Interaction of OIP5-AS1 with MEF2C mRNA promotes myogenic gene expression

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ABSTRACT

Long noncoding (Inc)RNAs potently regulate gene expression programs in physiology and disease. Here, we describe a key function for IncRNA OIP5-AS1 in myogenesis, the process whereby myoblasts differentiate into myotubes during muscle development and muscle regeneration after injury. In human myoblasts, OIP5-AS1 levels increased robustly early in myogenesis, and its loss attenuated myogenic differentiation and potently reduced the levels of the myogenic transcription factor MEF2C. This effect relied upon the partial complementarity of OIP5-AS1 with MEF2C mRNA and the presence of HuR, an RNA-binding protein (RBP) with affinity for both transcripts. Remarkably, HuR binding to MEF2C mRNA, which stabilized MEF2C mRNA and increased MEF2C abundance, was lost after OIP5-AS1 silencing, suggesting that OIP5-AS1 might serve as a scaffold to enhance HuR binding to MEF2C mRNA, in turn increasing MEF2C production. These results highlight a mechanism whereby an IncRNA promotes myogenesis by enhancing the interaction of an RBP and a myogenic mRNA.

INTRODUCTION

In the human genome, merely 2% of expressed transcripts are protein-coding RNAs, while the rest are noncoding (nc)RNAs. Among ncRNAs, the largest portion is comprised of long noncoding (Inc)RNAs, defined as being >200 nucleotides in length. IncRNAs potently regulate gene expression program at different levels, including chromatin remodeling, RNA transcription, mRNA transport, stability, and translation, as well as protein half-life, localization and function (1–3). In contrast to protein-coding RNAs (mRNAs), some IncRNAs show highly specific tissue distribution, suggesting that they may have regulatory roles restricted to certain tissues. In this regard, a growing number of studies have implicated IncRNAs in specific developmental and disease processes such as tumorigenesis, adipogenesis, neurogenesis, and myogenesis (1,2).

Myogenesis is a tightly regulated process that occurs during embryonic development and in adult skeletal muscle responding to injury (4). It begins when satellite cells are activated into myoblasts, which subsequently transform and fuse into long multinucleated myotubes, the contractile structures that enable skeletal muscle function. With advancing age, the progressive inability to maintain muscle homeostasis leads to sarcopenia and cachexia (5,6).

Protein regulators of myogenesis include a number of transcription factors, such as MYOD (myoblast determination protein D) and MYF5, which govern the initial steps in skeletal myoblast differentiation, and by myogenin (MYOG), myogenic regulatory factor (MRF)4, and myocyte-specific enhancer factors (MEF2A and MEF2C), which mediate later differentiation stages in myogenesis (7). A number of RNA-binding proteins have also been identified as key regulators of myogenic programs, including AUF1 (AU-binding factor 1), CUGBP1 (CUG triplet repeat RNA-binding protein 1), LIN28, KHSRP (K-homology splicing regulatory protein), and human antigen R (HuR) (4,8). HuR promotes myogenesis by binding the mRNAs encoding myogenic proteins such as MYOD and MYOG (9,10).

Besides regulatory proteins, a number of regulatory IncRNAs have been implicated in myogenesis (11). For example, lincMD1 acts as a competing endogenous RNA (ceRNA) that controls myogenesis by sponging the microRNAs miR-135 and miR-133a (12), lincMyoD regulates myoblast differentiation by suppressing translation of IMP2 (13), and linc-eng promotes myogenesis and prevents muscle atrophy by sequestering miR-125b (14). However, the functions of most muscle IncRNAs remain unexplored.

In a recent survey to identify IncRNAs differentially expressed in human myoblasts differentiating to myotubes, we found that the abundant and conserved IncRNA OIP5-AS1

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(15), was upregulated during myogenesis [GSE136004 and GSE92632]. OIP5-ASI was previously implicated in biological functions including neuronal activity, stem cell maintenance, and cancer cell proliferation (15–18). Although OIP5-ASI is most abundant in the brain (18), it is also highly expressed in skeletal muscle. In cancer cells, the association of OIP5-ASI with HuR was found to prevent HuR binding to mRNAs encoding cyclin D1 (CCND1) and SIRT1, and to reduce cell proliferation (16). Here, we report that OIP5-ASI increased early in myogenesis and that silencing OIP5-ASI potently attenuated myogenic differentiation, suggesting that OIP5-ASI promoted myogenesis.

The mechanism responsible for this regulation was linked to the interaction of OIP5-ASI with the 3′UTR of MEF2C mRNA. While HuR interacted with both transcripts in myoblasts, HuR bound to MEF2C mRNA optimally only in the presence of OIP5-ASI. In turn, HuR binding to MEF2C mRNA led to stabilization of MEF2C mRNA, raising MEF2C levels and enhancing myogenesis. Collectively, we propose that OIP5-ASI serves as a scaffold to recruit HuR to MEF2C mRNA, inducing MEF2C production and promoting skeletal muscle differentiation.

MATERIALS AND METHODS

Cell culture, myogenic differentiation, and creatine kinase activity

Mouse C2C12 myoblasts cells were cultured in growth medium (Dulbecco’s modified Eagle’s medium (DMEM), Life Technologies) supplemented with 20% fetal bovine serum (FBS, Gibco) and antibiotics (Life Technologies). Immortalized human AB1167 and AB678 myoblasts, developed as described (19), were cultured in growth medium (equal volume mixture of Ham’s F10 media with 20% FBS and Promocell Skeletal Muscle Cell Growth Medium). Mouse C2C12 and human AB1167 as well as human AB678 cells were induced to differentiate by growth to high density and replacement of the growth medium with differentiation medium (DMEM with 2% horse serum). Human kidney embryonic HEK293 fibroblasts were cultured in DMEM with 10% FBS and antibiotics. For silencing experiments, using a final concentration of 50 nM siRNA and Lipofectamine 2000 (Life Technologies), control small interfering RNA (Ctrl siRNA), OIP5-ASI siRNA or HuR siRNA was transfected 24 h before induction of differentiation. Creatine kinase (CK) activity was determined in cell lysates by using the EnzyChrom creatine kinase assay kit (BioAssay Systems) following the manufacturer’s protocol. Briefly, cell lysates (1 or 2 μg) were incubated with 10 μL substrate solution, 100 μL assay buffer and 1 μL enzyme mix at 37°C for 20 min; reactions were read 20 and 40 min later at 340 nm. CK activity was calculated by the equation CK = (OD20min – OD20min/ODCALIBRATOR – ODH20) × 150, and expressed as ‘units per μg of total protein’ or ‘fold change’.

Reverse transcription (RT) followed by real-time quantitative (q)PCR analysis

Total RNA from cultured cells was isolated using the Direct-zol™ RNA MiniPrep kit (Zymo Research), which includes a digestion step using DNase I. Total RNA from ribonucleoprotein immunoprecipitation (RIP) below and pulldown assays was isolated using TRIzol (Life Technologies) following the manufacturer’s protocol, and subsequently digested with DNase I. For cDNA synthesis, reverse transcription (RT) was performed for RNA prepared in TRIzol using Maxima reverse transcriptase following the manufacturer’s protocol (Thermo Fisher Scientific). qPCR analysis of RNA was performed according to the manufacturer’s instructions for KAPA SYBR FAST ABI Prism qPCR kit (KAPA Biosystems) with specific primers (Supplementary Table S1). RT-qPCR reactions were performed on QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) with a cycle setup of 2 min at 95°C and 40 cycles of 5 s at 95°C plus 20 s at 60°C; the fold change in abundance was calculated as described previously (20,21). In qPCR amplification reactions, control ‘RT minus’ (‘RT–’) reactions were routinely included.

Pulldown of endogenous lncRNA by biotinylated antisense oligo (ASO)

For affinity pulldown of endogenous human OIP5-ASI, human myoblast lysates were prepared using PEB (20 mM Tris–HCl [pH 7.5], 100 mM KCl, 5 mM MgCl2 and 0.5% NP-40) containing protease inhibitors (Roche) and RNase inhibitor (Thermo Fisher). The lysates were incubated with 100 pmol of biotin-labeled DNA oligomers complementary to OIP5-ASI for 2 h at 4°C as described previously (21). The biotinylated ASO is shown in Supplementary Table S2. RNA complexes were washed with NT2 buffer (50 mM Tris–HCl at pH 7.5, 150 mM NaCl, 1 mM MgCl2 and 0.05% NP-40) and isolated with Dynabeads M-280 Streptavidin beads (Invitrogen).

Western blot analysis and immunofluorescence

Total protein lysates were prepared in RIPA buffer containing protease inhibitors. Proteins were size-separated by SDS-PAGE and transferred onto nitrocellulose membrane (Life Technologies). For western blot analysis, primary antibodies recognizing HuR (3A2, #L1616), MYOG (F5D, #A6020), MYH (MHC; B-5, #B0620) or HSP90 (F-8, #C2017) were from Santa Cruz Biotechnology, and those recognizing MYOD1 (D8G3, Lot 2) or MEF2C (D80C1, Lot 1) were from Cell Signaling. After incubation with the appropriate secondary antibodies, protein signals were detected by SDS-PAGE and transferred onto nitrocellulose membranes (Life Technologies). For western blot analysis, protein signals were detected by chemiluminescence. Antibody B-5, #B0620 (Santa Cruz Biotechnology) was used to detect MYH by immunofluorescence; staining with DAPI (4′,6-diamidino-2-phenylindole) was used to identify nuclei.

Cloning and luciferase assay

Briefly, 3′UTR fragments of MEF2C mRNA were amplified with specific primers and the resulting products inserted into the psiCHECK2 plasmid downstream of the Renilla open reading frame (ORF). For reporter assays, psiCHECK2-MEF2C-3′UTR fragment plasmids were transfected using Lipofectamine 2000 (Invitrogen); 24 h
and reaching larger myotube diameters (Figure 1A; Supplementary Figure S1). AB1167 myogenesis was accompanied by the expression of myogenic markers MYOD, MEF2C and MYH, as determined by western blot analysis (Figure 1C). The activity of the myogenic marker enzyme creatine kinase also increased as myogenesis progressed (Figure 1D). We characterized other human myoblast lines, including AB678 (Supplementary Figure S2) and KM155 (not shown), observing similar results. During recent studies of myogenic noncoding RNAs, we found that the levels of the abundant lncRNA Oip5-as1 increased during C2C12 myogenesis (not shown). Here, RT-qPCR analysis revealed a rise in the levels of human OIP5-ASI during the early stages of human myogenesis and sustained high expression of OIP5-ASI afterwards (Figure 1E, Supplementary Figure S2D). In sum, along with changes in classic differentiation markers, the levels of lncRNA OIP5-ASI increase progressively and remain elevated in human myogenesis.

Silencing OIP5-ASI attenuates myogenesis in human myoblasts

To investigate a possible role for OIP5-ASI in myogenesis, we silenced OIP5-ASI in human AB1167 myoblasts using two different siRNAs (Figure 2A and Supplementary Figure S3A). Using siRNA #1, we found that silencing OIP5-ASI suppressed myogenesis, as determined by monitoring myotube formation, including reductions in the size and number of myotubes, as well as the number of nuclei per myotube after differentiating for 2 and 3 days (Figure 2B); similar results were observed using OIP5-ASI siRNA #2 (Supplementary Figure S3B). Quantification of the myotubes formed revealed that silencing OIP5-ASI reduced the fusion index from ~80% to 40% after 2 days of differentiation, and that the average of number of nuclei per myotube declined from 14 to 4 (Figure 2C). Beyond 3 days, control cultures displayed fusion of very large myotubes with few discrete edges, while distinct myotubes were still visible in the culture with silenced OIP5-ASI (Figure 2B).

Further evidence that myogenesis was suppressed after silencing of OIP5-ASI was found at 16 and 24 h of differentiation, when there was a marked decline in the levels of MYOG mRNA, encoding the early myogenic transcription factor MYOG, as measured by RT-qPCR analysis (Figure 2D); as MYOD mRNA and MYOD protein expression levels peaked before OIP5-ASI did, these markers were not significantly affected by OIP5-ASI silencing (not shown). However, silencing OIP5-ASI strongly delayed the production of the late differentiation marker MYH mRNA between 24 and 72 h, as assessed by RT-qPCR analysis (Figure 2D). In keeping with the fact that MYOG protein is an early differentiation marker and MYH protein is a late differentiation marker (Figure 1B, C), western blot analysis revealed that the levels of MYOG were lower in the OIP5-ASI-silenced culture starting from 24 h of differentiation, while MYH levels were decreased starting at 36 h of differentiation. In sum, MYOG mRNA and MYOG protein, as well as MYH mRNA and MYH protein showed a delayed pattern of expression in differentiating myoblasts after silencing OIP5-ASI compared with control cells (Figure 2D, E). Myogenic differentiation was also monitored by measuring creatine kinase activity. Compared with control myoblasts, silencing OIP5-ASI reduced creatine kinase activity as determined at 24, 36, 48 and 72 h (Figure 2F). These parameters were measured in other human myoblast cell lines with similar results (Supplementary Figure S3C–F). Taken together, these results support the notion that OIP5-ASI is required for the timely progression of skeletal myogenesis.
**Figure 1.** *OIP5-AS1* is upregulated early during myogenesis. (A) Fluorescent micrographs detecting MYH to monitor the progression of human (AB1167) myoblasts (day 0, when MYH is undetectable) to myotubes (day 4, which express high levels of MYH). Staining with DAPI was used to identify nuclei. (B–E) At the times indicated in differentiating AB1167 cultures, the relative levels of myogenic mRNAs were detected by RT-qPCR analysis and plotted as a percent of the maximum levels observed during myogenesis (B), the levels of myogenic proteins were assessed by western blot analysis (C), the levels of creatine kinase activity were measured enzymatically (Materials and Methods) (D), and the levels of *OIP5-AS1* were quantified by RT-qPCR analysis (E). Data in (B, D, E) are the means ±SEM from three or more biological replicates. Significance was established using Student’s *t*-test. *P* < 0.05; **P** < 0.01; ***P** < 0.001. Other data are representative of three or more biological replicates.
Figure 2. Silencing OIP5-AS1 attenuates myogenesis. (A) AB1167 myoblasts were transfected with Ctrl siRNA or OIP5-AS1-directed siRNA #1; 24 h later, they were placed in differentiation media, and collected at the times shown after the induction of differentiation. The levels of OIP5-AS1 were measured by RT-qPCR analysis. (B) AB1167 myoblasts were transfected with Ctrl siRNA or OIP5-AS1-directed siRNA #1 as described in panel (A), and differentiation was monitored by assessing MYH levels by immunofluorescence at the times indicated. (C) AB1167 myoblasts were transfected with Ctrl siRNA or OIP5-AS1-directed siRNA #1 as described in panel (A), and after differentiation for 2 days, the fusion index and the number of nuclei per myotube were quantified; five fields were assessed per experiment. (D) The levels of MYOG, MYOD, and MYH mRNAs were assessed in AB1167 cells transfected as in panel (A). (E) The levels of MYOG, MYOD, MYH, and loading control HSP90 were assessed by western blot analysis in AB1167 myoblasts transfected as in (A) Ctrl or OIP5-AS1-directed siRNA #1 and collected at the times shown after the induction of differentiation. (F) The levels of creatine kinase activity were assessed in cells transfected as in panel (A). Data in (A, C, E) are the means ± SEM from three or more biological replicates. Significance was established using Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001. Other data are representative of three or more biological replicates.
OIP5-ASI partially complements MEF2C 3′ UTR and promotes MEF2C mRNA stability

LncRNAs can play diverse roles in cellular events by interacting with multiple molecular partners including DNA, RNA, and protein (1,3). Cytoplasmic lncRNAs may have additional functions through interactions with other RNAs as well as with proteins (25). Given that OIP5-ASI was primarily localized in the cytoplasm during myogenesis (Supplementary Figure S4A) and was recently found to interact with a range of mRNAs (26), we tested systematically if OIP5-ASI was capable of associating with mRNAs encoding myogenic proteins, including MYH and myogenic regulatory factors (MYOD, MYOG and members of the MEF2 family). As shown in Figure 3A, using biotinylated Anti-sense Oligonucleotides (ASOs) to pull down OIP5-ASI from AB1167 cell lysates, only MEF2C mRNA was enriched in the pulldown material while other myogenic mRNAs were not. BLAST survey analysis identified nine sites of complementarity between OIP5-ASI and MEF2C mRNA. Notably, eight of these sequences were located in the MEF2C 3′ untranslated region (UTR) and only one site was present in the coding region (Figure 3B).

This interaction appeared to be important for the production of MEF2C, as silencing OIP5-ASI in AB1167 myoblasts not only delayed myogenic differentiation (Figure 2), but it also reduced the levels of MEF2C mRNA and MEF2C protein (Figure 4A). To investigate whether OIP5-ASI regulated MEF2C mRNA levels by binding specifically to the MEF2C 3′ UTR, we generated constructs spanning different segments of the MEF2C 3′ UTR. As shown in Figure 4B, luciferase constructs were prepared in which the renilla luciferase (RL) coding region was linked to fragments I, II or III (containing 1, 3 or 4 potential binding sites, respectively): the psiCHECK2 vector also encodes the firefly luciferase (FL) gene used to normalize the luciferase results and control for transfection. After transfection of the constructs into AB1167 myoblasts and induction of differentiation for 24 h, only fragment II, but not fragments I or III, increased luciferase activity significantly (Figure 4C), indicating that fragment II contains MEF2C sequences that promote luciferase production. In addition, the levels of RL mRNA relative to FL mRNA followed the same trend, indicating that the rise in luciferase activity was due to a relative increase in the levels of FL mRNA and not to changes in translation or luciferase activity (Supplementary Figure S4B). Importantly, OIP5-ASI contributed to the rise in expression driven by fragment II, as silencing OIP5-ASI lowered the luciferase activity that had been enhanced by MEF2C 3′ UTR fragment II (Figure 4D). These findings implicated OIP5-ASI in promoting MEF2C production through the MEF2C 3′ UTR fragment II.

To map more precisely the functional sites of OIP5-ASI interaction with MEF2C 3′ UTR fragment II, we sequentially deleted sequences in fragment II. As shown in Figure 4E, loss of the putative binding site 3 was sufficient to fully suppress reporter activity, suggesting that OIP5-ASI-complementary site 3 located in fragment II (Figure 4B) was important for promoting reporter expression. To test this hypothesis directly, we deleted complementarity fragment II-3 (21 nucleotides) of MEF2C 3′ UTR, creating a reporter that had fragment II but with region 3 deleted, MEF2C 3′ UTR II-3Δ. This deletion reporter exhibited significantly reduced luciferase activity than the reporter with the wild-type fragment II (Figure 4F), confirming the importance of complementary site 3 in the MEF2C 3′ UTR fragment II for the enhanced luciferase expression.

We tested the interaction between these two RNAs in an additional way. Among the transcript variants of OIP5-ASI expressed in myoblasts, the 1.9-kb OIP5-ASI [NR_026757.1, studied in (16) and fully contained in the longest and most abundant, 8.8-kb OIP5-ASI variant (not shown)] contained the region of complementarity with MEF2C 3′ UTR fragment II-3. We prepared a construct that expressed OIP5-ASI(s) tagged with MS2 RNA hairpins (24 total) OIP5-ASI-MS2, which was verified to have a similar localization as the endogenous OIP5-ASI (Supplementary Figure S4D). We co-expressed OIP5-ASI(s)-MS2 in AB1167 myoblasts with another construct that expressed a fusion protein (MS2-GST) that binds MS2 RNA hairpins and could be pulled down using GSH-containing beads (Figure 4G, schematic). As shown in Figure 4G, using lysates from transfected myoblasts, OIP5-ASI(s)-MS2 was effectively pulled down using GSH beads (Figure 4G, left graph); importantly, MEF2C mRNA was markedly enriched in this pulldown material (Figure 4G, right graph). Pulldown analysis indicated that OIP5-ASI-MS2 was able to interact with MEF2C 3′ UTR fragment II, while this interaction was dramatically reduced when testing the deletion mutant transcript MEF2C 3′ UTR II-3Δ (Figure 4H and Supplementary Figure S4C). Collectively, these findings support the notion that the OIP5-ASI lncRNA and MEF2C mRNA interact in myoblasts through complementarity sequences located in OIP5-ASI and the proximal region (segment II-3) of MEF2C 3′ UTR. Of note, while 8 MEF2C mRNA isoforms differing in the 3′ UTR have been described, region 3 of complementarity with OIP5-ASI was conserved among all of these isoforms (Supplementary Figure S4E).

To further investigate how OIP5-ASI promoted expression of the reporter RL mRNA bearing MEF2C 3′ UTR segment II, we tested the impact of OIP5-ASI abundance on MEF2C mRNA stability. As shown, endogenous MEF2C mRNA levels were reduced constitutively when OIP5-ASI was silenced (Figure 4I, left). We then measured the half-life of MEF2C mRNA by blocking de novo transcription using actinomycin D and measuring the time required for MEF2C mRNA to reach one-half the abundance measured at time 0. As shown, the half-life of MEF2C mRNA declined markedly faster in AB1167 myoblasts in which OIP5-ASI was silenced (t1/2 > 10 h) compared to control cells (t1/2 > 5 h) (Figure 4I, right). As a control, a stable transcript, GAPDH mRNA (encoding the housekeeping protein GAPDH), showed comparable half-lives whether OIP5-ASI was silenced or not. In keeping with the higher levels of OIP5-ASI in late myogenesis, the stability of MEF2C mRNA was also higher in myoblasts differentiating for 24 h (Figure 4I, right). These findings indicate that OIP5-ASI promotes MEF2C expression during myogenesis by associating with, and stabilizing, MEF2C mRNA.
Figure 3. OIP5-AS1 binds to and shares partial complementarity with MEF2C 3′UTR. (A) Left, schematic of biotinylated Ctrl and OIP5-AS1-directed ASOs. Right, after incubation of biotinylated ASOs with AB1167 lysates prepared 24 h after inducing differentiation, RNA complexes were pulled down using streptavidin beads (Materials and Methods). The presence of OIP5-AS1 and myogenesis-related mRNAs in the pulldown material was assessed by RT-qPCR analysis. The levels of test RNAs in the pulldown were normalized to the levels of GAPDH mRNA in the lysates. Data in (A,C,E) are the means ±SEM from three or more biological replicates. Significance was established using Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001. (B) Regions of potential complementarity identified between OIP5-AS1 and MEF2C mRNA.
Figure 4. Silencing OIP5-AS1 lowers MEF2C abundance by diminishing its RNA stability. (A) The levels of MEF2C mRNA (top) and MEF2C (bottom) in AB1167 myoblasts transfected with Ctrl or OIP5-AS1-directed siRNA #1 and processed as explained in Figure 2A were measured by RT-qPCR analysis (normalized to GAPDH mRNA) and western blot analysis (using HSP90 to monitor loading), respectively. (B) Schematic of the psiCHECK2 dual renilla luciferase (RL) and internal control firefly luciferase (FL) reporter constructs used to identify the region(s) of MEF2C 3′UTR regulated by OIP5-AS1. (C) Twenty-four hours after transfecting the plasmids shown, bearing MEF2C 3′UTR regions I, II, and III, AB1167 myoblasts were induced to differentiate for 24 h, and the relative RL/FL ratios were determined. (D) AB1167 myoblasts were co-transfected with MEF2C 3′UTR fragment II, along with either Ctrl siRNA or OIP5-AS1 siRNA; 24 h later they were replaced with differentiation media and cultured for an additional 24 h. The relative RL/FL ratios were determined. (E) AB1167 myoblasts were co-transfected with subsegments II-3, II-4 or II-5 of MEF2C 3′UTR fragment II; 24 h later they were replaced with differentiation media and cultured for an additional 24 h, whereupon the relative RL/FL ratios were determined. (F) AB1167 myoblasts were co-transfected with MEF2C 3′UTR fragment II or with fragment II lacking site 3 of interaction with OIP5-AS1 (II-3Δ); 24 h later they were replaced with differentiation media and cultured for an additional 24 h, whereupon the relative RL/FL ratios were determined. (G) Schematic of the MS2 pulldown assay, including plasmids pMS2 (a control vector expressing MS2 RNA), pOIP5-AS1(s)-MS2 (a vector expressing the chimeric RNA OIP5-AS1-MS2) and pMS2-GST, expressing a fusion protein (MS2-GST) which recognizes MS2 RNA tags and can be pulled down using glutathione (GSH) beads. Twenty-four hours after transfecting AB1167 myoblasts with either pMS2 or pOIP5-AS1(s)-MS2, as well as with pMS2-GST, cells were induced to differentiate. Twenty-four hours later, cell lysates were analyzed by pulldown using GSH-conjugated beads. The relative interaction of MS2 and OIP5-AS1(s)-MS2 with MEF2C mRNA was assessed by RT-qPCR analysis. (H) Twenty-four hours after transfecting plasmids expressing MEF2C 3′UTR fragment II or MEF2C 3′UTR fragment II-3Δ, or an empty vector control plasmid into AB1167 myoblasts, together with plasmids pMS2 or pOIP5-AS1(s)-MS2, as well as with pMS2-GST, cells were induced to differentiate. Twenty-four hours later, cell lysates were analyzed by pulldown using GSH-conjugated beads. The relative interaction of MS2 and OIP5-AS1-MS2 with MEF2C 3′UTR fragment II or MEF2C 3′UTR fragment II-3Δ was assessed by RT-qPCR analysis of the pulldown materials. (I) Twenty-four hours after transfecting Ctrl or OIP5-AS1 siRNAs, AB1167 myoblasts were placed in differentiation media for an additional 24 h, whereupon the steady-state levels of MEF2C mRNA were quantified (left). Cells were then treated with actinomycin D and the relative levels of MEF2C mRNA and normalization control transcript GAPDH mRNA were assessed by RT-qPCR analysis and normalized to 18S rRNA levels, also quantified by RT-qPCR analysis (right). mRNA half-lives ($t_{1/2}$) were calculated as the times required to reach 50% of the initial abundance of the mRNA at time 0 before adding actinomycin D. (J) AB1167 myoblasts that were either proliferating or induced to differentiate for 24 h were treated with actinomycin D to measure the stability of MEF2C mRNA as explained in panel (I). In panels (A, C–J) the data represent the means ± SEM from three or more independent experiments. Significance was established using Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001. Data in (A) are representative of three or more biological replicates.
**OIP5-AS1 recruits HuR to MEF2C mRNA, enhances MEF2C mRNA stability**

We sought to gain insight into the mechanisms leading to MEF2C mRNA stabilization by OIP5-AS1. We surveyed RNA-binding proteins (RBPs) that might associate with both transcripts and promote mRNA stability (27). Using the POSTAR2 database, a number of RBPs were found by PAR-CLIP (photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation) analysis to associate with MEF2C mRNA and with OIP5-AS1 (28,29), but only three RBPs — HuR/ELAVL1, WDR23 and CSTF2T— associated with both RNAs (Figure 5A). HuR was previously reported to associate with MEF2C and OIP5-AS1 (16,30), so we hypothesized that HuR may be a key mediator of the actions of OIP5-AS1 in stabilizing MEF2C mRNA.

We examined if HuR interacted with OIP5-AS1 in myoblasts using a range of methods. We performed RIP (ribonucleoprotein immunoprecipitation) analysis with an anti-HuR antibody and lysates prepared from differentiated AB1167 myoblasts using IP conditions that preserved ribonucleoprotein (RNP) complexes. After isolating RNA from the RNPs present in the IP material, the levels of OIP5-AS1, as detected using RT-qPCR analysis, were highly enriched in the HuR IP (Figure 5B, left), supporting the existence of these complexes in AB1167 myoblasts. We then employed OIP5-AS1-directed ASOs to pull down and identify interacting proteins; here too, OIP5-AS1 pulled down HuR far more effectively than a control (Ctrl) ASO (Figure 5B, middle). Finally, we tested the ability of HuR to associate with tagged OIP5-AS1(s)-MS2 RNA in AB1167 myoblasts co-expressing the GST-MS2 fusion protein; pull-down analysis revealed a similarly strong enrichment of HuR associated with the OIP5-AS1-MS2 relative to levels observed with a control MS2 RNA (Figure 5B, right).

Turning our attention to MEF2C mRNA, RIP analysis verified that MEF2C mRNA was capable of binding HuR (Figure 5C, left). In keeping with the ability of HuR to modulate the turnover and translation of many target mRNAs (31), silencing HuR in differentiated AB1167 myoblasts for 48 h reduced the levels of MEF2C mRNA and MEF2C protein, as determined by RT-qPCR and western blot analyses, respectively (Figure 5D, E), while reduced HuR expression did not affect OIP5-AS1 levels in AB1167 cells (Figure 5F). Interestingly, however, actinomycin D treatment revealed that HuR was required for stabilizing MEF2C mRNA, as silencing HuR reduced the half-life to ~10 h (Figure 5F).

Thus, we hypothesized that OIP5-AS1 and HuR might jointly regulate MEF2C mRNA stability. To test this possibility, we silenced OIP5-AS1 in HEK293 cells and monitored the interaction of HuR with MEF2C mRNA. Silencing OIP5-AS1 did not affect HuR levels (Figure 5G), but it significantly reduced the interaction of HuR with MEF2C mRNA (Figure 5H, left). This reduction in binding was specific for MEF2C mRNA, as other HuR targets like MMP9 mRNA, which does not have OIP5-AS1 complementarity sites, showed comparable binding to HuR regardless of OIP5-AS1 abundance (Figure 5H, right). These results indicate that HuR binds both MEF2C mRNA and OIP5-AS1 lncRNA, and that MEF2C mRNA stabilization leading to MEF2C protein production requires the presence of both OIP5-AS1 and HuR.

**OIP5-AS1 is required for HuR-MEF2C mRNA interaction**

To study if the promotion of human skeletal myogenic differentiation by OIP5-AS1 was linked to the enhanced production of MEF2C, we carried out a number of rescue experiments. A construct was made to express myc-tagged MEF2C protein from an mRNA that lacked 3’ UTR and hence did not have OIP5-AS1-interacting sites. After transfection of the vector into AB1167 myoblasts, exogenous MEF2C-myc and endogenous MEF2C proteins were detected by western blot analysis (Figure 6A). Silencing OIP5-AS1 reduced the levels of endogenous MEF2C, in keeping with the finding that silencing OIP5-AS1 lowered MEF2C mRNA levels and MEF2C mRNA stability (Figure 4H); importantly, however, overexpression of MEF2C-myc partially restored myogenesis as measured by the increased levels of MYH and creatine kinase activity, as well as by the elevated myotube formation (Figure 6A and Supplementary Figure S5A).

Similarly, the reduction in myogenesis elicited by silencing OIP5-AS1 [in this instance using an siRNA that was directed at the distal region of OIP5-AS1, outside of the region ectopically expressed, OIP5-AS1(s)]], evidenced by a loss of MEF2C protein levels and reduced creatine kinase activity (Figure 6B), was rescued by ectopic overexpression of the short OIP5-AS1(s) that bears the region of complementarity with MEF2C 3’UTR segment II-3 (Figure 6B). Importantly, however, ectopically expressing OIP5-AS1(s)Δ, which lacks the region that complements MEF2C 3’UTR segment II-3 and hence has reduced ability to bind MEF2C mRNA (Supplementary Figure S5B), did not rescue MEF2C protein production or creatine kinase activity (Figure 6B). Taken together, our findings support a model whereby the lncRNA OIP5-AS1, which is highly expressed in muscle and is elevated during myogenesis, functions as an interactive scaffold to recruit the RBP HuR for binding to the MEF2C 3’UTR, in turn stabilizing MEF2C mRNA and promoting MEF2C expression and myogenic differentiation (Figure 6C).

**DISCUSSION**

Muscle development and regeneration are tightly orchestrated through the strict coordination of gene expression programs. Myogenesis is regulated by major transcription factors, such as MYF5, MYOD, MYOG and MRF4 (4,32), as well as by a growing number of RBPs and noncoding RNAs (11,33). Here, we have identified OIP5-AS1, a human lncRNA highly expressed in muscle, as a major regulator of human myogenesis that appeared to be functionally important, as silencing OIP5-AS1 attenuated myogenesis and delayed myotube formation. Molecular details of this regulation were uncovered through the identification of MEF2C mRNA as a transcript that was partially complementary to OIP5-AS1 and was stabilized through its association with OIP5-AS1. Interestingly, this lncRNA-mRNA interaction facilitated HuR binding to MEF2C mRNA, leading to MEF2C mRNA stabilization and increased MEF2C.
Figure 5. HuR binds OIP5-AS1 and MEF2C mRNA, stabilizes MEF2C mRNA. (A) Venn diagram of RBPs from the CLIP database (Materials and Methods) binding OIP5-AS1 (red) and those binding MEF2C mRNA (blue); 3 RBPs binding both RNAs are listed in the intersection. (B) Binding of HuR to OIP5-AS1 in differentiating AB1167 cultures was verified by HuR RNP immunoprecipitation (RIP) analysis (left); after HuR RIP (Materials and Methods) using IgG or anti-HuR antibodies, the presence of OIP5-AS1 in the IP materials was measured by RT-qPCR analysis, normalized to the levels of GAPDH mRNA (a transcript that is not a target of HuR), and represented as the enrichment of OIP5-AS1 in HuR IP relative to the levels in IgG IP. Western blot analysis was performed to monitor the efficiency of the HuR RIP reaction. For OIP5-AS1 pulldown analysis (middle), differentiated AB1167 cultures were incubated with biotin-ASO-conjugated OIP5-AS1; HuR bound to the OIP5-AS1 ASO was detected by western blot analysis. A control (Ctrl) ASO was used to detect background binding of HuR. RT-qPCR analysis was performed to monitor the efficiency of the OIP5-AS1 pulldown. Further validation of this interaction was gained by assaying HuR binding to OIP5-AS1-MS2 (right); lysates prepared from differentiated AB1167 cultures expressing OIP5-AS1-MS2 or MS2 along with GST-MS2-binding protein (as described in Figure 4G) were subjected to western blot analysis to detect HuR in the GST pulldown material. Input, aliquots of the lysates before pulldown. RT-qPCR analysis was performed to monitor the efficiency of OIP5-AS1-MS2 pulldown. (C) HuR RIP analysis was performed as described in panel (B), and the presence of MEF2C mRNA was quantified by RT-qPCR analysis. (D–F) Twenty-four hours after transfecting Ctrl or HuR siRNAs, AB1167 myoblasts were differentiated for an additional 24 h and the steady-state levels of MEF2C mRNA (D) and MEF2C (E) were determined by RT-qPCR and western blot analyses, respectively. In these transfection groups, the steady-state levels of OIP5-AS1 were assessed by RT-qPCR analysis (F, left), and the half-life of MEF2C mRNA was measured after incubation with actinomycin D, as explained in Figure 4H (F, right). (G, H) Forty-eight hours after transfecting human embryonic kidney fibroblasts (HEK293 cells) with Ctrl siRNA or OIP5-AS1 siRNA, the levels of HuR were assessed by western blot analysis (G). The relative levels of OIP5-AS1 were shown by RT-qPCR analysis (H, left), and the binding of HuR to MEF2C mRNA was assessed by RIP analysis; data were normalized to the levels of GAPDH mRNA in each IP sample and represented as the enrichment of each mRNA relative to the levels in IgG IP (H, middle). MMP9 mRNA, a target of HuR lacking complementarity to OIP5-AS1, was included as control in RIP analyses; as shown, MMP9 mRNA remained enriched in HuR RIP regardless of OIP5-AS1 abundance (H, right). Western blot analysis was performed to monitor the efficiency of HuR IP. Data in (B–D,F,H) represent the means ± SEM from at least three independent experiments. Significance was established using Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001. Other data are representative of three or more biological replicates.
Figure 6. Rescue of myogenesis after silencing OIP5-AS1 by ectopic MEF2C expression. (A) AB1167 myoblasts were cotransfected with siRNAs (Ctrl or OIP5-AS1-directed) and with plasmids (either an Empty Vector control plasmid or a plasmid expressing myc-tagged MEF2C). Twenty-four hours after transfection, AB1167 myoblasts were induced to differentiate and 48 h after that the levels of MEF2C, MEF2C-myc, MYH, and HSP90 were determined by western blot analysis (left), and the activity of the myogenic differentiation marker creatine kinase was measured (right). (B) AB1167 myoblasts were cotransfected with siRNAs (Ctrl or OIP5-AS1-directed) and with one of these plasmids: MS2 only control plasmid, a plasmid expressing the short variant of OIP5-AS1-MS2(s) that bears the MEF2C 3’UTR interacting segment ‘(s)’, or the short variant of OIP5-AS1-MS2(s)Δ lacking the MEF2C 3’UTR interacting segment, ‘(s)Δ’ (schematic, top right). The endogenous (longer) OIP5-AS1 transcript depicting the siRNA recognition site outside of the segment in the ectopic vectors is indicated. In these transfection groups, the abundance of MEF2C was assessed by western blot analysis (left top), the levels of OIP5-AS1 by RT-qPCR analysis (left graph), and the activity of creatine kinase was measured (right graph). (C) Proposed model. In Proliferating (undifferentiated) myoblasts, lncRNA OIP5-AS1 levels are low. As myogenic differentiation progresses, OIP5-AS1 levels rise and through its complementarity with MEF2C mRNA, it helps recruit HuR to the MEF2C 3’UTR and enhances MEF2C mRNA stability and MEF2C production. Through this mechanism, OIP5-AS1 promotes MEF2C expression and enhances myogenesis. Data in (A, B) represent the means ± SEM from at least three independent biological replicates. Significance was established using Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001. Other data are representative of three or more biological replicates.
production in myoblasts. Conversely, loss of OIP5-AS1 prevented the timely accumulation of MEF2C and delayed myogenesis. In this paradigm, we propose that the lncRNA OIP5-AS1 functions to recruit HuR locally to MEF2C mRNA, ultimately promoting myogenesis.

**LncRNA–mRNA binding**

Interactions between a lncRNA and an mRNA have been reported to control mRNA stability and translation. For example, mRNA turnover is controlled by the interaction of 1/2-sbsRNAs with mRNAs, with the double-stranded RNA-binding protein STA1 (Staufen1) then inducing decay of the target mRNA (34). Another example is offered by BACE1-AS, which was shown to bind and stabilize BACE1 mRNA, encoding β-secretase, an enzyme important for generating the amyloid peptide Aβ42 (35,36). As examples of translational control, lineRNA-p21 partially complemented CTNNB1 mRNA and JUNB mRNA and recruited translational suppressors RCK/p54 and FMRP, lowering the translation of CTNNB1 and JUNB (37), 7SL RNA bound to TP53 3′UTR and repressed its translation by competing with HuR (38). In addition, the antisense lncRNA Uchl1-AS1 was capable of binding to the Uchl mRNA 5′UTR leading to increased translation of the protein UCHL1 (39). However, to our knowledge, the paradigm described here is the first example of a lncRNA (OIP5-AS1) recruiting an RBP to an unrelated mRNA (MEF2C 3′UTR) in order to enhance mRNA stability. In this instance, the positive impact of OIP5-AS1 on the expression of MEF2C mRNA led to enhanced skeletal muscle differentiation.

Many regulatory RBPs bind specific RNA sequences, although this binding is typically more flexible than that observed for DNA-binding proteins. In fact, only a small proportion of RNA sites for putative interaction with RBPs are occupied at a given point in time in cells (40), even though many RBPs are in excess. Thus, additional factors such as post-translational modifications of RBPs, local RNA secondary structure, and subcellular compartmentalization of the RNA or the RBP can influence the formation of RNPs (41–43). We propose that the interaction of OIP5-AS1 with the MEF2C 3′UTR through lncRNA-mRNA base pairing might modulate the MEF2C 3′UTR RNA structure to promote RBP binding. We further postulate that lncRNA OIP5-AS1, which binds HuR at multiple sites, can increase the local concentration of HuR within the cell; then, by specifically interacting with other RNAs such as MEF2C mRNA, OIP5-AS1 facilitates the formation of other HuR RNP complexes. As this work progresses, it will be interesting to expand the analysis and test if OIP5-AS1 might interact with other mRNAs during myogenesis and facilitate their association with HuR.

**HuR RNPs in myogenesis**

A survey of RBPs interacting with OIP5-AS1 revealed that OIP5-AS1 can associate with other RBPs besides HuR, including hnRNP C and FMR1. In cancer cells, OIP5-AS1 can bind to HuR and reduce the binding of HuR to mRNAs encoding proliferative proteins such as CCND1 and CCNA2 (cyclins D1 and A2), and in this manner, OIP5-AS1 can contribute to the reduced proliferation of cancer cells (16). Here, we found that OIP5-AS1 binding to the MEF2C 3′UTR led to MEF2C mRNA stabilization and promoted myogenic differentiation. Whether OIP5-AS1 also contributes to reducing myoblast proliferation to establish the fully differentiated state that favors myogenesis remains to be investigated. It is important to note that in early stages of myogenic differentiation, HuR was shown to bind to mRNAs encoding MYOD and MYOG and p21/CDKN1A, enhancing their early production during myogenesis and helping to establish the first steps in differentiation (9,10). Perhaps the subsequent rise in OIP5-AS1 helps to redirect HuR to other target mRNAs that must be translated later during the differentiation process, including MEF2C mRNA. We propose that HuR supports different stages of myogenesis by participating in different mRNPs and lncRNPs, as shown for the long intergenic noncoding RNA line-MD1 (44,45). It is important to note that the existence of many other factors capable of binding OIP5-AS1 and HuR helps to explain why the reduction of MEF2C mRNA stability seen after silencing HuR (Figure 5F) is different in magnitude from that seen after silencing OIP5-AS1 (Figure 4I). The mechanisms that regulate the increased OIP5-AS1 levels during myogenesis are not known, but the fact that the precursor transcript (pre-OIP5-AS1) increases during myogenesis (Supplementary Figure S6C) suggests that this elevation is at least in part due to enhanced transcription. Whether myogenic factors control OIP5-AS1 transcription also remains to be studied.

**Local conservation of OIP5-AS1**

While overall lncRNA sequences are poorly conserved among species, secondary structures, partial sequences, and interactions with molecular partners (e.g., miRNAs and RBPs) are more conserved. In this regard, OIP5-AS1 is not highly conserved but some segments have been identified as being functionally conserved among species. For example, a miR-7 binding site in OIP5-AS1 is conserved from zebrafish to human; in this regard, OIP5-AS1 binds to and regulates miR-7, influencing miR-7 actions in neuronal development of zebrafish and mice (15,18). Here, we found that silencing OIP5-AS1/Oip5-as1 attenuated both human (AB1167 cells) and mouse (C2C12 cells) myogenesis (Figure 2; Supplementary Figure S6A). OIP5-AS1 is highly abundant in muscle tissue and interacts abundantly with HuR in both human and mouse myoblasts (Supplementary Figure S6B). Interestingly, the short isoform of OIP5-AS1, which appears to be most abundant (Supplementary Figure S6D), also has the highest identity with murine Oip5-as1, including several sites complementary to mouse Mef2c mRNA and HuR-binding sites. Whether OIP5-AS1 has a conserved biological role in myogenesis by interacting with HuR and influencing MEF2C production in other species warrants further study.

**OIP5-AS1-HuR complexes beyond myogenesis**

OIP5-AS1 has also been implicated in cancer. It suppresses cell proliferation by reducing GAK levels, and acts as a
competing endogenous RNA for miR-218 binding to promote Kaposi’s sarcoma (17,46). MEF2C is also reported to promote the progression of lung cancer and osteosarcoma (47,48). Given that HuR is highly expressed in numerous cancers and contributes to carcinogenic gene expression programs (31), it will be important to determine if OIP5-ASI influences HuR binding to MEF2C mRNA or other cancer-associated mRNAs in the context of carcinogenesis.

In closing, the recruitment of IncRNA–RBP complexes to target nucleic acids has been studied in depth in the nucleus, where IncRNAs have been shown to recruit proteins to specific DNA regions to elicit changes in gene transcription. The paradigm presented here, OIP5-ASI recruiting HuR to MEF2C mRNA to coordinate myogenesis, illustrates a similar process at the post-transcriptional level. Akin to IncRNAs driving transcriptional programs, IncRNAs can also drive post-transcriptional programs by directing RBPs to subsets of mRNAs, thereby helping to orchestrate complex developmental processes such as myogenesis.

DATA AVAILABILITY
GEO: GSE136004 and GSE92632 (https://www.ncbi.nlm.nih.gov/geo/). Images of original blots are included in the Supplementary Figure S7.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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