hnRNP A2 Regulates Alternative mRNA Splicing of TP53INP2 to Control Invasive Cell Migration

Kim Moran-Jones,1 Joan Grindlay,1 Marc Jones,1 Ross Smith,2 and Jim C. Norman1

1Beatson Institute for Cancer Research, Glasgow, United Kingdom and 2School of Chemistry and Molecular Biosciences, University of Queensland, Queensland, Australia

Abstract

Largely owing to widespread deployment of microarray analysis, many of the transcriptional events associated with invasive cell migration are becoming clear. However, the transcriptional drivers to invasive migration are likely modified by alternative splicing of pre-mRNAs to produce functionally distinct patterns of protein expression. Heterogenous nuclear ribonucleoprotein (hnRNP A2) is a known regulator of alternative splicing that is upregulated in a number of invasive cancer types. Here, we report that although siRNA of hnRNP A2 had little influence on the ability of cells to migrate on plastic surfaces, the splicing regulator was clearly required for cells to move effectively on three-dimensional matrices and to invade into plugs of extracellular matrix proteins. We used exon-tiling microarrays to determine that hnRNP A2 controlled approximately six individual splicing events in a three-dimensional matrix-dependent fashion, one of which influenced invasive migration. Here, we show that alternative splicing of an exon in the 5′ untranslated region of a gene termed TP53INP2 is a key event downstream of hnRNP A2 that is necessary for cells to invade the extracellular matrix. Furthermore, we report that the consequences of altered TP53INP2 splicing on invasion are likely mediated via alterations in Golgi complex integrity during migration on three-dimensional matrices. [Cancer Res 2009;69(24):9219–27]

Introduction

One of the features of malignant cells, and one that makes cancer so difficult to treat, is their capacity to migrate invasively through the stroma to form metastases (1). Largely due to the use of microarrays to identify gene expression changes, many of the transcriptional events that drive cancer invasion are now clear. However, much of the complexity of gene expression is generated by mechanisms that act posttranscriptionally. One mechanism for deriving multiple proteins from a single gene is alternative splicing, and recent analyses have indicated that >90% of human genes are subjected to alternative splicing (2, 3). Alterations in mRNA splicing can alter gene expression in a way that contributes to acquisition of an invasive phenotype. A number of splicing-related mutations have been associated with malignancies, and some of these are within splice sites or splicing enhancers/silencers of cancer-related genes. For instance, “cis-acting” mutations have been identified within splice sites of genes for DNA repair (BRCA1; ref. 4), oncogenic kinases (c-kit; ref. 5), and cell-cell adhesion molecules (LI-cadherin; ref. 6), and these alter splicing in a way that likely contributes to malignant disease. Additionally, a number of altered splicing patterns that are not necessarily associated with mutations in the target gene itself are associated with cancer progression (reviewed in refs. 7–9). In some cases, these “trans-acting” events may drive cancer progression by increasing cell migration and invasion. For instance, Ron is a receptor tyrosine kinase that can promote cell migration, and alterations in its splicing produces a constitutively active form of the kinase (ΔRon) that increases invasiveness (10). Furthermore, Rac1b, an alternatively spliced form of the small GTPase Rac1, is generated by treatment of normal mammary epithelial cells with a matrix metalloproteinase (MMP-3), and this may contribute to enhanced cell migration as these cells undergo epithelial to mesenchymal transition (11). There are other examples of cancer-related splicing patterns of genes such as fibronectin, integrin β1, CD44, and uPAR, but the way in which these splicing events are regulated and how they might drive cancer cell migration is currently unclear.

The way in which genes are spliced is dictated by the specific binding of proteins to regulatory elements within their mRNAs. Heterogenous nuclear ribonucleoproteins (hnRNP) are a family of proteins with central roles in processes such as telomere biogenesis, mRNA stability and turnover, and cytoplasmic trafficking of mRNAs, and many hnRNPs participate in splicing control (12). Out of all the hnRNPs, a growing body of literature indicates that hnRNP A2 (and its splice variants hnRNP B1/A2b/B1b; hereafter referred to collectively as hnRNP A2) is upregulated or mislocalized in human cancers [lung (13), colon (14), breast (15), pancreatic (16), and stomach (17) carcinoma] and tumor-derived cell lines, and that hnRNP A2 can be used as a marker of poor prognosis in lung cancer (13). hnRNP A2 has been implicated in the splicing of a number of genes (18) and these events may contribute to the progression of tumors in which hnRNP A2 is upregulated. Here, we describe a requirement for hnRNP A2 in cancer cell invasiveness, and have used genome-wide exon-tiling microarrays to identify hnRNP A2–dependent splicing events that occur only when cells are plated into three-dimensional microenvironments. We have found that matrix-specific alternative splicing of an exon in the 5′ untranslated region (UTR) of tumor protein p53 inducible nuclear protein 2 (TP53INP2) is a key event downstream of hnRNP A2 that is necessary for cancer cells to invade the extracellular matrix.

Methods and Materials

Cell biological methods. The A2780 ovarian carcinoma cell line that was used for the exon array analysis, time lapse microscopy, immunofluorescence, and invasion assays (as indicated) was cultured and transfected as described previously (19, 20). The BE colon carcinoma cells that were used for invasion assays as indicated were transfected using GeneFector (Venn Nova). Inverted invasion assays were performed as described previously (21). Cell-derived matrix (CDM) was generated as described previously (22, 23).
**Time-lapse microscopy.** A2780 cells were seeded onto CDM-coated six-well plates and incubated at 37°C until cells adhered and began migrating (8–10 h). Cells were imaged with a ×10 objective and an inverted microscope (Axiovert S100, Carl Zeiss MicroImaging, Inc.) in an atmosphere of 5% CO2 at 37°C. Cells were imaged at 5-min intervals for 500 min. Andor IQ and Tracker software were used to track cell nuclei.

**Immunofluorescence.** Cells were fixed briefly in ice-cold methanol, permeabilized with 0.2% (v/v) Triton X-100 in PBS for 5 min, and blocked with 1% bovine serum albumin (BSA) in PBS for 1 to 2 h. Primary antibodies were γ-tubulin (T5192; Sigma-Aldrich) or gm130 (610822; BD) in blocking solution at 4°C overnight. Detection was with Alexa-fluor–conjugated secondary antibodies (488 and 594; Invitrogen), and confocal microscopy was performed (Fluoview FV1000, Olympus).

**siRNA duplexes.** Short RNA duplexes against hnRNP A2/B1 were A2#1: 5′GGAUUAUUAAUAACAUUA 3′ and A2#2: 5′GGAGAGUGUUGAGCCCAA3′, respectively. The exon-specific siRNAs were TP53INP2 exon 2 (E2#1): 5′UU-GAGUCCUAGAGCCU 3′ and TP53INP2 exon 2 (E2#2): 5′GGAGAUUGGUU-CACUU 3′. MTA3 exon 5 was [MTA3(E5)]: 5′GGAGAAGAACUCAACAAUU 3′. The EPB41L4A exon 11 [EPB41L4A(E11)] was 5′CCAAUUCACUGUCAAAGAAU 3′. The MAP9 exon 4 [MAP9(E4)] was 5′CCAAUUAUAUCACG-GUA3′. Dharmaco’s nontargeting smartpool was used as a negative control.

**Quantitative reverse-transcription–PCR.** cDNA was prepared using Promega’s ImpromII kit, and the oligo dT primer. Quantitative PCR was performed using SYBR green (Finnzymes, New England Biolabs) and a Chromo4 DNA Engine (Bio-Rad). ΔΔC(t) was determined as described by Livak and Schmittgen (24), using β-actin as a reference point. Primers were as follows:

- **β-actin**: Forward-5′AGCCATGTACGTAGCCATCC3′; Reverse-5′CTCTCAGCTGTGGTG GTGAA3′, amplicon 250 nt;
- **TP53INP2 exon5**: Forward-5′CCTGTTCCCTTATTC TTCACTTCCG 3′; Reverse-5′ATTCCCTCATCTTCTCCCT3′, amplicon 166 nt;
- **TP53INP2 Including exon 2**: Forward-5′CCTCACTGTACCTTGAAGTC3′; Reverse-5′CTGAAGAAGAGGCTGGAGAG3′, amplicon 149 nt;
- **MTA3 exon 5**: Forward-5′ATTCCCTCTTCAGGACGAGC 3′; Reverse-5′TTCCGCCAGAGTTTCTTTT 3′, amplicon 144 nt;
- **EPB41L4A exon 11**: Forward-5′GGCACCTGTTAGCC CGCCCTTTCTTT 3′; Reverse-5′CTGTGGAAGGAATGCTCCATT 3′, amplicon 136 nt;
- **MAP9 exon 4**: Forward-5′GGCGACTTGTTGCTTCTTTTG 3′, amplicon 120 nt.

**Exon-tiling microarray.** Triplicate samples were derived from A2780 cells nucleofected with either the Con or A2#1 siRNA, which had been plated onto CDMs for 18 h. RNA was extracted using Qiagen’s RNeasy kit.
Figure 2. hnRNP A2 knockdown disrupts Golgi morphology and migration of A2780 cells on CDM. A, A2780 cells transfected with either a control (Con) siRNA or one targeting hnRNP A2 (A2) were seeded onto cell-derived matrices, and allowed to adhere for ~10 h before time lapse microscopy. Frames were captured every 5 min over a 500-minute period, and these images were generated from Supplementary Movies S1 and S2. These images are representative stills from the movies. Bar, 50 μm. B, cell tracking software was used to determine cell displacement, migrational persistence, and average speed of migration of A2780 cells on CDM following either control siRNA or knockdown of hnRNP A2 using two independent oligonucleotides (A2#1 and A2#2). By defining a dither as a period of at least 30 min in which a cell moves <2 μm, the incidence and frequency of cells dithering within a 500-min period was determined. Also shown is the amount of time during the 500-min period that the cells spent dithering. Columns, mean; bars, SEM. *, significant difference of \( P < 0.005 \); **, \( P < 0.05 \) (Mann-Whitney test). C, A2780 cells were transfected with either a control or hnRNP A2–targeting siRNA, in combination with a fluorescently tagged Golgi marker and seeded onto cell-derived matrix. Approximately 10 h after seeding, time lapse microscopy was used to capture phase-contrast (phase) and fluorescent (Golgi) frames every 5 min for a 500-min time period, and frames were generated from Supplementary Movies S3 and S4. The presented images are representative stills from the movies. Bar, 50 μm. D, A2780 cells were transfected with either a control or hnRNP A2–targeting siRNA, seeded onto cell-derived matrix, and allowed to adhere for 24 h before being fixed and stained for γ-tubulin and the Golgi marker, gm130. Bar, 20 μm. The graph shows quantification of proportion of cells with scattered or fragmented Golgi complexes following transfection with either a control or two hnRNP A2–targeting (A2#1 and A2#2) siRNAs. Columns, mean; bars, SEM. *, statistical significance of \( P = <0.0001 \) (Mann-Whitney test).
Biotinylated target cRNA was generated and hybridized onto Affymetrix exon arrays using the HuEx-1_0-st-v2 chips by the Cancer Research UK Paterson Institute Microarray Service. Data were normalized using the Robust Multi-Array Average algorithm, and nonpaired t tests carried out between triplicates to find probe sets with expression differences of ±2 (P = 0.01). Splice indices were determined using the equation: splice index = (probe set intensity in sample 1/median gene intensity in sample 1)/(probe set intensity in sample 2/median gene intensity in sample 2), and probe sets with a splice index >1 or <−1 (which also fit the previous fold change and P value criteria) identified. We have deposited the array data generated on the Affymetrix MIAMEVIE web page.

Results

hnRNP A2 is required for invasive migration of tumor cells.

To suppress the cellular levels of hnRNP A2, we used two independent siRNA sequences (A2#1 and A2#2) that targeted all known isoforms of hnRNP A2. Both siRNAs suppressed the expression of hnRNP A2 in A2780 ovarian carcinoma (Fig. 1A) and BE colon cancer (data not shown) cells, and the knockdown was stable for at least 72 hours (Fig. 1A). We did not detect differences in A2780 cell migration on plastic surfaces following knockdown of hnRNP A2 (data not shown), indicating it was not required for the execution of basic processes of cell migration such as actin polymerization. But there are key differences between the characteristics of cells migrating in three-dimensional versus on two-dimensional matrices, and experimental systems measuring migration across plastic surfaces may not accurately model the type of motility that would be deployed by a tumor cell to move away from the primary tumor and form metastases at distant sites. To elucidate the potential contribution that hnRNP A2 may make to the invasive phenotype of aggressive tumors, we used an inverted invasion assay, in which cells migrate upward through Matrigel (predominantly a mixture of laminin and collagen IV) supplemented with fibronectin toward a gradient of serum and epithelial growth factor. Both A2780 (Fig. 1B) and BE cells (Fig. 1C) migrated efficiently into Matrigel/fibronectin plugs, and this index of invasiveness was strongly opposed by knockdown of hnRNP A2 with two independent siRNA sequences.

hnRNP A2 disrupts Golgi morphology and opposes cell migration on three-dimensional matrices.

To study the role of hnRNP A2 on cell migration in the context of a three-dimensional microenvironment, we used CDM, a relatively thick, pliable matrix composed mainly of fibrillar collagen and fibronectin, which recapitulates the extracellular environment. We therefore investigated the requirement for hnRNP A2 in Golgi complex and MTOC positioning during cell migration. To visualize the Golgi complex in migrating cells, we expressed a fluorescent version of galactosyl N-acetyl transferase, an enzyme that is resident to the Golgi complex (25). The asymmetric bipolar phenotype and reduction of pseudopod dominance induced by hnRNP A2 knockdown likely reflected a defect in establishment of cellular polarity. We therefore investigated the requirement for hnRNP A2 in Golgi complex (25) and MTOC positioning during cell migration. To visualize the Golgi complex in migrating cells, we expressed a fluorescent version of galactosyl N-acetyl transferase, an enzyme that is resident to the Golgi complex (26). In control cells, the Golgi complex was seen as a single condensed structure located in the perinuclear region anterior to the direction of migration (Supplementary Movie S3; Fig. 2C). However, knockdown of hnRNP A2 led to scattering of Golgi membranes with the fragmentation being most apparent when cells were dithering (Supplementary Movie S4; Fig. 2D). The positioning of the Golgi complex is thought to be dependent on the integrity of the MTOC. However, even in cells that displayed extensive disruption and scattering of Golgi membranes, the MTOC (Fig. 2D) and overall microtubule organization (data not shown) was not noticeably altered. Taken together, these data indicate that hnRNP A2 plays a key role in generating appropriate organization of the Golgi complex (and not the MTOC), and that this is required for polarized cell migration on cell-derived matrices and for tumor cell invasion into three-dimensional microenvironments.

Table 1. A2780 cells transfected with either a control or hnRNP A2–targeting siRNAs were seeded onto cell-derived matrices and allowed to adhere for 18 h before RNA was harvested

<table>
<thead>
<tr>
<th>Probe set ID</th>
<th>Gene ID</th>
<th>Gene name</th>
<th>Effect of hnRNP A2 knockdown</th>
<th>Fold change</th>
<th>Splice index</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>2478948</td>
<td>NM_020744</td>
<td>Metastasis-associated gene 3 (MTA3)</td>
<td>Skipping of exon 5</td>
<td>↓ 3.6</td>
<td>−1.77</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2790849</td>
<td>NM_001039580</td>
<td>Microtubule-associated protein 9 (MAP9)</td>
<td>Skipping of exon 4</td>
<td>↓ 3.4</td>
<td>−1.48</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>2871029</td>
<td>NM_022140</td>
<td>Erythrocyte membrane protein band 4.1-like 4A (EPB41L4A)</td>
<td>Skipping of exon 11</td>
<td>↓ 2.4</td>
<td>−1.01</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>3883017</td>
<td>NM_021202</td>
<td>Tumor protein p53 inducible nuclear protein 2 (TP53INP2)</td>
<td>Skipping of exon 2</td>
<td>↓ 2.2</td>
<td>−1.10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2749508</td>
<td>NM_021634</td>
<td>Relaxin receptor 1 (RXFP1)</td>
<td>Inclusion of exon 1</td>
<td>↑ 2.7</td>
<td>+1.50</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>2990076</td>
<td>NM_001007157</td>
<td>PHD finger protein 14 (PHF14)</td>
<td>Inclusion of exon 3</td>
<td>↑ 4.0</td>
<td>+2.00</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

NOTE: RNA was labeled and hybridized to Affymetrix HuEx-1_0-st-v2 chips. Genes containing probesets with ±2-fold change between the two conditions and with splice indices of >1 or <−1 were mapped to known exons, generating a list of six genes whose splicing is altered by the loss of hnRNP A2/B1.

When plated onto CDM, A2780 cells assumed a “slug-like” morphology (20), and migrated with a single leading lamellipodium and a rounded rear end (Supplementary Movie S1; Fig. 2A). However, following knockdown of hnRNP A2, A2780 cells had altered morphology (Fig. 2A) and reduced indices of migration (displacement, average speed, and persistence; Fig. 2B). Indeed, hnRNP A2 knockdown cells lacked a rounded cell rear and frequently were seen to extend pseudopods at both ends (Supplementary Movie S2; Fig. 2A). Furthermore, whereas control A2780 cells advanced consistently while maintaining front to back asymmetry, hnRNP A2 knockdown cells frequently stopped migrating and succumbed to prolonged episodes of hesitation or “dithering” during which pseudopod dominance was suppressed (Supplementary Movie S2; Fig. 1A). To obtain a quantitative index of hesitation, we defined a dither as an episode of at least 30 minutes during which a cell moved <2 μm. This analysis revealed that knockdown of hnRNP A2 increased the proportion of cells that dithered during the time-lapse period (500 minutes) cells by ∼2-fold (Fig. 2B). Moreover, hnRNP A2 knockdown cells dithered more frequently and for longer than those transfected with control siRNAs (Fig. 2B).

The positioning of the Golgi complex along with the microtubule organizing center (MTOC) is associated with generation of cell polarity during cell migration (25). The asymmetric bipolar phenotype and reduction of pseudopod dominance induced by hnRNP A2 knockdown likely reflected a defect in establishment of cellular polarity.

When plated onto CDM, A2780 cells assumed a “slug-like” morphology (20), and migrated with a single leading lamellipodium and a rounded rear end (Supplementary Movie S1; Fig. 2A). However, following knockdown of hnRNP A2, A2780 cells had altered morphology (Fig. 2A) and reduced indices of migration (displacement, average speed, and persistence; Fig. 2B). Indeed, hnRNP A2 knockdown cells lacked a rounded cell rear and frequently were seen to extend pseudopods at both ends (Supplementary Movie S2; Fig. 2A). Furthermore, whereas control A2780 cells advanced consistently while maintaining front to back asymmetry, hnRNP A2 knockdown cells frequently stopped migrating and succumbed to prolonged episodes of hesitation or “dithering” during which pseudopod dominance was suppressed (Supplementary Movie S2; Fig. 1A). To obtain a quantitative index of hesitation, we defined a dither as an episode of at least 30 minutes during which a cell moved <2 μm. This analysis revealed that knockdown of hnRNP A2 increased the proportion of cells that dithered during the time-lapse period (500 minutes) cells by ∼2-fold (Fig. 2B). Moreover, hnRNP A2 knockdown cells dithered more frequently and for longer than those transfected with control siRNAs (Fig. 2B).

The positioning of the Golgi complex along with the microtubule organizing center (MTOC) is associated with generation of cell polarity during cell migration (25). The asymmetric bipolar phenotype and reduction of pseudopod dominance induced by hnRNP A2 knockdown likely reflected a defect in establishment of cellular polarity. We therefore investigated the requirement for hnRNP A2 in Golgi complex and MTOC positioning during cell migration. To visualize the Golgi complex in migrating cells, we expressed a fluorescent version of galactosyl N-acetyl transferase, an enzyme that is resident to the trans-Golgi stacks (26). In control cells, the Golgi complex was seen as a single condensed structure located in the perinuclear region anterior to the direction of migration (Supplementary Movie S3; Fig. 2C). However, knockdown of hnRNP A2 led to scattering of Golgi membranes with the fragmentation being most apparent when cells were dithering (Supplementary Movie S4; Fig. 2C and D). The positioning of the Golgi complex is thought to be dependent on the integrity of the MTOC. However, even in cells that displayed extensive disruption and scattering of Golgi membranes, the MTOC (Fig. 2D) and overall microtubule organization (data not shown) was not noticeably altered. Taken together, these data indicate that hnRNP A2 plays a key role in generating appropriate organization of the Golgi complex (and not the MTOC), and that this is required for polarized cell migration on cell-derived matrices and for tumor cell invasion into three-dimensional microenvironments.
hnRNP A2 regulates three-dimensional matrix-dependent alternative splicing of TP53INP2. hnRNP A2 is a multifunctional protein, and one of its key roles is to control alternative splicing via both exon skipping and exon inclusion events (12). A recent study characterizing the requirement for a range of hnRNP proteins in alternative splicing of apoptotic genes has indicated that the splicing events controlled by hnRNP proteins varies between cell lines (27). These indications that hnRNP-regulated splicing is context dependent, in combination with our findings that hnRNP A2 affects cell migration in a three-dimensional matrix-dependent fashion, prompted us to screen for hnRNP A2–regulated alternative splicing events in both two-dimensional and three-dimensional matrix environments.

Figure 3. Knockdown of hnRNP A2 leads to altered mRNA splicing of TP53INP2. A, siRNAs were designed to target the following exons: MTA3(E5), MAP9(E4), EPB41L4A(E11), and TP53INP2(E2). siRNAs were transfected into A2780 cells and the cells plated onto cell-derived matrix. The efficacy of the siRNA knockdowns was confirmed using qRT-PCR to amplify the appropriate exons (qRT-PCR amplicon). Columns, mean; bars, SEM; *, significant difference of $P < 0.01$ (Mann-Whitney test).

B, siRNAs targeting MTA3(E5), MAP9(E4), EPB41L4A(E11), and TP53INP2(E2) were transfected into A2780 cells. These were then seeded onto cell-derived matrices and time-lapse microscopy was used as before to measure the incidence of dithering. C, fluorescence intensity of labeled RNA derived from control (black line) and hnRNP A2 (blue line) knockdown A2780 cells following hybridization to probe sets representing exons from the TP53INP2 gene. The schematic beneath indicates the exon/intron structure of two known TP53INP2 transcripts [ENST00000374810 (exon 2 inclusive; top) and ENST00000374809 (exon 2 skipping; bottom)]. Primers used for qRT-PCR to report on exon 2 inclusive (Incl. E2), exon 2 skipping (Skip E2), and exon 5 containing (Ex 5) are indicated in the light green boxes. The coding regions of the transcripts are in red. D, qRT-PCR validation of the exon-tiling microarray. RNA was extracted from control (black bars) and hnRNP A2 (blue bars) knockdown A2780 cells and analyzed for TP53INP2 transcripts using qRT-PCR. The primers indicated in the green boxes in C were used to amplify portions of exon 5 (common to both known TP53INP2 transcripts), and exon 2 skipping or exon 2 inclusive transcripts. Columns, mean; bars, SEM; *, significant difference of $P < 0.01$ (Mann-Whitney test).
environments. To do this, we plated control or hnRNP A2 knockdown cells onto either plastic or CDMs and compared their alternative splicing profiles using Affymetrix HuEx-1_0-st-v2 exon-tiling chips. These arrays comprise ~5 million probes grouped into 1.4 million probesets, interrogating over 1 million exon clusters (28). Using expression differences of ±2 (for individual probe sets), and splice indices of >1 or <−1 (relative to probe sets in different exons within the same gene), six genes were identified as having altered splicing patterns between control and hnRNP A2 knockdown cells plated onto CDM. Genes that displayed significantly altered splicing indices following hnRNP A2 knockdown are listed in Table 1 and splice maps corresponding to these data are displayed in Supplementary Fig. S1. No alterations in splicing of these genes was detected when cells were plated onto plastic (data not shown).

As can be seen from the data in Table 1 and their corresponding splice maps (Supplementary Fig. S1), knockdown of hnRNP A2 promoted 2 exon inclusion and 4 exon skipping events. To determine whether any of these four exon skipping events were responsible for the influence of hnRNP A2 on cell migration, we designed siRNA oligonucleotides to target the differentially spliced exons of MTA3 (exon 3), MAP9 (exon 4), EBP4H4A (exon 11), and TP53INP2 (exon 2) and transfected them into A2780 cells. Then, having confirmed that these siRNA duplexes were effective to reduce expression of the appropriate targeted exon (Fig. 3A), we assessed migration on CDM. Of these four validated siRNA oligonucleotides, only the one targeting exon 2 of TP53INP2 recapitulated the migratory phenotype (high incidence of dithering) seen following knockdown of hnRNP A2 (Fig. 3B).

Examination of the fluorescence intensity of the exon array probesets with reference to the genomic structure of TP53INP2 (Fig. 3C) indicated that siRNA knockdown of hnRNP A2/B1 (blue line) decreased the number of exon 2-containing transcripts. To confirm this, we designed primers (schematically indicated in the light green boxes in Fig. 3C) to amplify either a portion of exon 5 (Ex 5, common to both transcripts), exon 2–skipping (Skip E2) transcripts, or exon 2–including (Incl. E2) transcripts, and performed quantitative reverse-transcription–PCR (qRT-PCR). This quantitative analysis confirmed that knockdown of hnRNP A2 significantly reduced the number of TP53INP2 transcripts containing exon 2, and correspondingly increased the level of exon 2–excluding transcripts (Fig. 3D). Furthermore, as indicated by the use of PCR primers reporting on exon 5 (Ex 5), the total level of TP53INP2 transcripts (exon 2 inclusive plus exon 2 exclusive) was unaltered by hnRNP A2 knockdown (Fig. 3D).

The influence of hnRNP A2 on cell migration and invasion is mediated via alternative splicing of TP53INP2’s exon 2. To fully investigate the role of TP53INP2’s alternative splicing in cell migration, we needed to selectively suppress the expression of exon 2–containing TP53INP2 transcripts. We designed two siRNA oligonucleotides to target TP53INP2’s exon 2 (E2#1 and E2#2) and assessed their efficacy and selectivity using qRT-PCR. Both of these siRNAs significantly reduced the level of exon 2–containing TP53INP2 transcripts (Incl. E2), without affecting expression of those excluding exon 2 (Skip E2 and Exon 5; Fig. 4A). Using these two validated exon-specific siRNAs, we proceeded to investigate the role that exon 2–containing TP53INP2 transcripts had on cell migration and invasion. Interestingly, we found that selective suppression of exon 2–containing TP53INP2 transcripts recapitulated the same cellular phenotype as was seen following knockdown of hnRNP A2. Indeed, in cells with exon 2–specific TP53INP2 knockdowns, we consistently observed (a) reduced migration speed and

![Figure 4](image-url)
persistence on cell-derived matrices (Supplementary Movies S5 and S6; Fig. 4C); (b) increased dithering and dilatory migratory behavior comprising episodes of reduced back/front polarity (Supplementary Movies S5 and S6; Fig. 4B and D); (c) scattered Golgi membranes particularly in cells that were dithering (Supplementary Movies S7 and S8; Fig. 5A–C); and (d) reduced invasion into Matrigel for both the A2780 and BE tumor cell lines (Fig. 5D).

Discussion

The role of hnRNP A2 in alternative splicing of TP53INP2 transcripts. Although there is well-documented redundancy between the splicing activities of hnRNPs A1 and A2 (18), our data are consistent with a recent report indicating that the function of these two proteins do not completely overlap (27). Indeed, we find that suppression of hnRNP A2 alone is sufficient to generate a migratory phenotype without altering the levels or splicing of hnRNP A1 or by regulating splicing of established hnRNP A1 targets such as caspase-2 or c-Src (29, 30). hnRNP A2 promotes inclusion of the TP53INP2 exon 2, which is located in the 5′ UTR. Furthermore, this event was completely dependent on cells being within a flexible three-dimensional microenvironment, and was not detected when cells were plated onto a rigid substrate. It is now clear that alternative splicing is influenced by extracellular signals via the activation of various signaling pathways (31). Although examples of direct links between signaling kinases and the splicing machinery are somewhat...
rare, it has recently been reported that hnRNP A2 is phosphorylated by the Src-family kinase, Fyn (32). Src signaling is strongly promoted on CDMs (33), and it will be interesting to determine whether matrix-dependent phosphorylation of hnRNP A2 by Src family kinases and other pathways influenced by the extracellular matrix environment alters its capacity to promote exon 2–inclusive splicing of TP53INP2.

As 5’ UTRs are known to direct the efficiency of translation, it is likely that hnRNP A2 splicing will dictate the levels of TP53INP2 protein within the cell. Indeed, inspection of the TP53INP2 5’ UTR sequence indicates the presence of a CCUCCC motif, which could suggest that a functional IRES (34) may reside within exon 2. We have found that siRNAs targeting sequences within the coding region and 3’ UTR of TP53INP2 affect cell migration in a way that is similar to exon 2–specific knockdowns (data not shown). Such striking similarity between the consequences of transfecting siRNAs that specifically target exon 2 and those that target a coding exon (that would be expected to suppress overall TP53INP2 protein levels) suggests that exon 2–containing transcripts are more efficiently translated than those lacking this exon.

How does alternative splicing of TP53INP2 influence Golgi integrity and cell migration? TP53INP2 was originally described as a gene involved in mammalian craniofacial development (35). Subsequent independent studies have found TP53INP2 to be differentially expressed in diabetic rats, and possibly to influence the progression of diabetes by binding to the thyroid hormone receptor and functioning as a transcriptional coactivator within the nucleus (36). More recently, TP53INP2 has been found to have a role outwith the nucleus. Following induction of autophagy, TP53INP2 has been seen to relocate from the nucleus to autophagic vacuoles, and thereupon promote recruitment of the autophagy mediator, LC3, to these structures (37). Although this study highlights a potential role for TP53INP2 outwith the nucleus, the overexpression approaches used by these workers will have masked the influence of alternative splicing on its function. Our results highlight the need to determine the influence of regulated inclusion/knocking of exon 2 on TP53INP2 protein synthesis and localization with respect to cytoplasmic structures such as autophagosomes and the Golgi complex.

Although mislocalization of the Golgi complex often accompanies defects in polarized cell migration, it has long been debated whether this is a cause or a consequence of aberrant cell polarity. More recent defects in polarized cell migration, it has long been debated whether this is a cause or a consequence of aberrant cell polarity. More recent defects in polarized cell migration, it has long been debated whether this is a cause or a consequence of aberrant cell polarity. More recent defects in polarized cell migration, it has long been debated whether this is a cause or a consequence of aberrant cell polarity.
hnRNP A2 Regulates Alternative mRNA Splicing of TP53INP2 to Control Invasive Cell Migration

Kim Moran-Jones, Joan Grindlay, Marc Jones, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-1852

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2009/11/24/0008-5472.CAN-09-1852.DC1

Cited articles
This article cites 43 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/24/9219.full.html#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
/content/69/24/9219.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.