb/c nude mice (4 weeks old, female) were bought from Nihon Clea (Tokyo, Japan). Animals used for experimentation received humane care. Cells were injected into the nude mice subcutaneously (1×10^7 suspended in 0.2 ml of growth medium) and through tail vein (1×10^6 suspended in 0.2 ml of growth medium) injection. Tumor formation and mice health (body weight) was monitored every alternate day. The experiment was repeated twice, using three mice for each injection. Volume of the subcutaneous tumors was calculated as $V = L \times W^2/2$, where L was length and W was the width of the tumor, respectively. For metastasis assay, the recipient mice were sacrificed after 20 days of tail vein injection, and their lungs were evaluated for the presence of tumors. This study was carried out in strict accordance with the recommendations in the Animal Experiment Committee, Safety and Environment Management Division, National Institute of Advanced Industrial Science & Technology (AIST), Tsukuba, Japan. The project was approved by the AIST Committee on the Ethics of Animal Experiments.

Microarray Assay and Gene Expression Analysis—Control and hnRNP-K overexpressing NIH 3T3 cells were grown in a monolayer prior to RNA isolation. Total RNA was isolated using RNeasy Mini kit (Qiagen). Single-strand DNA was synthesized using Ambion WT expression kit (Applied Biosystems, Carlsbad, CA). Biotinylated single-strand DNA was prepared using GeneChip DNA Labeling Reagent (Affymetrix, Santa Clara, CA). The single-strand DNA was hybridized with DNA probes on a GeneChip Mouse Gene 1.0 ST array (Affymetrix). The raw fluorescence intensity of each probe was quantified using GeneChip Command console software (Affymetrix). Data obtained from the control and hnRNP-K overexpressing cells were normalized to the individual median of fluorescent intensity using GeneSpringGX software (Agilent Technologies, Santa Clara, CA). Up-regulated genes in normalized sample compared with normalized control were selected, and similar entities were found using GeneSpringGX software.

A



B

Name	Description	hnRNP-K/ Actin
Normal cell lines		
WI-38	Normal fetal lung fibroblasts	0.033
TIG-1	Normal skin fibroblasts	0.028
Tumorigenic cells		
YKG-1	Glioma	0.87
HT1080	Fibrosarcoma	0.86
U2OS	Osteogenic sarcoma	0.90
MCF7	Breast adenocarcinoma	0.84
HeLa	Epithelioid cervical carcinoma	3.96
U87	Glioblastoma	1.22
U118	Glioblastoma	1.47
A172	Glioblastoma	1.35
HCT116	Colon carcinoma	2.22
CMVJ	Lung carcinoma	1.45
MG-63	Osteosarcoma	1.46
IMR-32	Neuroblastoma	1.68

FIGURE 1. **hnRNP-K expression level in human normal and cancer cells.** Cancer cells showed higher level of expression as compared with the normal (WI-38 and TIG-1) cells as seen by Western blotting (*A*). Quantitation of the signal normalized against internal control, actin, is shown in *B*.

Bioinformatics and Pathway Analysis—Pathway analysis and biological process association were mapped using PANTHER expression data analysis (34, 35). Conceptually, it is a simple binomial test to compare classifications of multiple clusters of the selected list with a reference list (NCBI, *Homo sapiens* genes) to statistically determine over- or under-representation of PANTHER classification categories. Biological process result was selected based on over-representation value and p value < 0.5. Protein-protein interactions for target genes were performed with Osprey based on the General Respository for Interaction Data sets (The GRID) and Gene Onthology (GO) (36).

Expression Analysis of hnRNP-K-regulated Genes—RT-PCR was employed with the total RNA isolated with the RNeasy Mini kit (Qiagen) as mentioned above. First-strand cDNA was synthesized using 1 μg of RNA with oligo(dT) as the primer with MMLV reverse transcriptase. PCR was performed using specific primers listed as follows. PCR product (20 μl) was resolved on a 1% agarose gel, stained with GelRed (Biotium), visualized, and photographed under UV light (Bio-Rad). Primers were as follows: *Ptgs2*, 5′-ACACACTCTATCACTGGCACC-3′ (forward) and 5′-TTCAGGGAGAAGCGTTTGC-3′ (reverse); *Cck*, 5′-TGCAGCCTTCTCCGTGGAACTCG-3′ (forward) and 5′-TCCTCATTCCACCTCCTCCAAGCAGGG-3′ (reverse); *Mmp3*, 5′-TGTACCAGTCTACAAGTCCTCCA-3′ (forward) and 5′-CTGCGAAGATCCACTGAAGAAGTAG-3′ (reverse); *Ctgf*, 5′-CATGCTTGCAGACAGACCTG-3′ (forward) and 5′-GTCTAACAGACAAGGCTCTGACTC-3′ (reverse); *Mmp10*, 5′-AGTTGCTCCTGCATGTTCTG-3′ (forward) and 5′-TGCATCCTCTCACCTACTGC-3′ (reverse); *Tmsb4x*, 5′-GGCTGTCCACTGGTCTGAAA-3′ (forward) and 5′-GAACCACATCGATGGCGGAA-3′ (reverse); *Itga6*, 5′-TGGTCTTACAAATGATGCCTTGTT-3′ (forward) and 5′-GGCTGTGTCCTTTAAAGCCAGT-3′ (reverse); *Lilrb4*, 5′-AATAAAGCCAAATCCTCCTCAA-3′ (forward) and 5′-CATTGATTAGTGTGCCCTGG-3′ (reverse); *Rasa1*, 5′-TCAACTGACAAGGAAACGCA-3′ (forward) and 5′-TCTTGGACAATTCCAACAAAAT-3′ (reverse); and *Sdpr*, 5′-TGAGGTGAATAATGGGTGTTGA-3′ (forward) and 5′-TTATTTAATCTTCTTTCACACAGGC-3′ (reverse).

RESULTS

We had previously identified the role of hnRNP-K in *in vitro* migration of human cancer cells (33). To address the role of hnRNP-K in human carcinogenesis, we first examined its expression level in normal and cancer cells. As shown in Fig. 1, cancer cells showed many fold higher level of expression as compared with the control.

hnRNP-K Intracellular Antibody Causes Tumor Suppression and Loss of Metastasis In Vivo—HT1080 cells transfected with hnRNP-K

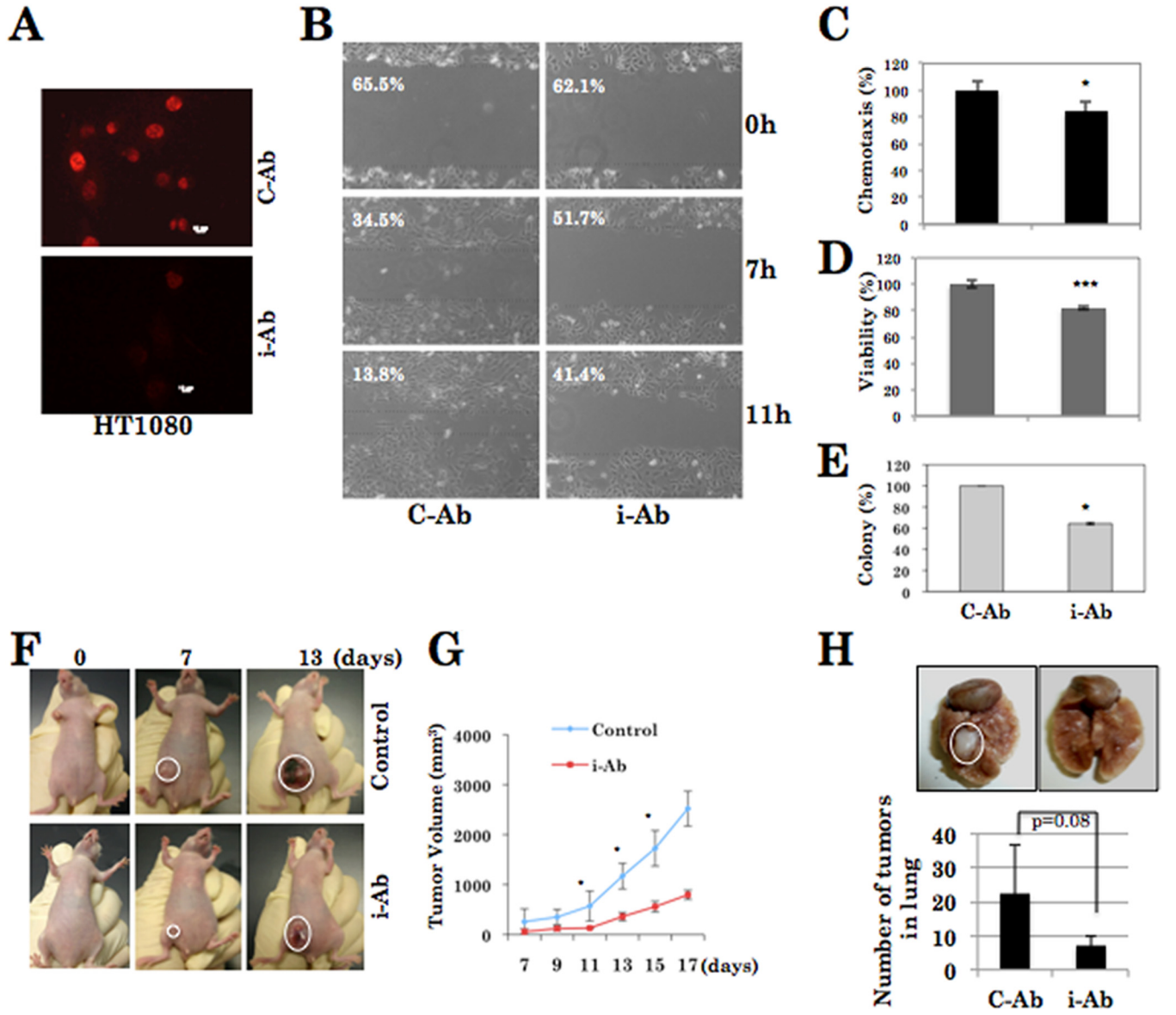


FIGURE 2. **hnRNP-K silencing by its iAb reduces its malignant properties in human fibrosarcoma (HT1080).** HT1080 cells transfected with hnRNP-K iAb show reduced expression by immunostaining (*scale bar*, 10 μm; *A*). hnRNP-K iAb-transfected cells showed reduced mobility in scratch wound (*B*) and chemotaxis assays (*C*). These cells also showed moderate reduction in cell viability (*D*) and colony-forming capacity (*E*). *In vivo* tumor formation in nude mice by injecting HT1080 cells and the tumor suppression by iAb transfection are shown (*F* and *G*). iAb-transfected cells reduced the metastasis in lung (*H*). Statistical significance as determined by Student's *t* test was as follows: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. *C-Ab,* control antibody, VHH (single heavy chain small antigen-binding fragment)-sfi expressing vector (pcDNA 3.1-Hygro) (33).

iAb expression vector were examined for hnRNP-K expression by immunostaining with specific antibody (Fig. 2*A*). hnRNP-K compromised cells were examined for their proliferation rate, colony-forming efficiency and invasion *in vitro* and *in vivo*. As shown, hnRNP-K-compromised cells showed delayed motility in scratch wound assays (Fig. 2*B*). Similar results were obtained in a chemotaxis assay. Quantitation from the three independent experiments showed that the hnRNP-K-compromised cells had 30% less chemotaxis (Fig. 2*C*), 22% less viability (Fig. 2*D*), and 40% less colony-forming efficiency (Fig. 2*E*). We next investigated *in vivo* tumor formation and metastasis of these cells in nude mice. As compared with the control cells, hnRNP-K compromised cells showed tumor suppression (Fig. 2, *F* and *G*), and most noticeably, the metastasis to lung was distinctly diminished (Fig. 2*H*). Whereas control cells formed big tumors in the lung, the hnRNP-K compromised cells showed either very small or no lung tumors in three independent experiments, suggesting that the hnRNP-K significantly contributes to the *in vivo* metastasis of cancer cells. Consistent with the decrease in metastatic capacity of iAb-expressing cells, mice injected with these cells looked healthier than the control mice. These mice maintained their body weight and showed increased survival rate as compared with the control cells (data not shown).

hnRNP-K Overexpression Contributes to the Malignant Properties of Cancer Cells—To address the functional significance of hnRNP-K in malignant and metastasis phenotype of cancer cells, we generated hnRNP-K-overexpressing derivatives of mouse immortal (NIH 3T3) and human osteosarcoma (U2OS) cells that are non-malignant and non-metastatic in the nude

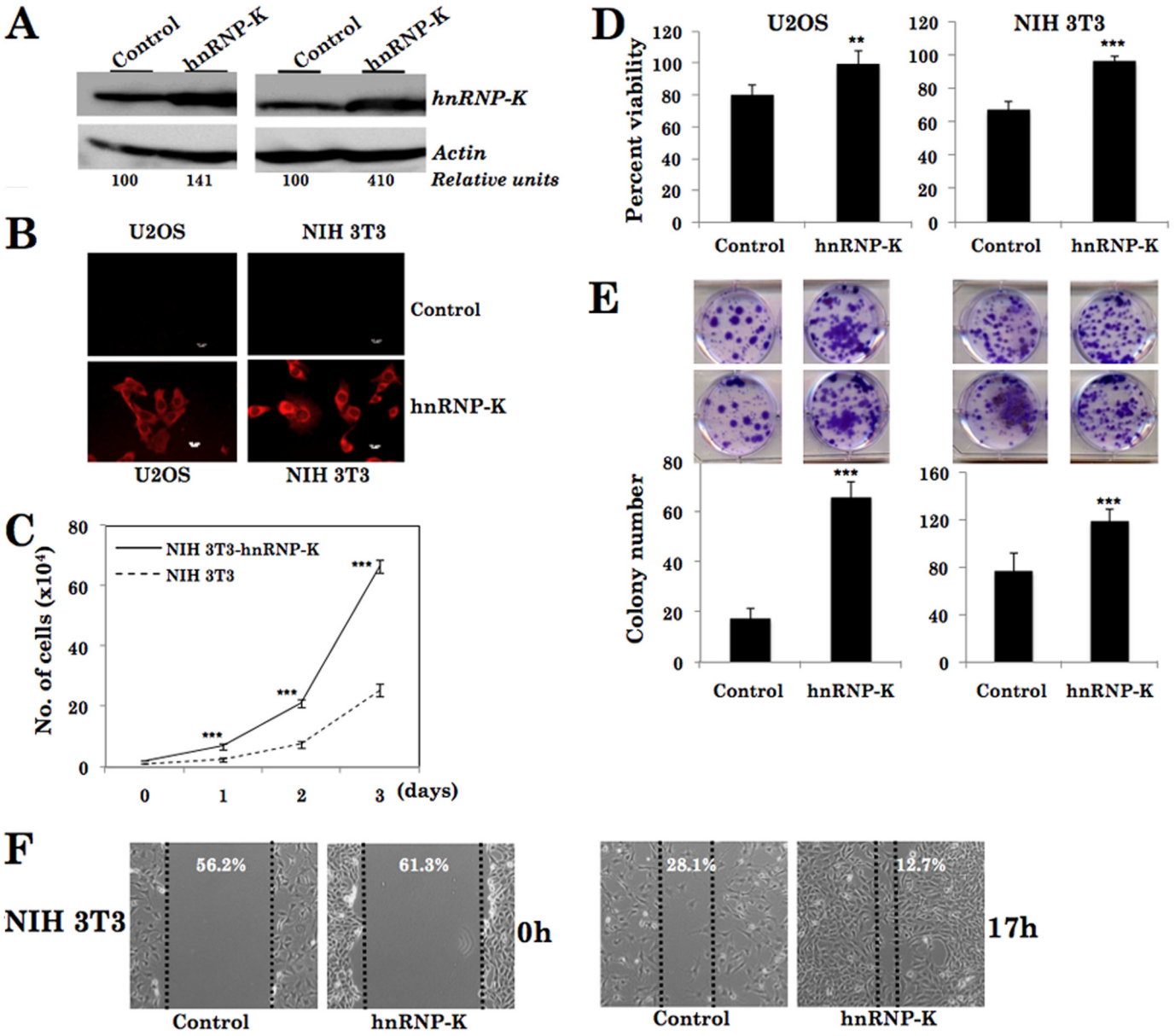


FIGURE 3. **hnRNP-K overexpression in NIH 3T3 cells increased their viability, colony-forming efficiency, and motility.** Expression level of hnRNP-K, in control and its overexpressing NIH 3T3 derivatives, is shown by Western blotting (*A*) and immunostaining (*B*). hnRNP-K-overexpressing cells showed higher proliferation (*C*) viability (*D*), colony-forming efficiency (*E*), and motility in wound-scratch assays (*F*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's *t* test).

mice model. Derivative cells were first analyzed for hnRNP-K expression by Western blotting and immunostaining (Fig. 3, *A* and *B*) and then subjected to proliferation, viability, colony-forming, and invasion assays. Growth curves of control and hnRNP-K-overexpressing cells revealed higher proliferation rate of the latter (Fig. 3*C* and data not shown). Furthermore, hnRNP-K-overexpressing, both NIH 3T3 and U2OS, cells showed higher viability, colony-forming efficacy, and invasion as compared with the control parent cells (Fig. 3, *D–F*, and data not shown). In the *in vivo* nude mice model, control NIH 3T3 cells neither formed tumors in subcutaneous xenografts nor showed any metastasis in lung in tail vein injection model (Fig. 4, *A* and *C*). hnRNP-K-overexpressing NIH 3T3 cells formed subcutaneous tumors and showed metastatic tumors in the lungs (Fig. 4*C*). Consistent with these results, Western blotting of lung tissues, isolated from mice injected with either control or hnRNP-K-overexpressing NIH 3T3 cells, revealed a higher level of expression of MMP-3 in the latter (Fig. 4*D*). Furthermore, we found that the hnRNP-K-overexpressing NIH 3T3 cells have a higher level of expression of VEGF, an established marker of angiogenesis (Fig. 4, *E* and *F*).

hnRNP-K-regulated Extracellular Matrix and Cell Motility Genes—To investigate the molecular mechanism of contribution of hnRNP-K in cancer cell malignancy and metastasis, we performed cDNA array analysis of control and malignantly transformed NIH 3T3 cells. As shown in Fig. 5, hnRNP-K-overexpressing malignant derivatives showed either up- (Table 1) or down-regulation (Table 2) of several genes. The most significant (~5-fold change in the expression level) of these genes, as identified by GeneSpringGX software, are shown in Table 1. Among these, matrix metallopeptidases (*Mmp-3* and *Mmp-10*) showed the highest up-regulation (6.35- and 4-fold, respec-

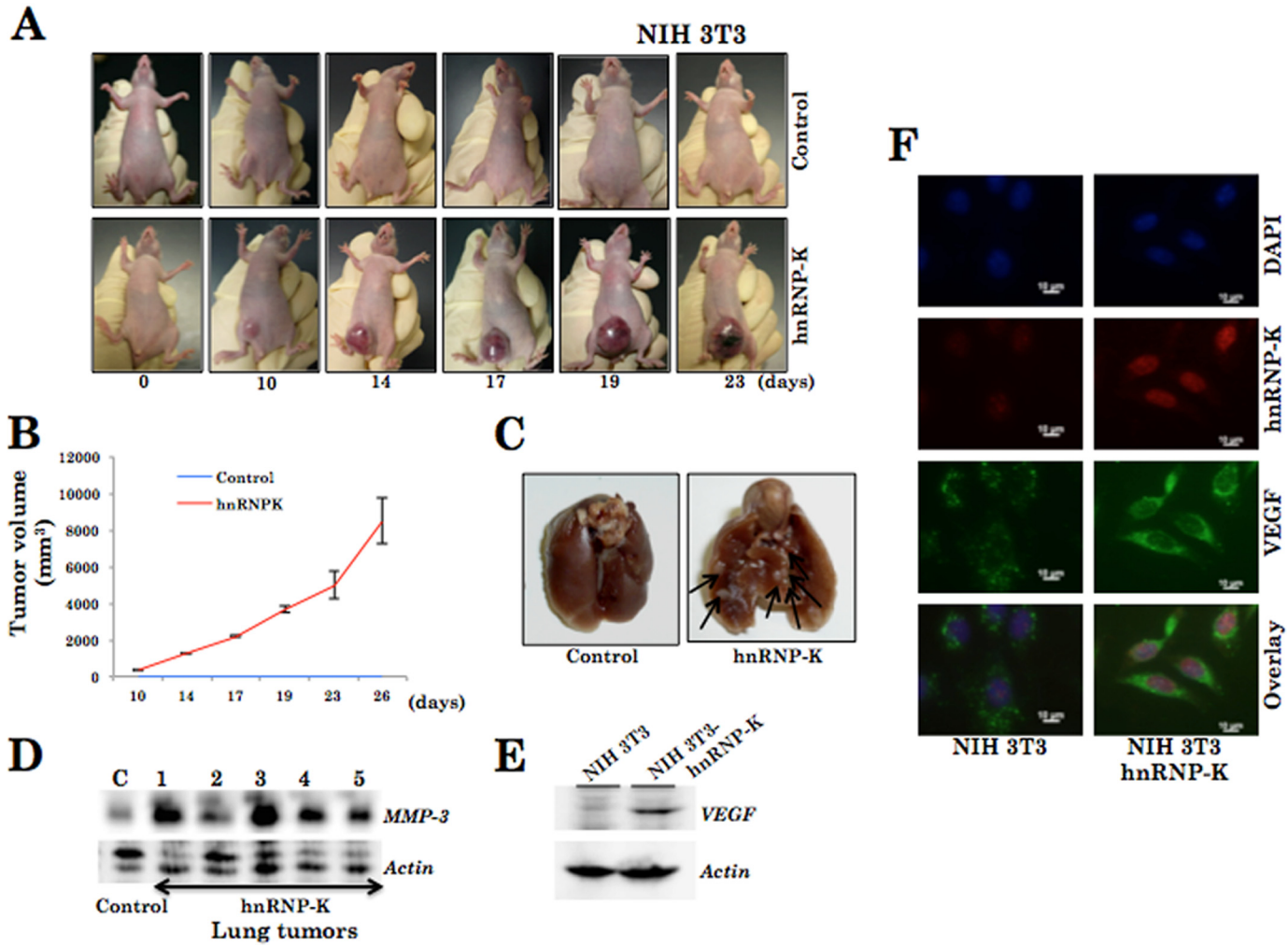


FIGURE 4. **hnRNP-K overexpression induced malignant transformation of NIH 3T3 cells.** hnRNP-K-overexpressing cells when xenografted in nude mice lead to tumor formation (*A*). *B*, average volume of hnRNP-K induced tumors from six mice is shown. Overexpression of hnRNP-K in NIH 3T3 cells resulted in lung metastasis (*C*). *D*, Western blot indicates a high level of expression of MMP-3 in lung tumor tissue extracts. A high level of expression VEGF (an angiogenesis marker) was detected in hnRNP-K-overexpressing NIH 3T3 cells by Western blotting as well as immunostaining (*F*). Actin was used as a loading control.

tively) followed by connective tissue growth factor, thymosin, integrin, and Ras-p21 (~3-fold increase). We also applied bioinformatics tools on the gene targets (Table 1 and Fig. 5) selected from the array analysis. The top pathways selected were plasminogen cascade, integrin and inflammation signaling along with biological processes, including cell communication and signal transduction (Fig. 5, *B* and *C*). The selected target genes could be grouped into six clusters. These genes indicated that the hnRNP-K is a multifunctional protein, and is involved in pathways regulating transcription, signal transduction, and extracellular matrix. For example, the Sdpr cluster signified up-regulation of genes involved in transcription, the *Il2rg* cluster signified proproliferation. The *Kng1-Ctgf* cluster signified anti-apoptosis and angiogenesis signaling, *Mmp3-Mmp10-Col1a1* and *Tmsb4x* gene clusters signified extracellular matrix signaling involved in cell motility. *Ptgs2* compose cluster for cell growth control (Fig. 5*D*). Of note, the analyses indicated that 32% of genes involved in extracellular matrix were regulated by hnRNP-K, suggesting the major contribution of extracellular matrix in hnRNP-K-mediated malignancy and metastasis (Fig. 5*E*).

We next validated the outcome of cDNA array and bioinformatics by gene-specific RT-PCR analysis. As shown, hnRNP-K-overexpressing NIH 3T3 cells showed up-regulation of all of the 10 identified genes (Fig. 6*A*). *Mmp-3* and *Mmp-10* showed highest up-regulation followed by *Tmsb4x*, *Rasa1*, *Cck*, *Lilrb4*, and *Itga6*. We also examined the protein expression level in control and hnRNP-K-overexpressing malignant cells and found that the selected genes such as *Ptgs2*, *Mmp-3*, *Ctgf*, and *Cck* were enriched in the latter (Fig. 6, *B* and *C*). Of note, as described above, tumors formed by hnRNP-K-overexpressing NIH 3T3 cells revealed a high level of MMP-3 expression as compared with the control (Fig. 4*D*).

Based on these data, we predicted that hnRNP-K might regulate cell migration in two ways. In the nucleus, it may cause transcriptional up-regulation of *Ptgs2*, *Mmp-3*, *Mmp-10*, *Tmsb4x*, *Cck*, and *Ras* (Fig. 6*A*). Activated Ras and MEK stabilize hnRNP-K in the cytoplasm either directly or through the ERK activation. In the cytoplasm, it might activate ERK in a regulatory loop that may in turn regulate cell migration by the MMP-3/MMP-10 pathway (Fig. 7). In addition to these molec-

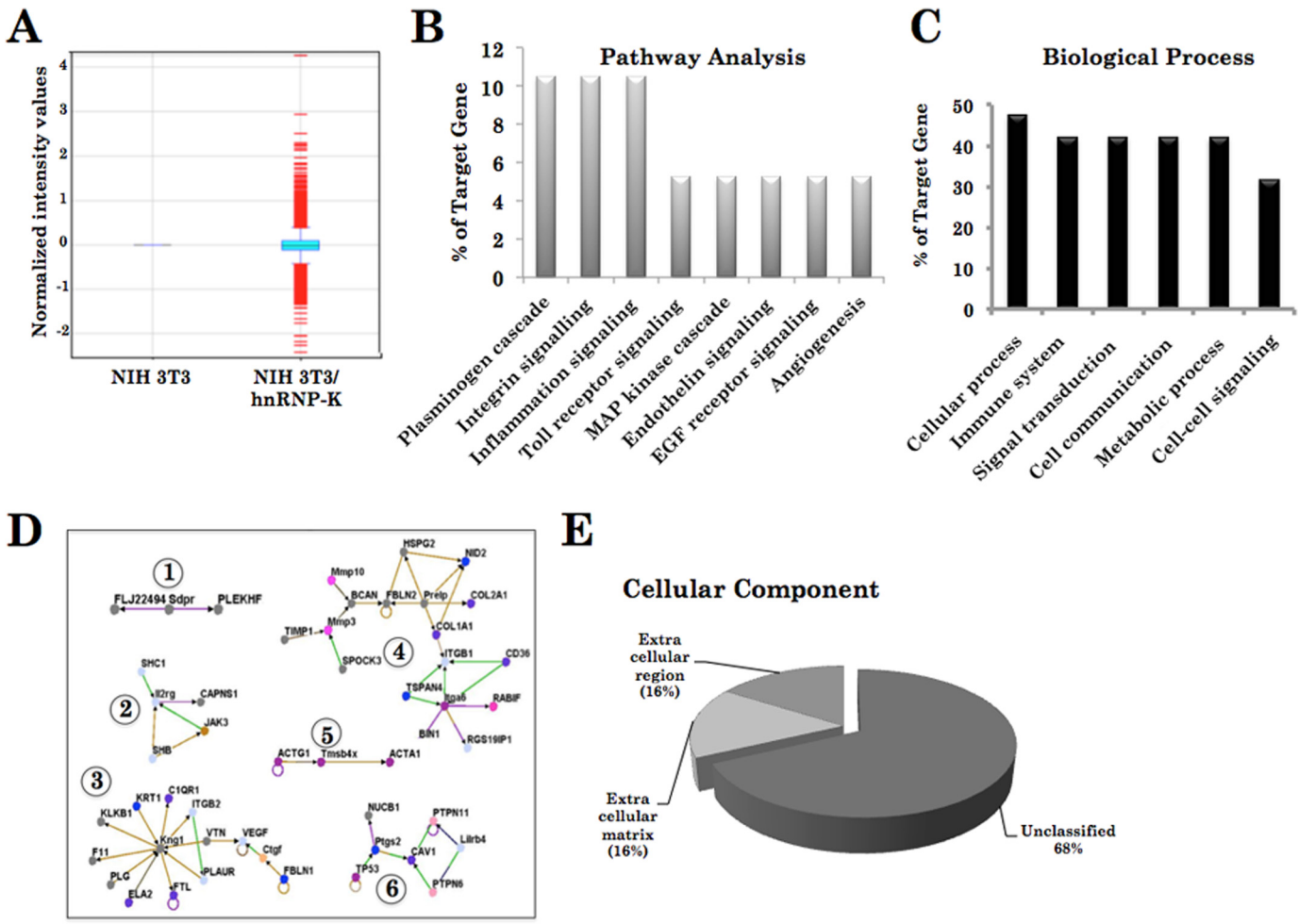


FIGURE 5. **cDNA array of control and hnRNP-K-overexpressing NIH 3T3 cells.** hnRNP-K overexpression showed up- and down-regulation of several genes (*A*). The pathway analysis on the up- and down-regulated genes (as shown in Tables 1 and 2) revealed that the hnRNP-K regulates pathways and biological processes as shown (*B–E*).

TABLE 1
The top 10 most up-regulated genes induced by overexpressing hnRNP-K in NIH 3T3 cells

Gene	Fold change	Biological progress
Mmp3	6.35	Collagen catabolic process; metabolic process; proteolysis
Mmp10	4.00	Collagen catabolic process; metabolic process; proteolysis
Ctgf	3.62	Angiogenesis; cell adhesion; cell differentiation; cell migration; cell-matrix adhesion; fibroblast growth factor receptor signaling pathway; integrin-mediated signaling pathway; lung development
Tmsb4x	3.51	Actin cytoskeleton organization; cytoskeleton organization; regulation of cell migration; sequestering of actin monomers
Itga6	3.32	Cell adhesion molecule activity; up regulation of cell-cell adhesion
Lilrb4	3.23	Immune response
Rasa1	3.19	Vasculogenesis; embryonic development; negative regulation of neuron apoptosis
Cck	2.91	Axonogenesis; eating behavior; neuronal migration
Sdpr	2.89	Up-regulation of transcription from RNA polymerase II promoter
Ptgs2	1.73	Prostaglandin biosynthetic process; cell motility; cyclooxygenase pathway; regulation of inflammatory response

TABLE 2
The top seven most down-regulated genes induced by overexpressing hnRNP-K in NIH 3T3 cells

Gene	Fold change	Biological progress
Col12a1	6.99	Cell adhesion
Prelp	5.14	Cell aging
Igsf10	5.03	Ossification; protein amino acid phosphorylation; vascular endothelial growth factor receptor signaling pathway
Slc47a1	4.56	Multidrug transport; transmembrane transport; transport monomers
Il2rg	3.87	Positive regulation of B cell differentiation; positive regulation of CD4-positive, CD25-positive, alpha-beta regulatory T celldifferentiation; positive regulation of T cell differentiation in the thymus
Tcn2	3.30	Cobalamin transport; cobalt ion transport; ion transport; transport
Kng1	2.97	Blood coagulation; inflammatory response; regulation of blood vessel size; vasodilation

ular events, hnRNP-K was also predicted to affect plasmin and integrin cascades that affect the PTGS2 signaling and cell migration (Fig. 7). According to this hypothesis, hnRNP-K was predicted to affect MMP-3/MMP-10 and cell migration through ERK signaling. We investigated this hypothesis by adopting the MAPK/ERK kinase inhibitor

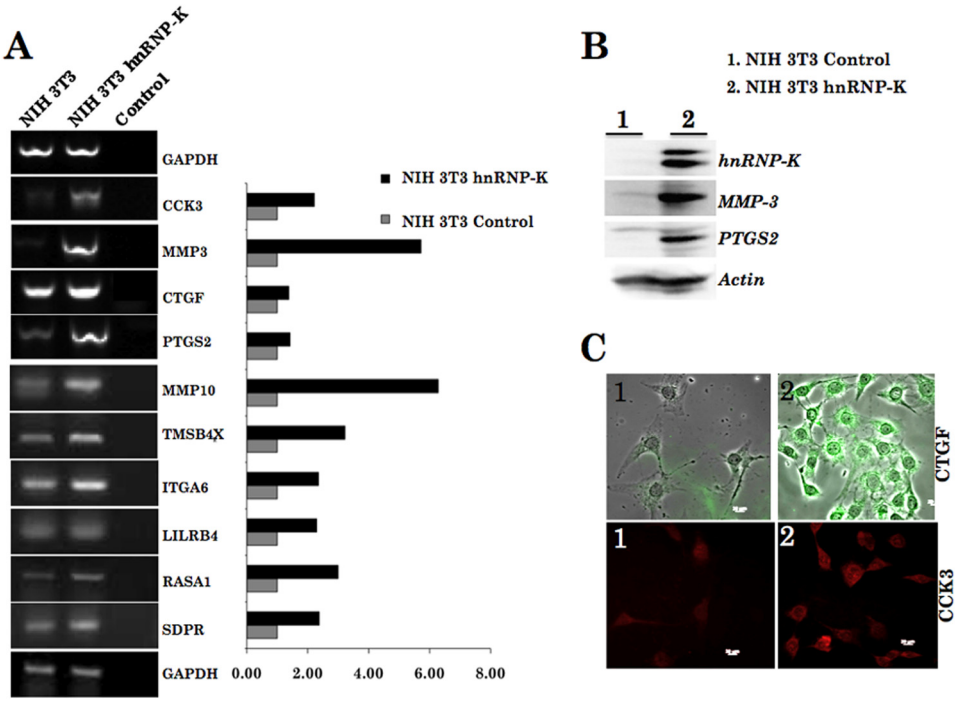


FIGURE 6. **Validation of the hnRNP-K-regulated gene targets identified by cDNA array.** Gene-specific RT-PCR in control and hnRNP-K-overexpressing cells confirmed the up-regulation of the downstream genes as shown (*A*). hnRNP-K-overexpressing cells showed higher expression level of MMP-3 and PTGS2 (Western blotting, *B*), CTGF, and CCK3 (immunostaining, *C*).

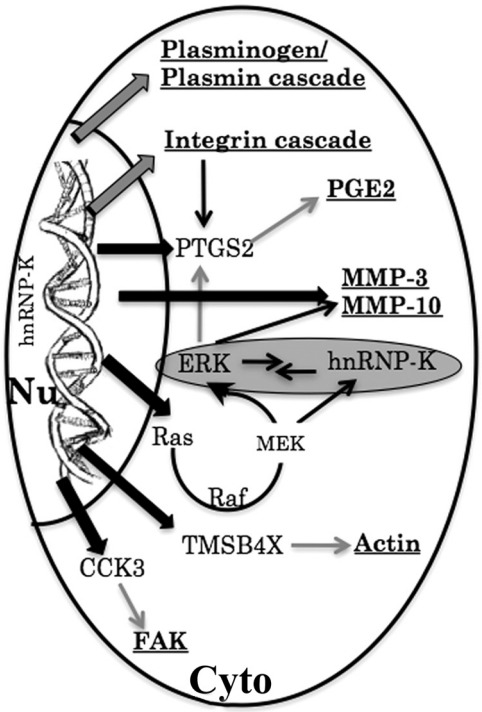


FIGURE 7. **Schematic model showing the role of hnRNP-K in regulation of cell migration through PTGS2, CCK3, RAS, ERK, and MMP-3.**

approach. As shown in Fig. 8*A*, control and hnRNP-K-overexpressing cells were treated with U0126 (1,4-diamino-2, 3-dicyano-1, 4-bis[2-aminophenylthio]butadiene), a highly selective inhibitor of MEK-1/MEK-2. As shown in Fig. 8*A*, U0126 caused inhibition of ERKp44/42, both in the control and hnRNP-K overexpressing NIH 3T3 cells. Of note, hnRNP-K-induced up-regulation of MMP-3 was compromised by treatment with U0126 in the hnRNP-K-overexpressing cells. The result endorses that the hnRNP-K up-regulates MMP-3 expression by activation of ERK kinase. Furthermore, treatment of cells with specific inhibitor of ERK1 and ERK2 (FR180204) led to reduced levels of hnRNP-K (Fig. 8*B*). hnRNP-K-overexpressing cells showed compromised cell migration when treated with MMP-3 inhibitor (MMP-3 inhibitor, Santa Cruz Biotechnology) (Fig. 7*C*), suggesting that hnRNP-K-induced cell migration occurs through ERK-MMP-3 activation.

DISCUSSION

hnRNP-K-overexpressing cells showed enhanced malignant and metastatic properties. Molecular analysis of the hnRNP-K-overexpressing NIH 3T3 derivatives revealed that it regulates a variety of biological cascades of which cell adhesion and invasion signaling pathways that regulate extracellular matrix, cell motility, and angiogenesis were most prominent (Table 1 and Fig. 6). Bioinformatics analysis on the up-regulated genes revealed that the plasminogen and integrin signaling were the most up-regulated pathways in hnRNP-K-overexpressing cells. It has been established that the activation of plasminogen cascade is required for the movement of tumor cells through an extracellular matrix, an essential step in metastatic spread of cancer from the primary tumor to a secondary remote site. Tumor cells induce breakdown of the extracellular matrix by a plasminogen/plasmin and matrix metalloproteinase system. Integrins have been shown to regulate adhesion and invasiveness of tumor cells and stimulate endothelial cells for angiogenesis. It is regulated by a cross-talk between the integrin family and other signaling proteins. Integrin $\alpha 3\beta 1$ was shown to up-regulate COX-2/PTGS in tumor cells (37). COX-2/PTGS

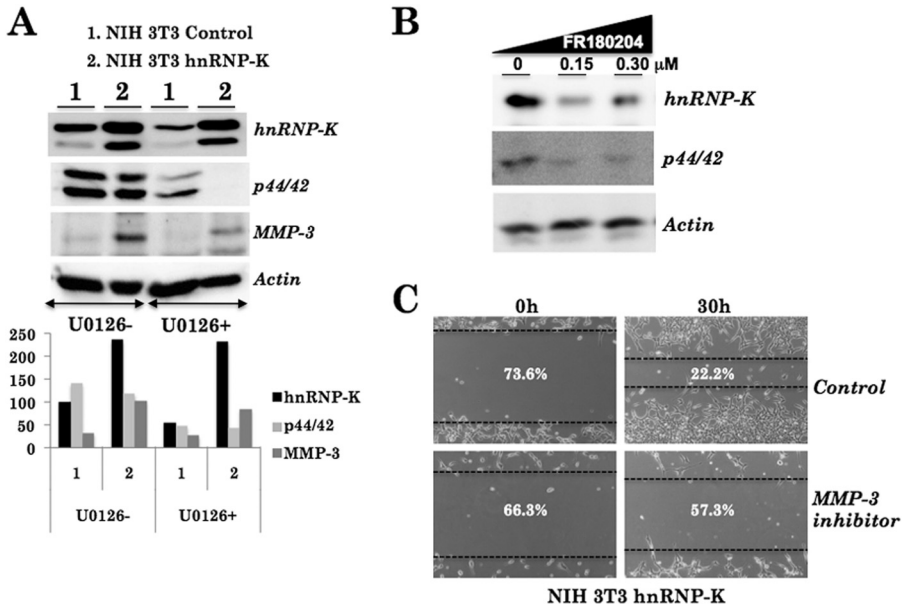


FIGURE 8. **Involvement of MEK and ERK in hnRNP-K-induced cell migration.** Cells treated with MEK1/MEK2 inhibitor, U0126, showed decrease in ERK (p44/42). Increase in MMP-3 in hnRNP-K-overexpressing cells was compromised by treatment with the inhibitor (*A*). ERK1/ERK2 inhibitor FR180204 resulted in decreased level of hnRNP-K confirming cross-talk between MEK, ERK, and hnRNP-K as proposed in the model (Fig. 7). Treatment of hnRNP-K-overexpressing cells with MMP-3 inhibitor resulted in their decreased invasion capacity.

expression is frequently enriched in premalignant, malignant, and metastatic tumors (38). It was also shown that COX-2 induces prostaglandin E2, which has a role in tumor cell invasion and cross-talk to endothelial cells for angiogenesis. COX-2 was also shown to be regulated by Akt and ERK1/2 pathways (39). Furthermore, COX-2 inhibition caused tumor suppression (40). The fact that the overexpression of hnRNP-K resulted in up-regulation of *Cox-2* RNA as well as protein suggesting that it might regulate *Cox-2* at the transcriptional level. A recent study showed that hnRNP-K binds to the *Cox-2* promoter as well as the UTR and regulates its stability (41). We also found that cholecystokinin (CCK3) was up-regulated in hnRNP-K-overexpressing malignant and metastatic cells. CCK3 has been shown to have role in the development of gastric, pancreatic, and colon cancers (42). It has been reported to activate focal adhesion kinase, involved in the progression of malignancies, invasion, and lymph node metastasis (43). These data revealed that the overexpression of hnRNP-K promoted metastasis by induction of signaling cascades involved in extracellular matrix, cell adhesion, invasion, and angiogenesis. Tmsb4x, an actin monomer-binding protein involved in cell migration and angiogenesis, was shown to be associated with tumor development (44, 45).

Matrix metalloproteinases MMP-3 and MMP-10 showed the highest up-regulation in hnRNP-K-overexpressing cells. Role of matrix metalloproteinases has been established in tumor metastasis. They, along with plasminogen and integrin family proteins, regulate degradation of extracellular matrix, invasion of cells through the matrix, and cross-talk with endothelial cells to generate new vessels for blood supply. Matrix metalloproteinases are known to be regulated by the RAS-MEK-ERK cascade. Because RAS also gets up-regulated in hnRNP-K-overexpressing cells, we examined the role of the RAS-MEK-ERK cascade in these cells by employing specific inhibitor of MEK1 and MEK2. As expected and shown in Fig. 8*A*, U0126 caused a reduction in the phosphorylation of ERK1 and ERK2. Similar data were obtained by treating the cells with another specific inhibitor of ERK1 and ERK2, FR180204 (Fig. 8*B*), further endorsing the involvement of the RAS-MEK-ERK cascade. Furthermore, NIH 3T3 cells treated with U0126 lost hnRNP-K-induced up-regulation of MMP-3, demonstrating that the hnRNP-K-regulated cell migration is by the Ras/MEK/ERK-MMP-3 pathway. Mikula *et al.* (46) used chromatin immunoprecipitations to study co-recruitment of hnRNP-K and ERK cascade activity along with the *Egr-1* gene and found that the spatiotemporal binding patterns of ERK cascade transducers (GRB2, SOS, B-Raf, MEK, and ERK) at the EGR-1 locus resemble both hnRNP-K and RNA polymerase II. Furthermore, knockdown of hnRNP-K by siRNA decreased the level of active MEK and ERK suggesting that hnRNP-K regulates MEK and ERK as seen in the present study. Taken together, in continuation to our first identification of hnRNP-K as a gene involved in cell migration in intracellular antibody screening system, we, in the present study, demonstrate that the hnRNP-K regulates metastasis *in vivo* by regulation of extracellular matrix components through the ERK signaling pathway. Thus the inhibitors of hnRNP-K such as siRNA, intracellular antibody or the small molecules might be useful for treatment of metastatic cancers.

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