A mRNA and Cognate MicroRNAs Localize in the Nucleolus

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We previously discovered that a set of 5 microRNAs are concentrated in the nucleolus of rat myoblasts. We now report that several mRNAs are also localized in the nucleoli of these cells as determined by microarray analysis of RNA from purified nucleoli. Among the most abundant of these nucleolus-localized mRNAs is that encoding insulin-like growth factor 2 (IGF2), a regulator of myoblast proliferation and differentiation. The presence of IGF2 mRNA in nucleoli was confirmed by fluorescence *in situ* hybridization, and RT-PCR experiments demonstrated that these nucleolar transcripts are spliced, thus arriving from the nucleoplasm. Bioinformatics analysis predicted canonically structured, highly thermodynamically stable interactions between IGF2 mRNA and all 5 of the nucleolus-localized microRNAs. These results raise the possibility that the nucleolus is a staging site for setting up particular mRNA-microRNA interactions prior to export to the cytoplasm.

Introduction

mRNA typically exits the nucleus by diffusion from its transcriptional birth and splicing sites culminating in engagement by nuclear pore complexes, without apparent stops at any intranuclear sites along the way.[1-6] However, there have been occasional reports of mRNAs being localized in nucleoli. Some of these observations were made during heat shock,[7,8] but there also have been reports of mRNAs as well as mRNA splicing factors localized in the nucleoli of healthy cells.[9-11] The possibility that some mRNAs might traffic through the nucleolus prior to export was first hinted in a study on the reactivation of the avian erythrocyte nucleus by their fusion with HeLa cells.[12] In the resulting heterokaryons, only when a nucleolus had formed in the activated erythrocyte nucleus did mRNAs transcribed from the chicken genome reach the cytoplasm and undergo translation. Although this result is subject to other interpretations, the notion that some mRNAs may circuit through the nucleolus prior to export has remained with us.

During a study of myogenic cell biology we discovered, to our surprise, that a substantial portion of a key microRNA, miR-206, is concentrated in the nucleoli of rat myoblasts,[13] and we subsequently found that at least 4 other microRNAs are also concentrated in the nucleoli of these cells.[14] More recently, microRNAs have also been found localized in the nucleoli of HeLa and MCF7 cells.[15,16] These findings, together with the aforementioned occasional reports of mRNAs in nucleoli of various cell types, led us to investigate the presence of mRNAs in the nucleoli of rat myoblasts. We find that particular mRNAs are concentrated in the nucleoli and have further characterized one of these, the mRNA for insulin-like growth factor-II (IGF2), a potent regulator of myogenic stem cell proliferation. RT-PCR revealed that the nucleolus-associated IGF2 mRNA is spliced, suggesting that it arrives in nucleoli from its nucleoplasmic sites of splicing. Bioinformatics analysis revealed that the 3′ UTR of IGF2 mRNA contains target sites for all 5 of the nucleolus-localized micro-RNAs.

Results and Discussion

To learn whether any mRNAs might be concentrated in the nucleoli of these cells, we isolated nuclei[17] and prepared highly purified nucleolar and nucleoplasmic fractions as detailed previously.[14] RNA from both nuclear fractions as well as from the cytoplasmic fraction were subjected to denaturing gel electrophoresis and the U3, U2 and U1 RNAs were identified based on their migration. As shown in **Figure S1**, the nucleolar fraction

Table 1. Nucleolus-enriched RNAs[1]

RNA	Abundance[2]	Chromosome	Transcript ID
small nuclear 7-2 (MRP RNase) RNA[3]	13.6	5	J05014
Rattus norvegicus 5.8S ribosomal rRNA[3]	13.3	–	V01270.1
U3B small nucleolar RNA[3]	12.7	10	K00780
actin, alpha 1, skeletal muscle (Acta1), mRNA	11.8	19	ENSRNOT00000024084 /// NM_019212
rRNA promoter binding protein (aka ribin), mRNA	11.6	6	NM_147136.1 ///LOC257642
Ba1-665 mRNA (rat specific-unknown function)	10.5	16	ENSRNOT00000041882
insulin-like growth factor 2 (Igf2), mRNA	9.4	1	ENSRNOT00000027602 /// NM_031511
high mobility group AT-hook I, related sequence 1 (hmga-1) mRNA	9.0	X	ENSRNOT00000038940
ribosomal protein L36-like mRNA	8.7	7	ENSRNOG00000033555
histone cluster 1, H2bm (Hist1h2bm) mRNA	8.3	17	ENSRNOT00000049785
rCG65904-like protein mRNA	7.3	2	ENSRNOT00000029028
heparan sulfate 6-sulfotransferase 3 mRNA	7.2	15	GENSCAN00000091390 /// XM_001078545
hydrocephalus-inducing protein (aka hydin) mRNA	6.5	19	GENSCAN00000040128 /// XM_001077360
olfactory receptor 1320 mRNA[3]	6.4	8	ENSRNOT00000048785 /// NM_001000471
homeo box D13 (Hoxd13) mRNA	6.3	3	ENSRNOT00000002155
keratin complex 1, acidic, gene 18, mRNA (predicted)	5.9	2	LOC683212
14-3-3 protein sigma mRNA (predicted)	5.7	6	GENSCAN00000032606 /// NM_001013941
peptidylprolyl isomerase (cyclophilin)-like 6 mRNA	5.7	20	GENSCAN00000029291 /// ENSRNOG00000043186
olfactory receptor 760 mRNA[3]	5.1	3	ENSRNOT00000030909 /// NM_001001069
ceroid-lipofuscinosis, neuronal 5 (Cln5) mRNA	4.5	2	ENSRNOT00000012928
mast cell protease 8 (Mcpt8) mRNA	4.4	15	ENSRNOT00000027950 /// NM_021598
vomeronasal 1 receptor, j5 mRNA[3]	4.3	1	ENSRNOT00000040738 /// NM_001008929

[1]Nucleolar/nucleoplasmic ratios >1.
[2]Relative nucleolar abundance (log$_2$).
[3]28S and 18S rRNAs, numerous small nucleolar RNAs, several olfactory receptor mRNAs and unannotated RNAs are omitted from this list for clarity.

contained no detectable U2small nuclear RNA and little if any U1, these 2 RNAs being markers for nucleoplasmic spliceosomes that are not known to appreciably traffic through nucleoli. In contrast, the nucleoplasmic fraction had no detectable U3 RNA, a RNA species that is involved specifically in pre-rRNA processing in the nucleolus.[18] Based on these and our previous quality control results[14] we regard these rat myoblast nucleolar and nucleoplasmic fractions to be of very high purity. Nucleolar and nucleoplasmic RNAs were then hybridized to rat genomic microarrays designed to detect the entire transcriptome including multiple splicing variants of both polyadenylated and non-polyadenylated RNAs.

As expected, the microarray analysis revealed small nucleolar RNAs as the most abundant species. Topping the list were 2 familiar, abundant small nucleolar RNAs, 7–2 and U3 and next in abundance were hundreds of less abundant small nucleolar RNAs (**Table 1**), as expected.[19] However, also present were several mRNAs ranging from between 5–10% of the abundance of 7–2 and U3 snoRNAs down to levels much lower (**Table 1**). The two most abundant mRNAs were α-actin mRNA (which may suggest its precocious transcription in these myoblasts- a point to which we shall return) and insulin-like growth factor-II (IGF2). Because IGF2 has been well-established as autocrine growth factor in myoblasts[20,21] we chose it for further study.

We sought to corroborate the nucleolar presence of IGF2 mRNA by performing fluorescence *in situ* hybridization. **Figure 1B** shows the *in situ* hybridization patterns obtained using probes to 28 S rRNA, as a positive control. As expected, strong signals were seen both in nucleoli and cytoplasm and, as can be seen, these were RNase-sensitive. No appreciable signal was seen

using a scrambled sequence hybridization probe (**Fig. 1C**). As shown in **Figure 1A**, IGF2 mRNA signal was observed in the nucleoli in many cells (white arrows), while other cells appeared to have both nucleoplasmic and nucleolar signal. The IGF2 mRNA hybridization was RNase-sensitive (**Fig. 1A**). An enlargement of a portion of the field is shown in Figure S2. In the course of multiple experiments and average of 70–80% of the cells displayed IGF2 mRNA hybridization signals in the nucleoli. It is possible that the absence of detectable nucleolar signal in the remaining cells reflects cell cycle variation although this was not pursued further. IGF2 mRNA hybridization signal was also present in the cytoplasm of all cells, and immunostaining confirmed the presence of IGF protein (**Figure S3**)as expected from previous work on the expression of this protein in myoblasts[20,21]. In additional experiments we confirmed the nucleolar localization of IGF2 mRNA using a second *in situ* hybridization protocol as described in the Materials and Methods and shown in **Figure S4**, again revealing strong, RNase-sensitive IGF2 signals in the nucleoli.

We next used RT-PCR to interrogate the state of splicing of the nucleolus-localized IGF2 mRNA. As shown in **Figure 2**, the IGF2 transcripts in the nucleolar fraction generated RT-PCR products consistent with them being spliced mRNAs (compare with the products generated from cytoplasmic RNA). It is to be noted that the nucleoplasmic IGF2 and transcripts also generated RT-PCR products consistent with them being spliced mRNAs, indicating that splicing of IGF2 pre-mRNA is very rapid, whether co- or post-transcriptional. (Our nucleoplasmic fraction contains the chromatin.) Even a method as sensitive as RT-PCR has its limits of detection and thus we cannot conclude that there
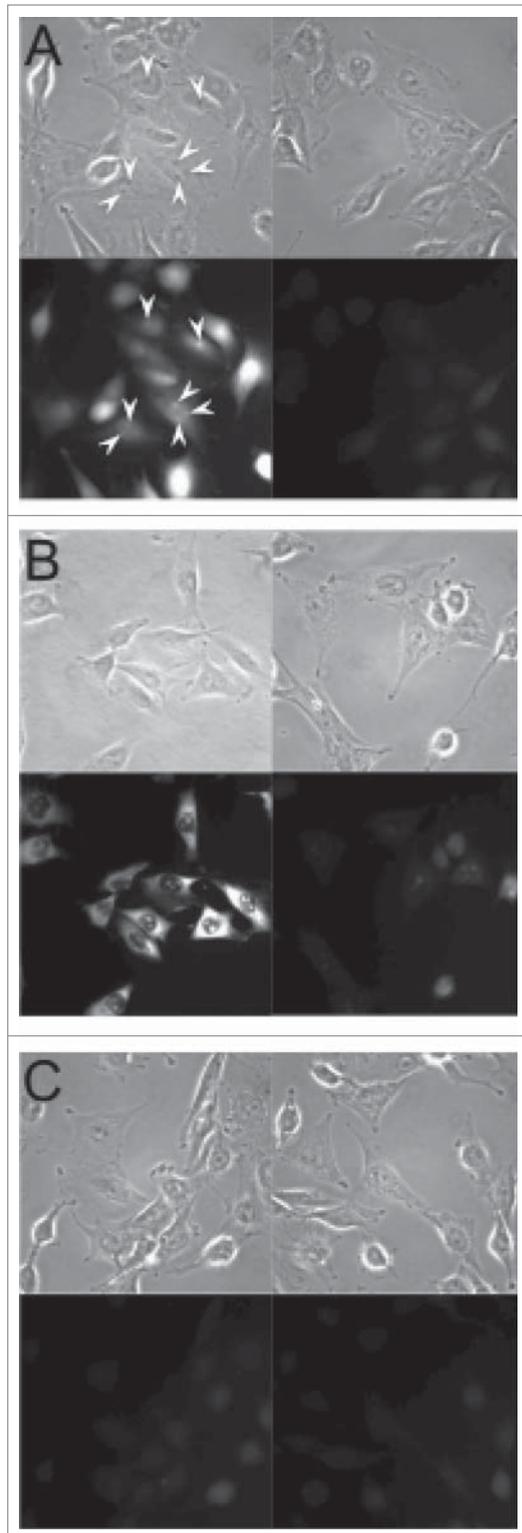
Figure 1. Fluorescence in situ hybridization. **-** : Cells incubated in buffer prior to hybridization; **+** : cells pre-incubated with RNase. A: IGF2 mRNA hybridization probes. B: 28 S rRNA probes. C: Scrambled sequence probes (see Materials and Methods). The upper panels in A-C are phase contrast images, the lower panels are the fluorescence microscopy images. Arrows indicate hybridization to IGF2 mRNA in nucleoli. (See also **Figure S2**).

is no unspliced IGF2 mRNA whatsoever in the nucleolar or nucleoplasmic fractions but it is nonetheless clear that the great majority of IFG2 RNA in both nuclear fractions is the spliced mRNA.

To explore the possibility that the nucleolar localization of IFG2 mRNA might be related to the earlier reported nucleolar abundance of 5 miRNAs in rat myoblasts, we searched the sequence of IGF2 mRNA for hybridization targets for these miR-NAs. As shown in **Figure 3**, there were predicted strong binding sites for both miR-206-3p and miR-206-5p in the 3′ UTR of IGF2 mRNA. (The terminology "strong binding sites" as used here derives from the rigorous themodynamic parameters incorporated into the Mirza software.[36]) In addition, strong binding sites in the 3′ UTR of IGF2 mRNA were also predicted for each of the other 4 nucleolus-associated microRNAs: mir-351 (**Figure 4**), miR-494 and miR-340 (**Fig. 5**) and miR-664 (**Fig. 6**). In the latter case, *i.e.* miR-664, a strong predicted binding site was predicted in both the 3′ and 5′ UTRs. Thus, even though this miRNA is probably generated from a snoRNA,[22] it nevertheless has strong target sequences in IGF2 mRNA. The relative strengths of the miRNA target sites are summarized in **Table 2**.

Translational regulation by microRNAs is typically negative. However, since IGF2 protein is abundantly present in these myoblasts (**Figure S3**) and continues to be present after myotube differentiation (**Figure S5**), the possibility of *positive* regulation of IGF2 mRNA by miR-206 arises. In this respect we have been struck by the fact that a well-established case of microRNA positive regulation, that of mRNA encoding the KLF-4 transcription factor in human mammary epithelial cells, has been demonstrated to involve none other than miR-206.[23] Even more striking is the fact that the 3′ UTR of human KLF-4 mRNA contains a miR-206 target site that is highly homologous to one we have detected in the rat IGF2 mRNA (**Fig. 7**). A second finding that is highly relevant here is that in murine myogenesis, IGF2 mRNA translation is indeed known to be under positive control.[24] The factor involved in establishing in this positive control is the murine homolog of the *C. elegans* RNA-binding protein Lin-28, which in that organism acts in collaboration with the microRNA lin-4.[25] This again points to positive regulation of IGF2 by a microRNA route. A third finding of relevance is that miR-206 has been implicated in the positive translational regulation of liver LXRα receptor mRNA in human macrophages.[26] While these findings together with the results presented here are not sufficient to conclude that

IFG2 mRNA translation in rat myoblasts is under positive control by miR-206, they are entirely consistent with this possibility and seem to constitute a provocative case.

It is to be emphasized that the 5 nucleolus-associated microRNAs have targets in mRNAs other than IGF2 mRNA. Thus, miR-206 was found to have targets in the 3′ UTR of α-tubulin mRNA, and the other 4 nucleolus-associated microRNAs were found to have targets in the 3′ UTRs of both histone 2A mRNA and ß-actin mRNA (Mirza program analyses, data not shown). This is consistent with the well-established fact that most microRNAs have targets in many different mRNAs.[27] We observed
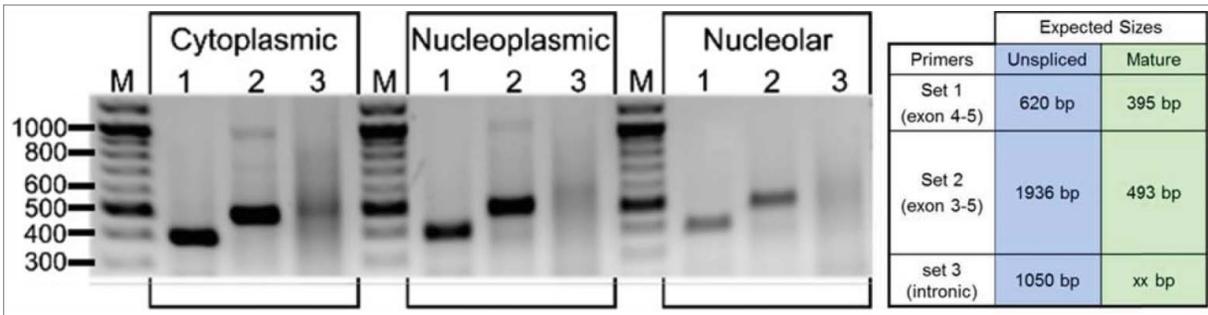
Figure 2. RT-PCR analysis. RNA was isolated from nucleolar, nucleoplasmic and cytoplasmic fractions and subjected to RT-PCR with the primers indicated in the box.

another very abundant mRNA in the myoblast nucleoli, *viz.* one encoding a hallmark of differentiated muscle, α-actin (**Table 1**). Like IGF2 mRNA, it contains strong miR-206 binding sites in its 3′-UTR (data not shown) and yet, in contrast to IGF2 mRNA, is not appreciably translated in growing myoblasts.[28] This suggests that its precocious transcription is accompanied by a temporary suppression of its translation, mediated perhaps by miR-206. Such negative regulation would be relieved later in the myogenic differentiation timetable. Thus, it is conceivable that both positive and negative translation-regulatory miRNA:mRNA interactions may be staged in the nucleolus, an intriguing hypothesis that warrants further investigation. Although in the studies on microRNA positive control of translation cited above,[23,24,26] the intracellular localization of the envisioned miRNA:mRNA complexes was not investigated, our results intriguingly point to the nucleolus as an intracellular site where the assembly of positive (and perhaps also negative) regulatory microRNA:mRNA complexes might be staged. Whether this might occur before the miRNA and/or the mRNA had already once visited the cytoplasm is a matter for speculation, which nothing inhibits us from posing here. The return of microRNAs to the nucleus is well documented.[29]

Since the plurifunctional nucleolus hypothesis was first articulated[30] (and where "pluri'" was used at that time to simply mean "more than one"), at least 2 roles of the nucleolus beyond ribosome synthesis have been established: assembly of the nascent signal recognition particle[31] and a ribosome synthesis-independent role in cell cycle progression.[32] On the basis of the present study it appears that there may be a third function of the nucleolus, the staging of certain mRNA-microRNA interactions, perhaps prior to any translational opportunity in the cytoplasm. We may therefore now consider prefixing "multi" (for at least 3) to the plurifunctional nucleolus concept.

One might ask why the formation of a positive (or negative) translational regulatory complex between a particular mRNA and a microRNA would need to be staged in the nucleolus, instead of just having the 2 molecules find each other in the cytoplasm (or in the nucleoplasm). We speculate that a nucleolus-localization factor or factors are bound to the miRNAs concentrated there (and perhaps these factor(s) brought them there) and that these special miRNPs then complex with nucleolar IFG2 mRNA (and likely other nucleolus-visiting mRNAs) to commit this transiently nucleolus-nested mRNA to translational up-regulation once in the cytoplasm. This idea has formal analogy with the exon-junction complex, which by its deposition on spliced mRNAs tags them for translational competency.[32] But in
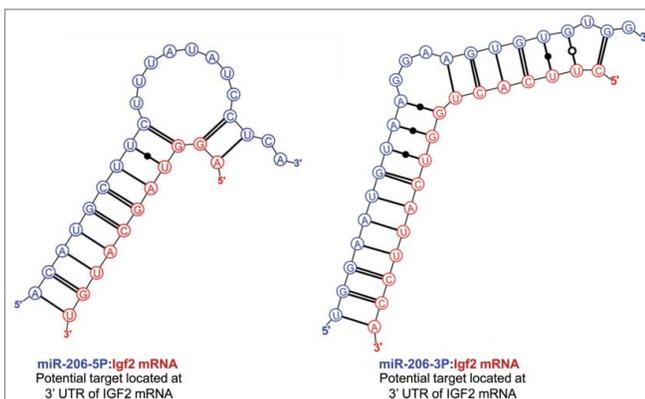
Figure 3. Predicted binding sites of miR-206 in the 3′-UTR of IGF2 mRNA.
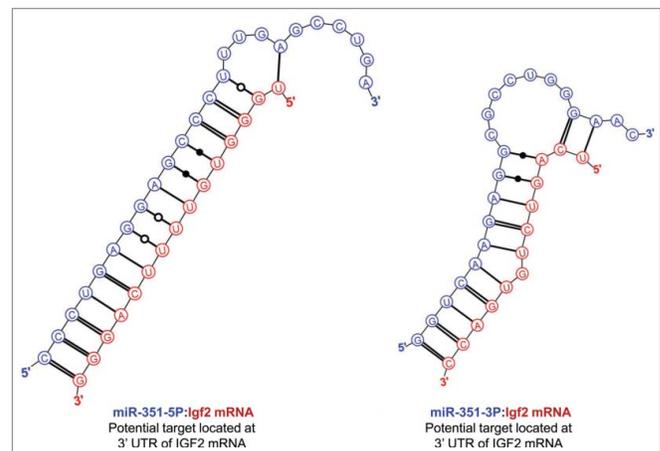
Figure 4. Predicted binding sites of miR-351 in the 3′-UTR of IGF2 mRNA.
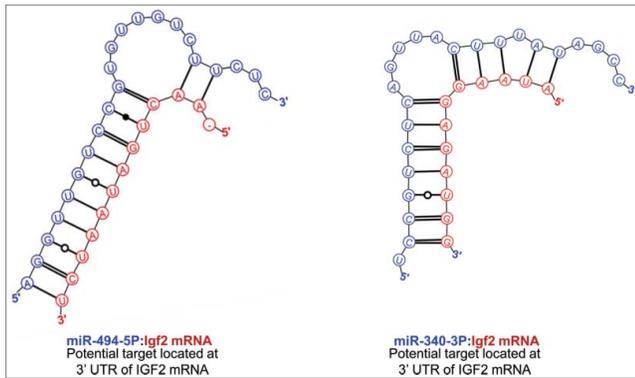
Figure 5. Predicted binding sites of miR-494 and miR-340 in the 3′-UTR of IGF2 mRNA.
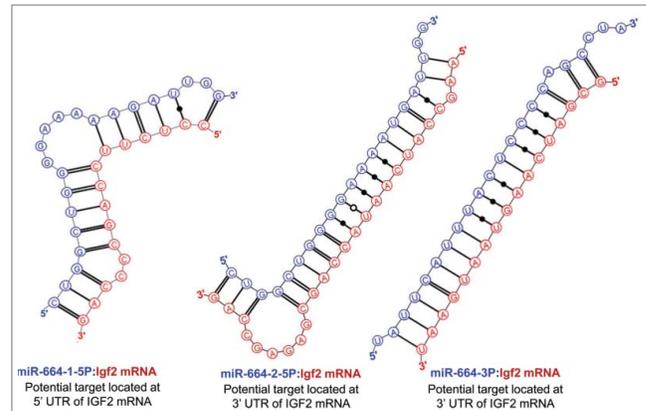
Figure 6. Predicted binding sites of miR-664 in the 5′- and 3'-UTRs of IGF2 mRNA.

addition, this hypothesis supposes that a visit to the nucleolus would allow the assembly of positive ribonucleoprotein regulatory complexes in a non-competitive environment, whereas formation of these complexes in the cytoplasm would be subject to competing binding reactions, for example ones dominated by high concentrations of other miRNPs that would negatively regulate IGF2 mRNA translation. The result would be a miRNA:mRNA complex that reaches the cytoplasm tagged and ready for rapid translation. Further studies will be needed to assess these provocative ideas.

Materials and Methods

Cell culture and fractionation

Rat L6 myoblasts were cultured as previously described[13,14] and nucleolar, nucleoplasmic and cytoplasmic fractions were isolated as detailed previously.[14] RNA from the nucleolar and nucleoplasmic fractions was electrophoresed in denaturing 10% polyacrylamide gels followed by staining with ethidium bromide to assess the quality of both nucleolar (U3 RNA) and nucleoplasmic (U1 and U2 RNAs) fractions. For myogenic differentiation of myoblasts (**Figure S5**), cells were transferred to medium containing 2% horse serum and cultured for 5 days.

RNA microarray analysis

RNA from the nucleolar and nucleoplasmic fractions was analyzed on Affymetrix rat genomic microarrays (GeneChip® Rat Exon ST Array) at the UMass Medical School Core RNA.

Sequencing Facility. Normalizations were performed using the Sketch-Quantile system within the Affymetrix Expression Console Program.

RT-PCR

RNA was purified from nucleolar, nucleoplasmic and cytoplasmic fractions of L6 cells with DNase I treatment using a Qiagen

RNeasy kit. First strand cDNAs were synthesized from RNA using the reverse primer IGF2-Rv1 (5′-TAATTTGGTTCACT-GATGGTTGCTGGAC-3′) and SuperScript III reverse transcriptase (Invitrogen, Grand Island, NY). The resulting cDNAs were used as templates for PCR reactions. IGF2 amplification from exon 4 to 5 was done with primer set 1: IGF2-Fw2 (5′-GCAGGCCTTCAAGCCGTG-3′) and IGF2-Rv1. Amplification of exons 3 to 5 was done with primer set 2: IGF2-Fw5 (5′-GTGCTGCATCGCTGCTTACC-3′) and IGF-Rv1. Amplification of unspliced IGF2 transcripts employed the primer set 3: IGF2-Fw3-Intr3 (5′-CAGGCTCAAGGTGACACCAGG-3′) and IGF2-Rv1. All reactions were run in presence of *Herculase* II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA). Standard PCR conditions consisted of initial denaturation at 95°C for 2 min, followed by 35 cycles at 95°C (30 s), 52°C (30 s) and 72°C (60 s) and final elongation at 72°C (5 min). Products were visualized by 1% agarose gel electrophoresis followed by ethidium bromide staining.

Fluorescence in situ hybridization

In situ hybridization to IGF2 mRNA was carried out using the DNA probes listed below. The underlined bases denote 5-aminohexylthymines which were labeled with Cy3 dye as previously described.[13]

IGF2-probe-1: 5′-GAG<u>T</u>CTCGC<u>T</u>GGGGCGG<u>T</u>AAGCAG CGA<u>T</u>GCAGC-3′

IGF2-probe-2: 5′-G<u>T</u>AGACACG<u>T</u>CCCTCTCGGAC<u>T</u>TG GCGGGGG<u>T</u>G-3′
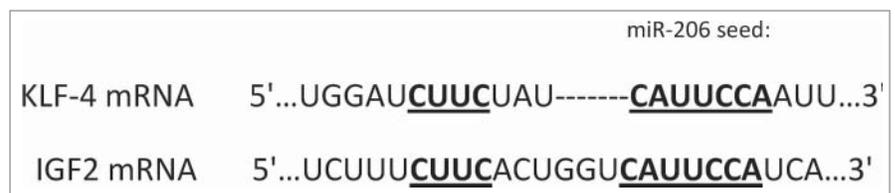
Figure 7. Comparison of the predicted rat myoblast miR-206:IGF2 mRNA interaction and that of miR-206 and KLF4 mRNA in human cells.[23]

Table 2. Summary of predicted miRNA Targets in IGF**2** mRNA[1]

Target ID	miRNA	miRNA Sequence 5'-3'	Target Quality	Target Sequence 5'-3'
ENSRNO_IGF2_mRNA_51	rno-miR-664-1-5p	CUGGCUGGGGAAAAAGAUUGG	23.071	CUUCAU**CCUCUUCCAGCCCCAG**CGGCCUCCUUAUCCAACUUCAGGUACCA
ENSRNO_IGF2_mRNA_1076	rno-miR-664-3p	UAUUCAUUUACUCCCCAGCCUA	123.88	UCAUAAUCCCAUUCCCUAUGUAACGGGGG**CAGCGAUCAAGUAAUGAAUGC**
ENSRNO_IGF2_mRNA_1601	rno-miR-351-5p	UCCCUGAGGAGCCCUUUGAGCCUGA	19.102	CGUGG**GAUGGGUGUUUUCAGGGG**CAUUUGCUGACCAUCCUCUGUGUCCCC
ENSRNO_IGF2_mRNA_1101	rno-miR-664-2-5p	CUGGCUGGGGAAAAUGAUUGG	24.847	AUC**AAGCCAUCAAUACCAGCGAGAGCCAG**UAACACCGGCUAGAGCCAUCA'
ENSRNO_IGF2_mRNA_651	rno-miR-206-3p	UGGAAUGUAAGGAAGUGUGUGG	76.522	AACAAUAGCCGCCCAAACUCUUU**CUUCACUGGUCAUUCCA**UCACAAAUGU
ENSRNO_IGF2_mRNA_351	rno-miR-351-3p	GGUCAAGAGGCGCCUGGGAAC	10.592	UAUGUGGUAAUUCUGCAAUGUAGUACCA**UCAGUCUGUGACC**UCCUCUUGA
ENSRNO_IGF2_mRNA_501	rno-miR-494-5p	AGGUUGUCCGUGUUGUCUUCUC	11.874	CAAAUUGGCUUAAGAAACUCCAU**AACUGAUAAUCU**AAAAAUUAAAUAACC
ENSRNO_IGF2_mRNA_601	rno-miR-206-5p	ACAUGCUUCUUUAUAUCCUCAU	15.066	AGAAGAAAGGAAGGGGACCCAAAAUUUUGC**AGGUAGCAUGU**CAUUGCUUC
ENSRNO_IGF2_mRNA_851	rno-miR-340-3p	UCCGUCUCAGUUACUUUAUAGCC	13.73	AGUCAGUUUGGCCAG**AUAAGGAGAUGG**CACUGCCAAGUGAUACAUGCUAC

[1]miRNA targets in IGF2 mRNA were located using MIRZA.

Oligos with the reverse polarity sequences of the IGF2 probes 1 and 2 were used as negative controls:

Scrambled-1: 5′-CGAGCTAGCGACGAATGGCGGGGTCGCTCTGAG-3′

Scrambled-2: 5′-GTGGGGGCGGTTCAGGCTCTCCCTGCACAGATG-3′

Positive control probes were for 28 S rRNA and SRP RNA as detailed in our previous studies.[33,34]

28 S-1: 5′-GTTACCGGCACTGGACGCCTCGCGGCGCCCATC-3′

28 S-2: 5′-ATGGCTCTCCGCACCGGACCCCGGTCCC-GACTC-3′

SRP-1: 5′-TTCCCACTACTGATCAGCACGGGAGTTTTGACCTT-3′

SRP-2: 5′-GCTCCCGGGAGGTCACCATATTGATGCC-GAACTT-3′

Cells on coverslips were fixed with 4% (v/v) formaldehyde and permeabilized with cold (4°C) acetone. *In situ* hybridization and RNase pre-treatment, when desired, were performed as detailed previously[13] except that after hybridization, cells were washed with 40% formamide/2× SSC (1× SSC = 0.15 M NaCl/0.015 mM sodium citrate, pH 7.0) and then 20% formamide/1× SSC for 30 min. each at 37°C followed by 3 15-min washes in 1× SSC at room temperature. An alternative *in situ* hybridization protocol was performed (**Figure S4**) in which cells were permeabilized with CSK buffer (10 mM PIPES, pH 6.8, 300 mM sucrose, 25 mM NaCl, 1 mM EGTA, 5 mM $MgCl_2$,) containing 0.5% Triton X-100 and 2 mM vanadyl ribonucleoside complex, and then fixed in 4% formaldehyde.

Coverslips were dehydrated through a 70%, 90%, 100% ethanol series and air-dried followed by incubation with ∼20 ng of each oligo in 40% formamide containing tRNA and salmon sperm DNA (5 μg/μl each). Cells were washed 3 times with 40% formamide/2× SSC, then 2× SSC for 5 min each and then once with 4× SSC/0.1% Tween-20. Coverslips were mounted in Prolong Gold Antifade (Invitrogen, Carlsbad, CA) and image acquisition and analysis was as previously described.[13]

Immunostaining

This was performed as previously detailed[13] using a polyclonal rabbit IgG for IGF2 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:25 dilution followed by visualization using a Cy3-conjugated donkey anti-rabbit IgG.

Bioinformatics

The genomic rat IGF2 sequence as well as that of rat IGF2 mRNA and its component exons were obtained from the UCSC genome database (http://genome.ucsc.edu), NCBI database and/or *Rat Genome* Database-RGD (http://rgd.mcw.edu*). The desired rat micro-RNA sequences were obtained from miRBase* (Kozomara and Griffiths-Jones, NAR, 2011) (http://mirbase.org). Searches for targets of the 5 nucleolus-associated microRNAs and IGF2 mRNA and other mRNAs were performed with the Mirza program[34], as refined in the University of Massachusetts Medical School Bioinformatics Core Facility and RNA Therapeutics Institute.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website: http://www.tandfonline.com/kncl

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