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Northwestern profiling of potential translation-regulatory proteins in human breast epithelial cells and malignant breast tissues: evidence for pathological activation of the *IGF1R* IRES

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Abstract

Genes involved in the control of cell proliferation and survival (those genes most important to cancer pathogenesis) are often specifically regulated at the translational level, through RNA-protein interactions involving the 5'-untranslated region of the mRNA. *IGF1R* is a proto-oncogene strongly implicated in human breast cancer, promoting survival and proliferation of tumor cells, as well as metastasis and chemoresistance. Our lab has focused on the molecular mechanisms regulating *IGF1R* expression at the translational level. We previously discovered an internal ribosome entry site (IRES) within the 5'-untranslated region of the human *IGF1R* mRNA, and identified and functionally characterized two individual RNA-binding proteins, HuR and hnRNP C, which bind the *IGF1R* 5'-UTR and differentially regulate IRES activity. Here we have developed and implemented a high resolution northwestern profiling strategy to characterize, as a group, the full spectrum of sequence-specific RNA-binding proteins potentially regulating *IGF1R* translational efficiency through interaction with the 5'-untranslated sequence. The putative *IGF1R* IRES *trans*-activating factors (ITAFs) are a heterogeneous group of RNA-binding proteins including hnRNPs originating in the nucleus as well as factors tightly associated with ribosomes in the cytoplasm. The *IGF1R* ITAFs can be categorized into three distinct groups: (a) high molecular weight external ITAFs, which likely modulate the overall conformation of the 5'-untranslated region of the *IGF1R* mRNA and thereby the accessibility of the core functional IRES; (b) low molecular weight external ITAFs, which may function as general chaperones to unwind the RNA, and (c) internal ITAFs which may directly facilitate or inhibit the fundamental process of ribosome recruitment to the IRES. We observe dramatic changes in the northwestern profile of non-malignant breast cells downregulating *IGF1R*

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expression in association with acinar differentiation in 3-D culture. Most importantly, we are able to assess the RNA-binding activities of these putative translation-regulatory proteins in primary human breast surgical specimens, and begin to discern positive correlations between individual ITAFs and the malignant phenotype. Together with our previous findings, these new data provide further evidence that pathological dysregulation of *IGF1R* translational control may contribute to development and progression of human breast cancer, and breast metastasis in particular.

Keywords

Breast carcinoma; Sequence-specific RNA-binding proteins; Regulation of gene expression at the translational level

Introduction

Many genes involved in the control of cell proliferation and survival (i.e. those most important to cancer biology) are now known to be regulated specifically at the translational (RNA to protein) level {Gray, 1998 #32; Rajasekhar, 2003 #56}. The mRNAs associated with these genes contain very long 5'-untranslated regions, which function essentially as RNA "promoters", with structural features and regulatory protein binding sites that provide mechanisms by which the translational efficiency of individual mRNAs can be specifically modulated. One of the most intriguing of these translation-regulatory features is the internal ribosomal entry site (IRES), which allows the 40S ribosomal subunit to bypass the highly structured regions of the 5'-UTR and arrive in the immediate vicinity of the initiation codon (Baird et al., 2006; Coleman and Miskimins, 2009; Prats and Prats, 2002). These gene-specific translation-regulatory mechanisms may be pathologically altered in malignant disease (Chappell et al., 2000; Shi et al., 2008), however this area, and in particular its relevance to breast cancer, remains vastly underexplored.

The type 1 insulin-like growth factor receptor (*IGF1R*) is a potent proto-oncogene strongly implicated in the development and progression of human breast cancer through its powerful mitogenic, anti-apoptotic, and potential transforming activities (Belfiore and Frasca, 2008; Jones et al., 2007; Kim et al., 2007; Law et al., 2008; LeRoith and Roberts, 2003; Romanelli et al., 2007; Yanochko and Eckhart, 2006; Zhang and Yee, 2000). *IGF1R* overexpression contributes significantly to the resistance of tumor cells to cytotoxic and targeted therapeutic agents (Gooch et al., 1999; Guix et al., 2008; Kurmasheva et al., 2009; Kurmasheva and Houghton, 2006; Miller et al., 2009; Resnicoff et al., 1995; Rexer et al., 2009; Scotlandi et al., 2002; Shi et al., 2005; Turner et al., 1997; Yuen et al., 2009; Zeng et al., 2009), as well as to the metastatic properties of the malignant cells (Lopez and Hanahan, 2002; Sachdev et al., 2004; Sachdev et al., 2009); chemoresistance and metastasis are two of the most significant clinical problems currently facing breast cancer treatment.

Our lab determined that translation of the human *IGF1R* mRNA is controlled by an IRES (Meng et al., 2005; Meng et al., 2008; Meng et al., 2010). We have positively identified and extensively characterized two of the sequence-specific RNA-binding proteins that interact specifically with the *IGF1R* 5'-UTR and differentially modulate IRES function. Our results established HuR as a potent IRES repressor (Meng et al., 2005), while hnRNP C appears to compete with HuR and activate the *IGF1R* IRES (Meng et al., 2008). However, we realized that there are multiple additional RNA-binding proteins interacting with the *IGF1R* 5'-untranslated sequence and potentially contributing to *IGF1R* translational regulation. The RNA recognition motif is one of the most common protein domains in the eukaryotic genome (Varani and Nagai, 1998), and approximately 8% of all human genes encode RNA-binding proteins, yet relatively few of these have been characterized in any detail (Pullmann et al., 2007). We

set out to examine, as a group, the full spectrum of sequence-specific RNA-binding proteins which may be involved in regulating IGF1R translation, and the IRES in particular.

Here we have categorized the putative *IGF1R* translation-regulatory proteins according to intermolecular interactions within the cell, factors affecting affinity for the 5'-untranslated RNA, whether they bind within or outside of the core functional IRES, and relationship to IRES activation. We observe dramatic alterations in the pattern of protein binding to the *IGF1R* 5'-UTR / IRES accompanying differentiation of non-malignant breast epithelial cells in 3-D culture. Most importantly, northwestern profiles of primary human breast surgical specimens provide evidence for pathological dysregulation of *IGF1R* translational control in malignant breast epithelial cells, and particularly in breast metastases.

Materials and Methods

Recovery of sequence-specific RNA-binding proteins from cells

We tested multiple individual variables and two major protocols (A and B described below) for preparation of whole cell extracts. A series of hybrid (A/B) protocols were compared, and the most optimal of these (Protocol H) selected for use with primary breast surgical specimens.

Whole cell extract Protocol A: Cells were scraped from the surface of the flask and the cell pellet resuspended in 3X volumes of hypotonic lysis buffer (10 mM Tris, pH 7.8; 10 mM KCl, 3 mM MgCl₂, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.25% NP-40, supplemented with AEBSF, leupeptin, aprotinin, and phosphatase inhibitor cocktail (Sigma)) and incubated on ice for 55 min with frequent gentle agitation. The suspension was brought to 2 mM CaCl₂ and DNase I (60 u/ml) and micrococcal nuclease (500u/ml) were added and the incubation continued for 45 min at 4°C. Glycerol was added (to 10%) and NaCl added very slowly with stirring to a final concentration of 500 mM, and the incubation continued for an additional 35 min. Triton X-100 (1%), deoxycholate (0.5%), and SDS (0.1%) were added and incubated further for 35 min. Finally, 5 mM EDTA and 5 mM EGTA (to inactivate MNase) were added and incubated 15 min, then microfuged at top speed for 10 min and the supernatant recovered. Microdialysis was performed using Slide-a-lyzer units (Pierce) in 40 mM Tris pH 7.8; 50 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, 7 mM 2-mercaptoethanol, 0.1% NP-40. Glycerol (10%) was re-added and the samples frozen rapidly in dry ice/ ethanol and stored at -80°C.

Whole cell extract Protocol B: The cell pellet was resuspended in 5X volumes of cold RNP lysis buffer (10 mM Tris, pH 7.4; 100 mM NaCl, 2.5 mM MgCl₂, 14 mM 2-mercaptoethanol, 0.5% Triton X-100, supplemented with protease and phosphatase inhibitors). The suspension was incubated on ice for 7 min, then sonicated (Branson) on ice for 5 seconds × 2 at a setting of 2. The sample was then microfuged at top speed for 10 min at 4°C, the supernatant recovered, rapidly frozen, and stored at -80°C.

Whole cell extract Protocol H (hybrid, human): Begin as in Protocol B. After sonication, glycerol was added to 10% and NaCl added slowly to 500 mM final concentration with stirring. The sample was microfuged, and microdialysis performed on the supernatant as described above in Protocol A. This hybrid protocol also effectively extracts IGF1R itself, enabling both northwestern and western analysis to be performed on the same sample (in fact the same blot).

High resolution Northwestern profiling of sequence-specific RNA-binding proteins

Extracts prepared from cultured cells or surgical specimens were separated by SDS/PAGE (10%) and transferred to nitrocellulose membrane using carbonate buffer. Proteins were renatured by sequential incubation of the blot first in isotonic detergent-free buffer (1X TBS, overnight), followed by a complex physiological buffer (Ren/Block solution: 40 mM Tris-Cl, pH 7.4, 60 mM KCl, 1 mM MgCl₂, 10 mg/ml BSA, 6% glycerol, 0.25 mM spermidine, 50

µg/ml tRNA (as non-specific competitor), 75 µM ATP, 75 µM GTP, 0.01% NP-40, 250 mM beta-mercaptoethanol) for 2 hours at room temperature and then overnight at 4°C. The radiolabeled RNA probe (sense orientation) representing the human *IGF1R* 5'-untranslated sequence (including the IRES) was synthesized by in vitro transcription using T7 RNA polymerase (Promega) in the presence of ³²P-UTP. The radiolabeled probe was added to the Ren/Block solution, MgCl₂ concentration adjusted to 1 – 5 mM as indicated, and incubated with the blot for 2 hours at room temperature on a rotating platform. The blot was washed once in fresh Ren/Block solution plus 1 mg/ml heparin for 10 min, and then twice more in Ren/Block solution for 5 min each, then wrapped and exposed to autoradiographic film with intensifying screens.

Three-dimensional culture of breast cell lines

MCF-10A and T47D cells (obtained from ATCC) were placed in three-dimensional culture essentially as described (Debnath et al., 2002; Petersen et al., 1992). Briefly, 10⁶ cells were resuspended in 1.2 ml Matrigel (B-D) and plated in 35 mm dishes with 2 ml media provided above the matrix. Development of acinar structures (MCF-10A) or cell clusters (T47D) were followed over time by phase contrast microscopy. Following 15 days incubation, embedded cells were harvested by depolymerization of the matrix using Cell Recovery Solution (B-D) according to the manufacturer's instructions, and used for preparation of whole cell extracts (Protocol A) and northwestern profiling. Alternatively, 10⁵ cells were seeded in 1 ml of 5% Matrigel on top of 0.5 ml 100% Matrigel. Northwestern results obtained from surface plated cells were identical to those embedded within the Matrigel. For confocal microscopic examination of 3-D cultures, cells were seeded on top of small quantities of Matrigel spread over the surface of 8-well chamber slides.

Acquisition of human breast surgical specimens

A series of primary invasive breast carcinoma specimens or metastases (snap frozen or embedded in OCT) was acquired through the UAB Comprehensive Cancer Center Tissue Procurement Facility under an IRB-approved protocol. Normal breast specimens from reduction mammoplasty were acquired for use as controls. All specimens were examined by a Board-certified pathologist with special expertise in breast cancer (A.F.) to assess the prevalence and distribution of normal or malignant breast epithelium. Gross microdissection was performed where appropriate to enrich for the epithelial component. From three to twelve consecutive sections were transferred directly from cryostat to cold lysis buffer with protease and phosphatase inhibitors and subjected to whole cell extraction (Protocol H) optimized for recovery of the *IGF1R* translation-regulatory proteins.

Results

IGF1R translational regulation

The human *IGF1R* mRNA contains an extraordinarily long 5'-untranslated region (5'-UTR) of 1,040 nucleotides (Fig. 1, contrast with an "average" 5'-UTR of ~75-250 nucleotides) (Cooke et al., 1991). The *IGF1R* 5'-UTR is projected to adopt a complex secondary structure with a high degree of internal base-pairing and an overall thermodynamic stability of greater than -500 kCal / mole (Meng et al, 2010). Indeed, we have found that this highly structured sequence represents a substantial impediment to ribosomal scanning and IGF1R translation initiation (Meng et al., 2008). In addition, an upstream open reading frame (uORF) within the 5'-UTR, encoding a 28 amino acid peptide with no relationship to the IGF1R protein, tends to derail the ribosome before it reaches the IGF1R coding sequence. An extremely G-C rich sequence immediately following the uORF termination codon decreases even further the efficiency of IGF1R translation initiation mediated by conventional ribosomal scanning.

However, we have also discovered an internal ribosomal entry site (IRES) contained within the 5'-UTR which allows the ribosome to bypass the obstacles presented by the highly structured 5'-UTR, arriving in the immediate vicinity of the IGF1R initiation codon (Meng et al., 2005). Importantly, the IRES is not constitutively active (that would obviate the physiological function of the complex 5'-UTR), but rather is regulated through dynamic interactions with a series of sequence-specific RNA-binding proteins (Meng et al, 2005; Meng et al, 2008). In this manner, the IRES allows the translational efficiency of the *IGF1R* mRNA to be modulated independently of the global controls on the overall rate of protein synthesis in the cell.

Characterization of sequence-specific translation-regulatory proteins by the Northwestern assay: Recovery of functional RNA-binding proteins from cells

There have been two major factors impeding progress in the study of gene-specific translational regulation. First, to our knowledge, no systematic efforts had been made to establish an optimal protocol for extraction and recovery of sequence-specific RNA-binding proteins from cells. Second, the UV-crosslinking methods we have relied on to covalently capture sequence-specific RNA-binding proteins on their preferred substrates are effective only for U residues or uridine-derivatives, thus RNA-binding proteins with affinity for A-, C-, G-rich, or mixed sequences without neighboring U residues are very likely to be missed by these technologies. We set out to address each of these limitations.

We utilized the northwestern assay to characterize the RNA-binding proteins potentially regulating internal ribosomal entry and translational efficiency of the *IGF1R* mRNA. The northwestern is a solid phase functional assay which is complementary to UV crosslinking, capable of measuring the RNA-binding activities of individual proteins in a sequence-specific manner, but without constraints on target sequence composition. The northwestern is performed under highly stringent conditions, with inclusion of a large excess of tRNA as a non-specific competitor throughout the protocol, and addition of the polyanion heparin to the first wash, to eliminate weak or unstable RNA-protein interactions. While RNA-binding activity may reflect the abundance of the RNA-binding protein, it is well established that RNA-binding activity can be dynamically modulated e.g. through post-translational modifications to the RNA-binding protein, and there are documented instances in which RNA-binding activity does not correlate, and may even be inversely correlated, with abundance of the RNA-binding protein (Abdelmohsen et al., 2007; Jo et al., 2008; Meng et al., 2005). Having performed ~50 different northwestern experiments over the course of these studies, we came to recognize a very reproducible pattern of proteins interacting specifically with the *IGF1R* 5'-untranslated sequence. We have numbered the *IGF1R* northwestern bands from 1 through 10, beginning with highest molecular weight (Band 1 series ~125->200 kDa) to the lowest molecular weight (Band 10 ~25 kDa), using letter suffixes to designate additional bands in similar molecular weight range (i.e. Band 4b has a slightly lower apparent molecular weight than Band 4).

We evaluated three interventions to assist in biochemical extraction of these *IGF1R* translation-regulatory proteins from the nucleus: salt to disrupt ionic interactions, nuclease digestion to facilitate dissociation from high molecular weight nucleic acids, and detergents (particularly deoxycholate) to disintegrate microsomes associated with the rough ER, a proportion of which will tend to co-fractionate with the nucleus through attachment to the nuclear membrane. Incubation with 500 mM NaCl alone was sufficient for extraction of a significant number of the *IGF1R* 5'-UTR-binding proteins, however, a further increase in NaCl to 1M, digestion with DNase and micrococcal nuclease, or supplemental detergents were each effective in facilitating recovery of additional sequence-specific RNA-binding proteins from the nucleus (Fig. 2A).

In general, cytoplasmic lysates yielded substantially fewer and less intense bands on northwestern than were recovered from the nucleus. Surprisingly, we found that the interventions which enhanced recovery of RNA-binding proteins from the nucleus were actually associated with loss of RNA-binding activity in the cytoplasm (Fig. 2B). Certain predominantly high molecular weight proteins were sensitive to micrococcal nuclease digestion, while a group of lower molecular weight proteins were sensitive to addition of high salt. To explain these findings, we have postulated that incubation with high salt or digestion with nuclease results in dissociation of cytoplasmic RNA-binding proteins from their native target mRNAs, and that a system apparently exists within the cytoplasm to rapidly inactivate (or degrade) any such free (unbound) translation-regulatory proteins (see Discussion).

Therefore, we began experimenting with preparation and analysis of whole cell extracts, utilizing the same interventions described above, but eliminating the differential centrifugation step that would separate the cytoplasmic and nuclear components. Indeed we found that there were a number of proteins which yielded relatively intense bands on the *IGF1R* northwestern when recovered from whole cell extracts, but were not detected at all, or with much lower intensity, in either the nucleus or the cytoplasm. This suggested that extraction of cytoplasmic proteins in the presence of nuclear components may protect these RNA-binding proteins from inactivation.

Sequence-specificity of RNA-binding proteins recognizing the *IGF1R* 5'-UTR: internal vs. external ITAFs

To assess the sequence-specificity of these RNA-protein interactions, we bisected the *IGF1R* 5'-UTR probe into two fragments. Several of the most intense bands detected by northwestern bound selectively to the smaller probe (90 nucleotides) representing the core functional IRES (Fig. 3). A distinct subset of bands was detected with the larger probe representing the ~950 nucleotides outside of the core IRES sequence. The term ITAF (IRES *trans*-acting factor) has been coined to describe RNA-binding proteins involved in regulation of IRES-mediated translation initiation (Pilipenko et al., 2000). As applied to *IGF1R* translational regulation, it appears there is a series of "external" ITAFs that bind the 5'-untranslated sequence outside of the IRES, as well as a group of "internal" ITAFs which bind within the core functional IRES.

Note that the pattern obtained using the full-length 5'-UTR (including the IRES) as probe is a composite of that obtained using the two individual probes, but that variation in magnesium concentration affects considerably the relative affinity of the external and internal ITAFs for the RNA. When magnesium concentration is low, the 5'-UTR is inherently less structured, and the core functional IRES becomes more accessible to the internal ITAFs (e.g. Bands 2, 4, and 5). Conversely, the higher degree of secondary structure that accompanies higher magnesium concentration favors binding of the external ITAFs (e.g. Bands 1, 7, and 9).

We have recently completed a systematic dissection of the sequence elements critical for IRES function, and found that point mutations or deletions of critical residues within the core functional IRES are associated with dramatic changes (positive or negative) in affinity of the internal ITAFs (but not the external ITAFs) for the *IGF1R* 5'-UTR (Meng et al, 2010), further confirming the sequence-specificity of these RNA-protein interactions.

Characterization of the *IGF1R* ITAFs by intermolecular association and subcellular fractionation

We felt it was important to further characterize the *IGF1R* 5'-UTR-binding proteins as to their relationship to other molecules and structures within the cell. Perhaps the best characterized group of RNA-binding proteins are the hnRNPs, which originate in the nucleus and appear to

be heavily involved in processing of nascent mRNA (hnRNA). They are designated hnRNP A through U, roughly correlating with their molecular weights, and exist in a complex of well-defined stoichiometry (Dreyfuss et al., 1993). Dreyfuss and coworkers have determined that the entire complex of ~20 hnRNP proteins can be immunoprecipitated using monoclonal antibody 4F4 to hnRNP C, one of the core components of the complex and one of the most prevalent of the hnRNPs (Pinol-Roma et al., 1988). To determine whether some of the *IGF1R* ITAFs might belong to the hnRNP family, we used the 4F4 antibody to IP the hnRNP complex, and then separated those proteins by SDS / PAGE and performed northwestern analysis using the *IGF1R* 5'-UTR probe (Fig. 4A). We found that select bands amongst the northwestern profile were immunoprecipitated with the 4F4 antibody. One of these was Band 8d, which we have determined by western analysis performed sequentially on the same blot to be hnRNP C itself, and which we had previously identified using crosslinking technology as an *IGF1R* 5'-UTR-binding protein and potent IRES-activator (Meng et al, 2008). Bands 2 and 4b which co-immunoprecipitate with hnRNP C are also, by definition, members of the hnRNP family of RNA-binding proteins. However, none of the remainder of the *IGF1R* 5'-UTR-binding proteins detectable by northwestern appear to be hnRNPs.

We were also very interested to determine whether some of the cytoplasmic *IGF1R* ITAFs detected by northwestern might be inherently associated with the translation machinery (Fig. 4B). We performed standard fractionation procedures to separate the 100,000 × g supernatant (S100) from the polysomal pellet, then salt washed the polysomes, and used puromycin with high salt to disassemble polysomes into individual ribosomal subunits. Each of these fractions was then separated on SDS/PAGE and analyzed by northwestern using the *IGF1R* IRES probe. Bands 2 and 8a are absolutely restricted to the nuclear fraction. However, Bands 4 and 5, two internal ITAFs which are among the most dominant features of the *IGF1R* northwestern profile, co-fractionate with polysomes. Curiously, Band 4 is quantitatively recovered in the ribosomal salt wash, while Band 5 remains closely associated with the ribosomes even after incubation with puromycin. It appears that a proportion of Bands 9 and 10 may also be tightly associated with ribosomes. Note that the *IGF1R* 5'-UTR-binding proteins are not generally present free in the S100 cytoplasmic supernatant.

There are now several known examples of canonical ribosomal proteins shown to be capable of sequence-specific interactions with non-ribosomal RNAs (including viral IRESs) and even extra-ribosomal regulatory functions (Fukushi et al, 2001; Chiocchetti et al, 2005; Wanzel et al, 2008). Therefore, we repeated the northwestern analysis on these same fractions using a 17.5% polyacrylamide gel to determine whether any of the canonical integral ribosomal proteins might function as ITAFs for *IGF1R*. Only one very low molecular weight (~15 kDa) protein was detected by northwestern, however, and it fractionated with the nucleus.

We also performed an IP-Northwestern analysis using an anti-phosphoserine antibody to determine which of the *IGF1R* ITAFs might be targets for serine phosphorylation. We observed that a subset of the high molecular weight external ITAFs (the Band 1 series) as well as Band 9 appear to contain phosphoserine residues (data not shown). A number of reports have demonstrated that the RNA-binding activity and/or subcellular localization of certain RNA-binding proteins can be influenced by signal transduction culminating with phosphorylation of serine residues (Abdelmohsen et al., 2007; Jo et al., 2008).

In conclusion, the putative *IGF1R* ITAFs detected by northwestern are a remarkably heterogeneous group of RNA-binding proteins, varying in their subcellular localization, intermolecular associations, and biochemical properties.

Distinctions in the northwestern profiles of T47D and MCF-10A cells: correlation with pathological activation of the *IGF1R* IRES and the malignant phenotype

We had previously demonstrated differences in the pattern of protein binding to the *IGF1R* 5'-UTR as detected by crosslinking, comparing the non-transformed human mammary epithelial cell line MCF-10A, in which IGF1R expression is low (normal) and IRES activity borders on the threshold of detection, to the breast carcinoma cell line T47D, in which IGF1R protein levels are elevated ~4 fold and IRES activity is substantially increased (Meng et al., 2008). We had observed differences in the relative RNA-binding activities of HuR (IRES repressor) and hnRNP C (IRES activator), along with two additional crosslinked proteins of ~60 and ~85 kDa which mirror HuR and hnRNP C respectively.

However, the northwestern assay reveals multiple RNA-binding proteins not detectable by crosslinking, particularly with use of the optimized whole cell extraction protocol, so we felt it was important to re-examine the status of the *IGF1R* translation-regulatory proteins in these two model cell lines using this technology. Many of the bands on the northwestern profiles of the T47D and MCF-10A cells exhibit very similar molecular weights and relative intensities, however, certain distinctions are observed (Fig. 5). The relative RNA-binding activities of Bands 2, 5, and 7 are considerably higher in the malignant T47D cell line than in the non-malignant MCF-10A cells, while just the reverse is true for Band 4, which is considerably more intense in MCF-10A. These results suggest that Bands 2, 5, and 7 may be positive ITAFs, involved in promoting IRES activity, while Band 4 may represent a negative ITAF, capable of inhibiting IRES function.

Alterations to the northwestern profile of MCF-10A cells undergoing acinar differentiation in 3-D culture

The distinction between the normal and malignant phenotype is not evident in traditional monolayer tissue culture, where even normal breast epithelial cells fail to differentiate, having no contact with basement membrane material and no opportunity to become spatially organized. However, a number of investigators have now established the utility of a three-dimensional culture system to demonstrate the capacity of non-malignant breast epithelial cells to undergo a normal process of differentiation (Debnath et al., 2002; Petersen et al., 1992). We wished to determine whether *IGF1R* translational regulation might be altered when breast cells differentiate in 3-D culture. MCF-10A and T47D cells were seeded in Matrigel, and proliferation of the cells over time was assessed by both phase contrast (Fig. 6A,B) and confocal microscopy (Fig. 6C,D,E). As expected, the non-transformed MCF-10A breast epithelial cells formed relatively uniform small spherical clusters resembling acini of normal breast tissue, and polarization of cells on the circumference of these acinar structures was evident from the basal localization of the nuclei. Many of these acinar structures showed evidence of luminal clearing via separation and apoptosis of centrally-located cells. In contrast, the malignant T47D breast tumor cells provided the same 3-D culture conditions formed large heterogeneous amorphous clusters, with no evidence of differentiation, polarity, apoptosis, or limitation of proliferation.

Cells cultured in 3-D were recovered by dissolution of the matrix and extracts were prepared in parallel from both standard monolayer and 3-D cultures of both cell lines. The changes in northwestern profile accompanying acinar differentiation of MCF-10A cells in 3-D were dramatic (Fig. 6F). The high molecular weight Band 1 series, as well as Bands 2, 7, and 8a were completely lost. Notably, Band 4, the internal ITAF which we had speculated might be an IRES inhibitor, was selectively retained in the northwestern profile of the MCF-10A cells grown in 3-D culture. At the same time, IGF1R protein levels, measured by western performed sequentially on the same blot, decreased precipitously. Alpha-tubulin levels increased substantially when MCF-10A cells were grown in 3-D, confirming that the decreases in IGF1R

levels and northwestern band intensities were not the result of limited recovery of cells from 3D culture or underloading of protein on the gel. In contrast, changes in the northwestern profile of T47D cells grown in 3-D culture were limited, and IGF1R levels decreased only modestly, indicative of the failure of these breast tumor cells to differentiate appropriately in this environment. These results demonstrate the utility of the northwestern assay to discern changes in activities of sequence-specific RNA-binding proteins in association with phenotypic alterations, and a clear distinction in *IGF1R* translational regulation between the malignant and non-malignant breast epithelial cells.

Northwestern profiling of primary human breast surgical specimens: correlation with malignancy and metastasis

Our ultimate objective was to extend these studies to primary human breast specimens. First it was necessary to establish that functional RNA-binding proteins could be successfully extracted from surgical specimens, and that the northwestern technique would be sensitive enough to yield informative patterns from limited quantities of biological material. Recognizing the inherent cellular heterogeneity of both normal breast tissue and malignant breast tumor specimens, we attempted to use laser microdissection to isolate the epithelial component for extract preparation. We found that even minimal processing (i.e. hematoxylin staining) required for recognition of tissue architecture was incompatible with recovery of functional RNA-binding proteins. However, informative high resolution northwestern patterns were obtained from unstained specimens which had been grossly microdissected on the basis of distribution of normal or malignant epithelial cells as assessed by H&E staining of an adjacent cryosection. (Fig. 7A). We observed in the primary tumor specimen a discrete subset of the bands normally seen with cultured T47D cells. In particular, Band 4b, which is typically only a minor component of the northwestern profile of cultured breast tumor cells, was very intense in the primary tumor, along with Bands 7, 9, 9b, and 10, while a number of other bands usually prominent in the northwestern profile of cultured cells were completely absent in the primary tumor.

We extended this study to include a series of primary breast tumors, breast metastases, and non-malignant breast tissues (Fig. 7B). Band 4b was again one of the more dominant bands in 3 out of 6 of the malignant specimens (2 primary tumors and 1 metastasis), while Band 7 (an external ITAF) was highly active in two of the malignant specimens (1 primary tumor and 1 metastatic lesion), both of which had been described as poorly differentiated. Interestingly, Bands 2 and 8d (hnRNP C) were evident in each of the breast metastases, but none of the primary breast tumors or normal specimens. We have characterized hnRNP C as a potent activator of the *IGF1R* IRES. IGF1R protein levels were substantially increased in each of the metastatic lesions, approaching that observed in the T47D cells.

With the exception of Band 9, a low molecular weight external ITAF, the northwestern profiles of the two normal breast specimens (obtained from reduction mammoplasty) were negative. While the relatively low density of breast epithelial cells within the normal architecture of the breast tissue may be partly responsible, these results are also compatible with the relative inactivity of the *IGF1R* IRES expected in these normal tissues. However, the positivity of the northwestern profile obtained from the adjacent uninvolved tissue of specimen F, and its similarity (particularly Band 5) to that of the frankly malignant tissue is notable. This result is suggestive of a field effect (Heaphy et al., 2009), or perhaps a germ-line alteration predisposing this patient to dysregulation of IGF1R translation and the development of breast malignancy; this patient presented at age 47 with bilateral multifocal invasive carcinoma.

The clinical / pathological data associated with each of the human breast specimens are summarized in Table 1. Representative H&E images of each of the breast specimens are shown in Fig. 7C.

Discussion

RNA-binding translation-regulatory proteins: cytoplasmic susceptibility and nuclear protection

It would be dangerous to the phenotypic integrity of the cell for translation-regulatory proteins to retain their RNA-binding activities and have an opportunity to associate with other RNA molecules once freed from their original intended target mRNAs in the cytoplasm where translation is actively taking place. Such an occurrence could transform highly orchestrated translational regulation into chaotic protein synthesis. Thus it may benefit the cell to have in place a quality control system to rapidly and efficiently inactivate or destroy these RNA-binding proteins (through post-translational modification or targeted degradation) once they become free in the cytoplasm. Such a system could be responsible for the “cytoplasmic susceptibility” we have observed, wherein recovery of functional sequence-specific RNA-binding proteins from the cytoplasm is highly inefficient, particularly if interventions which favor dissociation of RNA-binding proteins from their native target sequences are utilized.

Within the nucleus, however, RNA-binding proteins are purposefully becoming associated with individual mRNA molecules for the first time in a controlled fashion. This cotranscriptional tagging of nascent mRNA with specific RNA-binding proteins likely serves to indicate the suitability of the mRNA for translation, the appropriate intracellular location for that mRNA to be translated, and the relative efficiency with which it should be translated. Thus it would be important for conditions within the nucleus to favor retention of RNA-binding activity, and indeed, recovery of active RNA-binding proteins from the nucleus is relatively efficient. In the preparation of whole cell extracts, where nuclear and cytoplasmic components are recovered together, we have observed that nuclear protection is generally dominant over cytoplasmic susceptibility, though this varies somewhat for individual proteins and between individual cell lines. In practical terms, this experience has taught us that cytoplasmic RNA-binding proteins are typically recovered much more efficiently in the context of a whole cell extract. However, if our interpretation is correct, the cellular systems responsible for nuclear protection and cytoplasmic susceptibility may carry far greater biological relevance.

A two-component model of *IGF1R* translational regulation: internal and external ITAFs

We have begun to characterize a series of sequence-specific RNA-binding proteins which may be involved in regulation of *IGF1R* expression at the translational level. On the basis of our northwestern results, we are able to divide the putative translation-regulatory proteins interacting with the *IGF1R* 5'-untranslated RNA sequence into two distinct categories: External ITAFs, which bind outside of the core functional IRES (nucleotides 1 – 959), and Internal ITAFs, which bind within the core IRES (nucleotides 951 – 1040).

The Band 1 series of proteins are high molecular weight external (~125->200 kDa) ITAFs which we have determined to be targets for serine phosphorylation. These proteins likely modulate the overall conformation of the 5'-untranslated region of the *IGF1R* mRNA, ultimately controlling the accessibility of the core functional IRES to the requisite internal ITAFs and the 40S ribosomal subunit. The large size of these cytoplasmic proteins and their sensitivity to micrococcal nuclease implies that they may contact a relatively large surface area of the RNA, perhaps bringing together disparate segments of the 5'-untranslated sequence, serving as a scaffold to coordinate intermolecular interactions involved in assembly of the translation-regulatory complex on the *IGF1R* 5'-UTR. Band 7 is a relatively low molecular weight external ITAF which disappears in association with downregulation of *IGF1R* expression when MCF-10A cells differentiate in 3-D, and its appearance in the northwestern profiles of human breast surgical specimens correlates with poor differentiation. The other low molecular weight external ITAFs (Bands 9 and 10), which are somewhat less sequence-

specific, may serve as general RNA chaperones or helicases, facilitating IRES function by helping to untangle the complex 5'-UTR sequence.

The internal ITAFs, which generally occupy the intermediate molecular weight range of the northwestern profile, likely participate directly in the fundamental process of ribosome recruitment, either facilitating or interfering with IRES-mediated translation initiation. Band 5 is tightly associated with ribosomes, displays elevated RNA-binding activity in T47D cells (in which the IRES is pathologically upregulated (Meng et al, 2008)), and is detectable in the northwestern profiles of nearly all of the breast tumors and metastatic lesions, thus correlating with the malignant phenotype. Band 2 is a nuclear protein which displays greater RNA-binding activity in T47D cells, disappears completely when MCF-10A cells downregulate IGF1R and cease proliferation upon differentiation in 3-D culture, and is detectable in the northwestern profiles of each of the breast metastases analyzed in this study. Band 8d, an internal ITAF which we had previously characterized as the potent IRES activator hnRNP C, is also observed in the northwestern profiles of each of the metastatic lesions of breast tumor origin, in association with substantial increases in IGF1R protein levels.

In contrast, Band 4, which we have characterized as a component of the ribosomal salt wash, is unique amongst the internal ITAFs detected by northwestern in demonstrating an apparent negative correlation with *IGF1R* IRES activity. The RNA-binding activity of Band 4 is considerably higher in the non-malignant MCF-10A cells, which express low (normal) levels of IGF1R and borderline detectable IRES activity (Meng et al, 2008), than in malignant T47D cells. Furthermore, Band 4 selectively retains its RNA-binding activity upon terminal differentiation of MCF-10A cells in 3-D culture, when IGF1R protein levels drop precipitously and many of the other northwestern bands are disappearing. It is conceivable that these positive and negative IRES-regulatory proteins may initiate assembly of distinct ribonucleoprotein complexes on the *IGF1R* 5'-UTR, consistent with the model we originally proposed to explain how *IGF1R* translational regulation could be dynamically modulated (Meng et al., 2003). The properties of the *IGF1R* ITAFs are summarized in Table 2.

Clinical / pathological implications: Dysregulation of *IGF1R* translational control in breast malignancy and metastasis

Our results suggest that cumulative changes in the activities of RNA-binding translation-regulatory proteins may occur during development and progression of human breast cancer, contributing to altered patterns of gene expression and the molecular pathogenesis of the disease. It is conceivable that such RNA-binding translation-regulatory proteins may have the capacity to function as oncogenes (or tumor suppressors) themselves, and that aberrant translational control of IGF1R may contribute to initiation and progression of breast malignancy. As little as 50% increase in IGF1R protein levels may allow cells to proliferate in an anchorage-independent manner (i.e. adopt a transformed phenotype) (Rubini et al., 1997). Furthermore, IGF1R is essentially required for establishment and maintenance of the malignant phenotype, as numerous oncogenes including SV40 large T antigen and *ras* fail to transform *IGF1R*^{-/-} cells (Sell et al., 1993). Pathological activation of the IRES could represent a previously unrecognized mechanism for altered *IGF1R* expression in breast cancer, one which would not be evident from standard microarray analyses, and which may be even more important in metastatic lesions than in the primary tumor.

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Abbreviations

IGF1R type 1 insulin-like growth factor receptor

IRES	internal ribosomal entry site
ITAF	IRES <i>trans</i> -activating factor
5'-UTR	5'-untranslated region
uORF	upstream open reading frame

Fig 1. *IGF1R* translational regulation: Architecture of the 5'-untranslated region of the human *IGF1R* mRNA

The structural and regulatory features contained within the 5'-untranslated region of the human *IGF1R* mRNA are indicated (drawn to scale, coordinates refer to distance in nucleotides). The conventional mechanism for translation initiation in eukaryotes involves a process of scanning by the 40S ribosomal subunit from the beginning of the mRNA (blue arrow) to the initiation codon. The complex *IGF1R* 5'-UTR presents multiple obstacles to ribosomal scanning. Domains 2 and 3 are highly stable secondary structures with individual free energies of -98.4 and -159.4 kCal/mol and a combined free energy of -257.8 kCal/mol. (For reference, a structure with $dG > -60$ kCal / mol is considered a significant obstacle to ribosomal scanning.) The upstream open reading frame (uORF) encodes a 28 amino acid peptide with no relationship to *IGF1R*. The uORF initiation codon is in good Kozak consensus, thus scanning ribosomes which reach this point will tend to initiate translation (upon joining of the 60S ribosomal subunit), and then dissociate from the mRNA upon reaching the uORF termination codon (red arrow). Ribosome dissociation is facilitated by the G-C rich element which immediately follows the uORF termination codon. We have referred to this G-C rich segment (56 out of 59 residues are G or C) as the "third obstacle". Finally, however, there exists an internal ribosomal entry site (IRES, nucleotides 951 – 1040) positioned just upstream of the authentic *IGF1R* initiation codon. The IRES provides an alternative mechanism for *IGF1R* translation initiation, recruiting the 40S ribosomal subunit directly to the vicinity of the AUG (green arrow), allowing it to bypass the obstacles presented by the remainder of the 5'-UTR. Importantly, the IRES is not constitutively active, but rather is specifically regulated through competitive and cooperative interactions with multiple RNA-binding proteins recognizing specific sequence elements within or outside of the *IGF1R* IRES. The RNA-binding proteins regulating *IGF1R* translation are the subject of this investigation.

Detergent	--	--	--	--	--	--	P	T/D/S	P/S
DNase	--	--	--	--	+	+	--	--	--
MNase	--	--	--	+	--	+	--	--	--
NaCl (M)	.15	.5	1.0	.5	.5	.5	.15	.5	1.0

Fig. 2. Characterization of Sequence-specific RNA-binding proteins by the Northwestern assay: Development of a protocol for optimal recovery of functional RNA-binding proteins from cells
A: Northwestern assay evaluating recovery of sequence-specific RNA-binding proteins from the nucleus. Following hypotonic lysis of T47D breast tumor cells in the presence of 0.25% NP-40, pelleted nuclei were resuspended in an extraction buffer containing various concentrations of NaCl (lanes *a* through *c*). For lanes *d* through *f*, nuclei were incubated with DNase I and / or micrococcal nuclease prior to 500 mM NaCl. For lanes *g* through *i*, nuclear remnants following extraction with 150, 500, or 1000 mM NaCl respectively were resuspended and extracted with additional detergents: T-PER (Pierce), 1% Triton X-100/ 0.5% deoxycholate/ 0.1% SDS, or 50% T-PER/ 1% SDS respectively. Numerical designations for the proteins interacting specifically with the *IGF1R* 5'-untranslated RNA are indicated to the left. Note that the majority of bands detected by *IGF1R* northwestern required at minimum 500 mM NaCl for efficient extraction from the nucleus, while several individual RNA-binding

proteins (particularly Bands 4b and 8a, see arrows) required additional NaCl, nuclease digestion, and/or supplemental detergent for efficient recovery. **B:** Cytoplasmic lysates obtained by hypotonic lysis of T47D cells were incubated with 500 mM NaCl and / or micrococcal nuclease, and then analyzed by northwestern. Note that the same manipulations which enhance recovery of RNA-binding proteins from the nucleus result in loss of RNA-binding activity in the cytoplasm. This phenomenon, which we refer to as “cytoplasmic susceptibility”, may be explained by targeted inactivation of RNA-binding translation-regulatory proteins once they become dissociated from their intended target sequences in the cytoplasm.

Fig. 3. Sequence-specificity of RNA-binding proteins regulating IGF1R translation: internal and external ITAFs

Northwestern assays were performed to assess the sequence-specificity of the RNA-protein interactions taking place on the 5'-untranslated region of the *IGF1R* mRNA. Three different RNA probes were synthesized: the full length 5'-UTR (nucleotides 1 – 1040, panels A and B), the isolated 90 nucleotide sequence (nucleotides 951-1040) to which the core functional IRES has been delimited (panel C), and the 5'-untranslated sequence from which the core functional IRES sequence has been deleted (nucleotides 1-959, panel D). Distinct patterns of protein binding are observed for the core IRES and the remainder of the 5'-UTR, while the pattern of protein binding to the full-length 5'-UTR (including the IRES) is a composite of those two series of bands. Potential translation-regulatory proteins binding within the *IGF1R* core functional IRES are designated “internal” ITAFs (IRES *trans*-acting factors), while those binding 5'-untranslated sequence outside the core functional IRES are referred to as “external” ITAFs. The relative affinity or accessibility for the internal versus the external ITAFs can be modulated by varying the magnesium concentration (and thereby the level of secondary structure of the RNA). Molecular weight markers are shown on the left, and the positions of the *IGF1R* ITAFs are indicated on the right. Whole cell extracts: lanes i, MCF-10A, Protocol A; lanes ii, T47D, Protocol A; lanes iii, T47D, Protocol B.

Fig. 4. Characterization of *IGF1R* ITAFs by intermolecular association

A: T47D whole cell extract (Protocol B) was subjected to immunoprecipitation using the 4F4 monoclonal antibody (mouse IgG1) to hnRNP C, which immunoprecipitates the hnRNP complex (composed of >20 proteins). Aliquots of the input, pellet, and supernatant were separated by SDS / PAGE and analyzed by northwestern using the full-length *IGF1R* 5'-UTR / IRES probe. Three of the *IGF1R* northwestern bands are selectively recovered by 4F4 immunoprecipitation (lane b): Band 8d, which is hnRNP C itself (as confirmed by western analysis performed sequentially on the same blot), as well as Bands 2 and 4b. Significant immunodepletion of hnRNP C (8d) and Band 2 is observed in the corresponding supernatant (lane b of right panel). Lanes a and c of each panel are negative control immunoprecipitations and supernatants (no immunodepletion) obtained using antibodies to two other RNA-binding proteins (3A2 mouse monoclonal IgG1 to HuR and goat polyclonal C-18 to TIAR). The experiment was repeated with identical results **B:** MCF-10A and T47D cells were subjected to conventional fractionation of the cytoplasm by differential ultracentrifugation to yield an S100 supernatant (lanes S) and polysomal pellet (lanes P). Aliquots of polysomal pellets were incubated in 500 mM KCl, and centrifuged again to yield a supernatant (ribosomal salt wash, lane A) and pellet (salt-washed ribosomes, lanes R). Finally, an aliquot of salt-washed ribosomes was incubated with puromycin and 500 mM KCl, and centrifuged again to yield free ribosomal subunits (lane U). Each of these fractions was analyzed by northwestern using the *IGF1R* IRES probe. Note that Bands 2 and 8a are exclusively nuclear, Band 5 is tightly associated with ribosomes, while Band 4 is quantitatively recovered in the ribosomal salt wash. A proportion of Bands 9 and 10 are also tightly associated with the ribosomes.

Fig. 5. Distinctions in the pattern of protein binding to the *IGF1R* 5'-UTR / IRES between non-malignant and malignant cultured breast cells

Whole cell extracts (Protocol B) of T47D (malignant) or MCF-10A (non-malignant) breast epithelial cells were prepared and subjected to northwestern analysis (4×10^5 cell equivalents per lane) using the *IGF1R* 5'-UTR / IRES probe. Though the patterns are generally quite similar, certain bands exhibit significant differences in relative intensity (RNA-binding activity). In particular, Bands 2, 5, and 7 are considerably more intense in T47D, correlating with the substantially higher IRES activity (Meng et al, 2005) and IGF1R protein levels in these cells. Band 4 is considerably more intense in MCF-10A cells, suggesting this may represent an IRES inhibitor.

Fig. 6. Alterations in RNA-binding activities of *IGF1R* translation-regulatory proteins accompanying differentiation of non-malignant breast epithelial cells in 3-D culture

A, B: Single-cell suspensions of MCF-10A and T47D cells were embedded in Matrigel in 35 mm dishes and allowed to proliferate or differentiate for 15 days. Representative phase contrast micrographs reveal the distinction between the small, relatively uniform acinar structures formed by the non-transformed MCF-10A breast epithelial cells versus the large, amorphous clusters formed by the T47D breast tumor cells. **C, D:** MCF-10A and T47D cells were also plated on the surface of a thin layer of Matrigel on 8-well chamber slides to facilitate examination by confocal microscopy. Following fixation, nuclei were stained with DAPI, and a series of confocal images was captured demonstrating the organized morphology of the

MCF-10A acinar structures versus the disorganized clusters of T47D cells. The immunofluorescent micrographs are shown in reverse image (nuclei are dark). Bar = 50 μ m. **E:** MCF-10A cells were seeded on an 8-well chamber slide in which Matrigel had been intentionally spread so as to cover only a portion of the surface of the well, forming a “beach” with the inorganic growth surface. This allowed us to capture, in a single image, acinar structures on the surface of the Matrigel and a continuously proliferating monolayer on the adjacent uncoated portion of the slide. This illustrates the capacity of the non-transformed MCF-10A cells to differentiate and cease dividing when provided the appropriate environmental context, versus indefinite proliferation in standard monolayer culture. **F:** Northwestern analysis revealing changes in the pattern of protein binding to the *IGF1R* 5'-UTR / IRES accompanying differentiation of non-malignant MCF-10A breast epithelial cells in 3-D culture. MCF-10A and T47D cells were recovered from 3-D culture following dissolution of the matrix (see Materials and Methods) and whole cell extracts prepared (Protocol A). Extracts were also prepared in parallel from cells harvested from standard monolayer culture (2-D). Equal numbers of cell equivalents of these extracts were analyzed by northwestern using the *IGF1R* 5'-UTR / IRES probe. Results of western analyses for IGF1R (C-20 rabbit polyclonal, Santa Cruz) and alpha tubulin (B-5-1-2, Sigma) performed sequentially on the northwestern blot are shown below.

Fig. 7. Northwestern analysis of sequence-specific translation-regulatory proteins binding to the *IGF1R* 5'-UTR in primary human breast tumors, metastases, and normal tissues

A: A primary human breast tumor specimen (poorly differentiated ductal carcinoma, specimen D, Table 1) was cryosectioned and subjected to varying degrees of processing to enable laser microdissection. Processed materials were then recovered from microdissection cap or slides and subjected to whole cell extraction (Protocol H) before performing northwestern analysis. Lane **g**: cryosections washed in water and immediately recovered from slides and transferred to lysis buffer (containing protease and phosphatase inhibitors). Lane **h**: cryosections (still in OCT) directly transferred to lysis buffer with no histological processing. Lane **i**: Sections stained with hematoxylin (standard protocol, but omitting final xylene rinse), then immediately

recovered from slides and transferred to lysis buffer. Lane *j*: Sections stained with hematoxylin including final xylene rinse. Lane *k*: Sections stained with both hematoxylin and eosin. Lane *l*: Section stained with H&E and subjected to laser microdissection, with captured malignant epithelial cells selectively transferred to lysis buffer. Reference samples: Lane *a*: HeLa nuclear extract; Lane *b*: K-1 (T47D derivative) Protocol H whole cell extract; Lane *c*: T47D Protocol H whole cell extract (2.5×10^5 cell equivalents); Lane *d*: same as Lane *c*, one-fourth sample loaded; Lane *e*: same as Lane *c*, one-tenth of sample loaded; Lane *f*: same as Lane *c*, one-twenty-fifth of sample loaded. **B**: Human breast surgical specimens (snap frozen or embedded in OCT) were examined by H&E staining to assess distribution of epithelial cells and guide gross microdissection as appropriate. Relative proportion of malignant breast epithelial cells within each tumor sample ranged from ~85 to near 100%. Consecutive cryosections (unprocessed) were then transferred directly to lysis buffer and used for preparation of whole cell extract (Protocol H). Equivalent aliquots were subjected to northwestern analysis using the full-length *IGF1R* 5'-UTR / IRES probe. Specimens A through C are metastatic lesions of breast tumor origin. Specimens D through F(T) are primary invasive breast tumors. Specimen F(N) is adjacent histologically uninvolved tissue obtained from the same block as primary tumor F(T). Specimens G and H are normal breast tissue obtained from reduction mammoplasty. The results are representative of four replicate northwestern analyses performed on the same series of specimens. Molecular weight markers are indicated on the left, while the positions of *IGF1R* ITAFs are indicated adjacent to each panel. Results of western analyses for IGF1R (N-20 rabbit polyclonal, Santa Cruz) and alpha tubulin (B-5-1-2, Sigma) performed sequentially on the northwestern blot are shown below. **C**: Representative H&E stained sections of each of the human breast surgical specimens. Bar = 50 μ m.

Table 1

Clinical / pathological characteristics of human breast surgical specimens

Age/Race/Gender	Diagnosis	ER PR Her2	Differentiation	Size	Lymph nodes	Notes
<u>Metastatic lesions of breast tumor origin</u>						
A. 59WF	Ductal carcinoma Metastatic to Lung	ER-Neg PR-Neg Her2-Non-ampl	Poor			previous history breast carcinoma (1 yr prior); tumor nodules in lung morphologically identical to original tumor (also ER,PR-Neg)
B. 60F	Lobular carcinoma Metastatic to Ovary	ER-Pos PR-Pos Her2-Non-ampl	Moderate			previous history breast carcinoma (7 yr prior); involves both ovaries fallopian tube, omentum; ascites
C. 50F	Adenocarcinoma Metastatic to Ovary	ER-Pos PR-Pos	Moderate			involves both ovaries alpha-lactalbumin-positive CA-125-negative
<u>Primary invasive breast carcinoma</u>						
D. 52BF	Ductal carcinoma invasive	ER-Neg PR-Neg Her2-Non-ampl	Poor	8cm	2/22-pos	
E. 50WF	Ductal carcinoma invasive	ER-Pos PR-Neg Her2-Amplified	Poor	7.5cm	16/19-pos	angiolymphatic invasion
F. 47WF (T)	Bilateral, multifocal carcinoma	ER-Pos PR-Pos Her2-Non-ampl	Moderate Moderate	3.5cm (Left) 9.0cm (Right)	4/28-pos 0/3 (+)	ductal and lobular features predominately lobular features
<u>Adjacent uninvolved or Normal breast tissue</u>						
F. (N)	Adjacent uninvolved tissue from primary tumor F					histologically non-malignant
G. 35WF	Normal breast (reduction)					benign parenchyma no mass lesions
H. 26WF	Normal breast (reduction)					fibrosis no mass lesions

Table 2

Properties of Sequence-specific RNA-binding proteins interacting with the IGF1R 5' -UTR / IRES

Designation	MW	Relation to IRES	Intracellular Localization	Status in Breast cell lines	Change with 3-D culture MCF-10A	Status in Breast Specimens	Inter-molecular Association	Nuclear extraction/Cytoplasmic Susceptibility	Affect of Mg ⁺⁺ on Affinity
Band 1 series	~125-250 kDa	External ITAFs	Cytoplasmic		Lost upon acinar differ.		serine phosph.	susceptible to MNase in cytoplasm	favored by ↑ Mg ⁺⁺ (tRNA structure)
Band 2	~105 kDa	Internal ITAF	Nuclear	T47D>MCF-10A	Lost upon acinar differ.	positive 3 / 3 breast metastases	hnRNP		favored by ↓ Mg ⁺⁺
Band 3	~95 kDa	Internal ITAF	Nuclear	MCF-10A> T47D				Lost upon microdialysis	
Band 4	~85kDa	Internal ITAF	Cyto/Nucl	MCF-10A>T47D (inverse corr. IRES activity)	Selectively retained upon acinar differ.	positive 3 / 3 breast metastases	Ribosomal salt wash		favored by ↓ Mg ⁺⁺
Band 4b	~75kDa	Internal ITAF	Nuclear	T47D>MCF-10A		highly active 1/3 metastases 2/3 1° tumors (corr. w/ poor diff)	hnRNP	recovery ↑ by 1M NaCl, DOC or nuclease	
Band 5	~70 kDa	Internal ITAF	Cyt>Nuc	T47D>MCF-10A		active in 3/3 metastases 2/3 1° tumors (corr. malignant phenotype)	Tightly associated Ribosomes	susceptible to MNase in cytoplasm	favored by ↓ Mg ⁺⁺
Band 6 series	~60 kDa	Internal ITAFs	Cyto / Nucl			active in 2/3 metastases 2/3 1° tumors	highly labile	susceptible to high salt in cytoplasm	
Band 7	~ 50 kDa	External ITAF	Nuc>Cyt	T47D>MCF-10A	Lost upon acinar diff.	highly active 1/3 metastases 1/3 1° tumors (corr. w/ poor diff)			favored by ↑ Mg ⁺⁺ (↑ RNA structure)
Band 8a	~45 kDa	Internal ITAF	Cyto / Nucl		Lost upon acinar diff.			recovery ↑ by 1M NaCl, DOC or nuclease	
Band 8d (hnRNP C)	42 kDa	Internal ITAF	Nuc>Cyt			active in 3/3 metastases	hnRNP	susceptible to high salt in cytoplasm	
Band 9	~37 kDa	External ITAF	Nuc>Cyt	Retained		readily detectable	serine phosph; asso Ribosomes		favored by ↑ Mg ⁺⁺
Band 10	~25 kDa	External ITAF	Cyto / Nucl			readily detectable	asso Ribosomes	recovery ↑ by high salt	