The *IGF2* intronic miR-483 selectively enhances transcription from *IGF2* fetal promoters and enhances tumorigenesis

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Insulin-like growth factor 2 (*IGF2*), a developmentally regulated and maternally imprinted gene, is frequently overexpressed in pediatric cancers. Although loss of imprinting (LOI) at fetal promoters contributes to increased *IGF2* in tumors, the magnitude of *IGF2* expression suggests the involvement of additional regulatory mechanisms. A microRNA (miRNA) screen of primary Wilms' tumors identified specific overexpression of miR-483-5p, which is embedded within the *IGF2* gene. Unexpectedly, the *IGF2* mRNA itself is transcriptionally up-regulated by miR-483-5p. A nuclear pool of miR-483-5p binds directly to the 5' untranslated region (UTR) of fetal *IGF2* mRNA, enhancing the association of the RNA helicase DIXH9 to the *IGF2* transcript and promoting *IGF2* transcription. Ectopic expression of miR-483-5p in *IGF2*-dependent sarcoma cells is correlated with increased tumorigenesis in vivo. Together, these observations suggest a functional positive feedback loop of an intronic miRNA on transcription of its host gene.

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Human insulin-like growth factor 2 (*IGF2*), a maternally imprinted gene, is transcribed from five promoters whose activity is both tissue-specific and developmentally regulated. In the fetus, *IGF2* mRNA is highly transcribed primarily from the paternal promoters P2, P3, and P4, which are largely silenced in most tissues after birth. In the adult liver, *IGF2* expression is driven by biallelic activation of the promoter P1 [Supplemental Fig. 1; Monk et al. 2006]. Expression of *IGF2* is frequently elevated in malignancies, including Wilms' tumor [a pediatric kidney cancer] [Huff 2011], sarcomas, and some colorectal cancers. In these cancers, the fetal promoters P2, P3, and P4 are often preferentially used, and loss of imprinting (LOI) commonly leads to their biallelic expression [Pollak 2008]. LOI at fetal promoters has been widely considered the cause of the elevation of *IGF2* expression in tumors [Jelicic and Shaw 2007], but additional mechanisms are likely to contribute to the very high levels of *IGF2* expression seen in many cancers.

The global expression pattern of microRNAs (miRNAs) is typically much higher in differentiated tissues than in cancers, consistent with a broad role for miRNAs in suppressing cellular proliferation and enhancing differentiation [Chang et al. 2008]. However, in comparing miRNA expression patterns in the pediatric kidney cancer Wilms' tumor versus normal renal tissues, we observed selective up-regulation of miR-483-5p in the cancer cells. We demonstrate that miR-483-5p, which resides in an intron of the *IGF2* gene, enhances transcription of fetal but not adult *IGF2* transcripts by binding to its 5' untranslated region (UTR), thereby up-regulating expression of its host gene. This unexpected nuclear function for a miRNA points to the complexity of mechanisms regulating the expression of the *IGF2* gene, implicated in both normal development and cancer.

**Results and Discussion**

The *IGF2* intronic miR-483 is overexpressed in Wilms’ tumor

In screening 431 miRNAs for differential expression in Wilms’ tumor compared with normal adult and fetal kidneys, we identified miR-483 (comprising two distinct miRNAs: miR-483-5p and miR-483-3p) as being the most dramatically and specifically up-regulated miRNA [Fig. 1A; Supplemental Tables 1, 2]. Although most miRNAs are more abundantly expressed in mature nonproliferating tissues than in embryonic tissues or cancers [Chang et al. 2008], both miR-483s were expressed >10-fold higher in the highly proliferative Wilms’ tumor (N = 20) compared with adult differentiated kidneys (N = 3). miR-483 expression was also more than threefold higher in the tumor compared with fetal kidney tissue (N = 5), which contains proliferative renal precursors thought to be the cells of origin for this embryonic malignancy [Fig. 1B]. The primary miR-483 lies in intron 2 of the *IGF2* transcript and encodes two miRNAs on opposite strands of the premiR-483 hairpin [Fig. 1C]. The levels of both miRNAs parallel the expression of the *IGF2* host transcript in Wilms’ tumor, fetal kidneys, and adult kidneys [Fig. 1B; Veronesi et al. 2010; Ma et al. 2011]. miR-483-5p has also recently been reported among miRNAs, conferring an adverse prognosis in both Wilms’ tumor and adrenal cortical carcinoma [Patterson et al. 2011; Watson et al. 2013].
miR-483-5p enhances IGF2 transcription

Although both miR-483s are highly conserved among placental mammals (Supplemental Fig. 2), we were unable to validate 3' UTR target sequences that had been predicted by available computational algorithms using traditional luciferase reporter experiments. We therefore tested the effect of ectopic miR-483-5p or miR-483-3p expression on endogenous transcripts by microarray profiling. Given the scarcity of Wilms' tumor cell lines, we selected MHH-ES-1 Ewing's sarcoma cells (Wisniewski et al. 2002), derived from a highly aggressive pediatric tumor expressing endogenous IGF2 (Fig. 2A,B). This observation indicates promoter-specific 5' UTRs or 3' UTRs, black boxes indicate coding regions, and exons are numbered. The miR-483 gene encodes two miRNAs: miR-483-5p (blue) and miR-483-3p (red).

![Figure 1](image1)

**Figure 1.** The IGF2 intronic miR-483s are highly expressed in Wilms' tumor along with the host IGF2 mRNA. [A] Box plots showing higher miR-483 expression in Wilms' tumors compared with normal adult and fetal kidney tissues. In each box, the central mark (red) indicates the median, the edges of the box represent the 25th and 75th percentiles, and the deviations extend to the most extreme data points. The Y-axis is in units of fluorescence intensity. [B] qRT–PCR measurement of coexpression of IGF2 mRNA and the miR-483s in fetal kidneys, adult kidneys, and Wilms' tumors. Expression is relative to levels in adult kidney tissues. [C] Localization of miR-483s within the human IGF2 gene. Arrows denote promoters P0-P4. Gray boxes indicate promoter-specific 5' UTRs or 3' UTRs, black boxes indicate coding regions, and exons are numbered. The miR-483 gene encodes two miRNAs: miR-483-5p (blue) and miR-483-3p (red).

![Figure 2](image2)

**Figure 2.** miR-483-5p induces promoter-specific IGF2 transcription. [A] Scatter plot (log) of mRNA profiles of MHH-ES-1 cells transfected with miR-483-5p (Y-axis) versus scrambled control (X-axis). Transcripts expressed in both samples (red), one sample (blue), or neither sample (yellow) are shown [as determined by default settings of the Affymetrix GCOS software], with green lines indicating twofold, threefold, 10-fold, and 30-fold differential expression between the two samples. The arrow points to signals from three independent IGF2 mRNAs probes. [B] Genes that are changed [either up-regulated or down-regulated] more than twofold in MHH-ES-1 cells transfected with a miR-483-5p mimic compared with scrambled control. [C] Time course of IGF2 mRNA expression after transfection of MHH-ES-1 with a miR-483-5p mimic, as measured by qRT–PCR. Expression is relative to levels at day 0. [D] Induction of IGF2 mRNA by miR-483-5p in two human sarcoma cell lines [MMH-ES-1 and RD] and two mouse sarcoma lines [F4328 and F4864], as measured by qRT–PCR. The asterisk denotes a significant difference (P < 0.01) compared with mock treatment, whose measurement is set to 1. [E] Stability of IGF2 mRNA in miR-483-5p transfected MHH-ES-1 cells treated with the transcriptional inhibitor DRB. Signal is normalized to total RNA and expressed as percentage of IGF2 mRNA in untreated controls. [F] Nascent IGF2 mRNA transcripts, measured by nuclear run-on assays in MHH-ES-1 cells transfected with a miR-483-5p mimic. Expression is relative to levels of nascent GAPDH mRNA. The asterisk denotes a significant difference (P < 0.01) compared with scrambled control. [G] Expression of promoter-specific IGF2 transcripts in MHH-ES-1 cells transfected with a miR-483-5p mimic. P1/P0 refers to the common transcript from these promoters (see the Supplemental Material for promoter information). The asterisk denotes a significant difference (P < 0.01) compared with mock treatment, whose expression is set to 1. [H] Linear regression of expression of each IGF2 promoter-specific transcript (Y-axis; expression relative to that of ACTB mRNA) with miR-483-5p (5p for short) expression (X-axis; expression relative to that of RNU48 small RNA) in human primary tissues [adult or fetal livers, hearts, kidneys, muscles, and brains; umbilical] and Wilms' tumors, as measured by qRT–PCR. The coefficient of determination (R²) and associated P-value are shown.
MHH-ES-1 cells treated with the transcriptional inhibitor 5,6-dichloro-1-β-D ribofuranosylbenzimidazole (DRB) or actinomycin D showed no difference following transfection with either a miR-483-5p mimic, a scrambled mimic, or mock transfected controls (Fig. 2E), indicating that mRNA stability is not altered by the miRNA. In contrast, nuclear run-on assays showed increased nascent IGF2 transcripts in MHH-ES-1 nuclei overexpressing miR-483-5p compared with a scrambled mimic, suggesting that miR-483-5p expression enhances transcription of the IGF2 mRNA (Fig. 2F).

To test which of the five IGF2 promoters are affected by miR-483-5p, we used specific qRT–PCR primers that distinguish between individual transcripts. miR-483-5p specifically increased IGF2 mRNA from the grouped fetal promoters P2, P3, and P4 but not from the far upstream P0 and P1 promoters that regulate IGF2 expression in adult tissues (Fig. 2G). IGF2AS, a 2-kb antisense RNA whose promoter lies adjacent to P3 [Supplemental Fig. 1], was also up-regulated by miR-483-5p. Consistent with the promoter-specific effect of miR-483-5p, the expression of this mRNA in a panel of human fetal and adult tissues and Wilms’ tumors correlated with P2-, P3-, and P4-derived IGF2 transcripts ($R^2 > 0.5$) but not with the P0- and P1-initiated mRNAs [Fig. 2H]. Enhanced expression of IGF2 mRNAs driven from the fetal P2–P4 promoters by miR-483-5p is also accompanied by a slight relaxation of gene imprinting [Supplemental Fig. 4].

Nuclear miR-483-5p interacts with the 5’ UTR of the major fetal IGF2 transcript

Given the positive regulatory effect of miR-483-5p on IGF2 mRNA transcription, rather than the characteristic role of miRNAs in regulating the stability of mature mRNA, we tested whether the miRNA localizes to the nucleus. Using fluorescence in situ hybridization [FISH] with digoxigenin [DIG]-conjugated locked nucleic acid [LNA] probes and confocal microscopy, we observed multiple punctate foci of endogenous miR-483-5p within the nuclei of MHH-ES-1 cells, along with a diffuse cytoplasmic signal [Fig. 3A]. The nuclear miR-483-5p signal was comparable with that of the small nuclear RNA U6. Mutant miR-483-5p probes with three mismatched nucleotides in the seed region (see Supplemental Table 5 for sequences) failed to detect the endogenous miR-483-5p in MHH-ES-1 cells. The wild-type probes failed to detect the signal in a fibroblast cell line that lacks IGF2 mRNA expression, and no nuclear staining was evident with the control miRNAs miR-675 and miR-31, which have well-defined cytoplasmic functions [Valastyan et al. 2009; Keniry et al. 2012]. Further analysis of nuclear and cytoplasmic RNA fractions from three different cell lines confirmed the presence of miR-483-5p in both compartments (Supplemental Fig. 5).

To search for potential miR-483-5p target-binding sites, we scanned the entire 30-kb human IGF2 gene, from the P1 promoter to the 3’ UTR region, identifying two strong potential binding sites [site 1 and site 2] within the 5’ UTR of the major P3 fetal transcript [Fig. 3B]. A single such site is also present in the 5’ UTR of the mouse and rat IGF2 mRNA encoding the comparable transcript (variant 1) [Supplemental Fig. 5A]. Although miR-483-3p does not...
modulate IGF2 levels, a conserved miR-483-3p-binding site exists within the P3 5' UTR region. Since these candidate target sequences are present in potential promoter regulatory DNA elements as well as in the 5' UTR of the mRNA transcript, we used biotinylated miRNA affinity pull-down assays (Lal et al. 2011, Kang et al. 2012, Yoon et al. 2012) to test for binding of the miRNAs to genomic DNA versus RNA. The assays were conducted separately, with nuclear versus cytosolic extracts derived from cells that had been transfected with the various biotinylated miRNA baits, followed by qRT–PCR analysis of specific regions of IGF2 DNA or mRNA. Neither biotinylated miR-483-5p nor miR-483-3p showed specific enrichment for the relevant genomic DNA sequences (Supplemental Fig. 5B,C). However, specific IGF2 mRNA sequences were coprecipitated with the miRNA baits: miR-483-5p pull-down experiments revealed >50-fold enrichment of the P3 mRNA transcript containing the P3-driven 5' UTR region compared with the pull-down using a mutant miR-483-5p with a three-nucleotide change in the seed sequence or a miR-483-5p biotinylated on the opposite strand (Fig. 3C; Supplemental Fig. 6). The P3 IGF2 mRNA pull-down by miR-483-5p was observed from the nuclear but not the cytosolic fraction. Biotinylated miR-483-3p failed to pull down the IGF2 transcript. Thus, miR-483-5p selectively binds to the nuclear IGF2 P3 transcript.

To determine whether miR-483-5p binding to its predicted target sites within the 5' UTR is critical for up-regulation of IGF2 mRNA expression, we cotransfected 2'-O-methyl-modified antisense oligonucleotide protectors (Giraldez et al. 2006) complementary to either site 1 or site 2 to block binding by the 5p miRNA. Cotransfection of the antisense oligonucleotide targeting site 2 abolished miR-483-5p-mediated induction of IGF2 (Fig. 3D). A mutant antisense oligonucleotide against site 2 (17 out of 34 sequence mismatches) or an antisense targeting the GAPDH 5' UTR had no effect on IGF2 mRNA induction. In contrast to antisense protectors targeting site 2, targeting site 1 had no effect on IGF2 mRNA expression. Thus, only one of the two miR-483-5p-binding sites within the 5' UTR of the IGF2 mRNA appears to mediate a positive feedback loop, leading to increased transcription. Moreover, the antisense oligonucleotide targeting site 2 alone, but not its mutant version, significantly lowers the baseline endogenous IGF2 mRNA level (Fig. 3D). This suggests that the effect that we observed is not restricted to ectopic overexpression of miR-483-5p but that the native miRNA positively regulates the steady-state levels of its host mRNA.

To determine whether induction of IGF2 transcription occurs through nuclear miR-483-5p directly interacting with IGF2 mRNA, we preformed nuclear run-on assays where either the miR-483-5p sequence is mutated or its target sequence is blocked. Expression of miR-483-5p increased the levels of nascent IGF2 transcripts in MHH-ES-1 cells. This effect was suppressed by antisense protectors targeting site 2 but was not affected by mutant protectors or observed following expression of mutant miR-483-5p (Fig. 3E). These results suggest that the interaction of miR-483-5p with IGF2 mRNA P3 site 2 alters IGF2 mRNA transcription.

miR-483-5p promotes the association of IGF2 mRNA with the RNA helicase DHX9

To better understand the mechanisms involved in miR-483-5p-mediated regulation of IGF2 expression, we searched for associated proteins using complementary strategies. First, we used biotinylated 5' UTR of the IGF2 P3 transcript as bait to isolate interacting proteins from the nuclear extracts of MHH-ES-1 cells transfected with miR-483-5p or a scrambled control. As an additional control, we also transfected cells simultaneously with miR-483-5p together with an antisense protector targeting site 2 or a mutated antisense protector. Electrophoresis of affinity-purified proteins followed by silver staining identified a 140-kDa protein that was enriched in cells transfected with miR-483-5p compared with cells transfected with either control sequences or antisense sequences targeting site 2 (Fig. 4A; Supplemental Fig. 7). Targeted sequencing of this protein identified it to be ATP-dependent RNA helicase A (DHX9). Western blot analysis confirmed the coprecipitation of DHX9, and we note that DHX9 protein expression increases upon overexpression of miR-483-5p (Fig. 4B). The interaction between miR-483-5p, IGF2 mRNA, and DHX9 was confirmed using a miRNA–protein affinity purification assay as a second approach (Newman et al. 2008). Biotinylated miR-483-5p or a scrambled control was used as bait to pull down associated proteins from MHH-ES-1 nuclear extracts, followed by mass spectrometric analysis. In this analysis, DHX9 was one of the predominant proteins specifically associated with miR-483-5p (Fig. 4C; Supplemental Table 4).

To further test the potential interaction between DHX9, the IGF2 mRNA, and miR-483-5p, we immuno-precipitated endogenous DHX9 to see whether it directly binds to the RNAs. We observed that DHX9 is bound specifically to IGF2 mRNA P3 but not to P2 or P4, the two IGF2 mRNA variants that lack the miR-483-5p-binding site, or H19, a mRNA transcribed from the same genomic locus (Fig. 4D). Blocking site 2 using antisense sequences but not mutant sequences against the site significantly reduced but did not completely abolish DHX9 binding to IGF2 P3, suggesting that miR-483-5p increases the association between DHX9 and IGF2 mRNA, and DHX9 may also interact with other regions of the mRNA besides the miR-483-5p-binding site. Cross-linking assays to determine whether DHX9 directly interacts with miR-483-5p were inconclusive due to high levels of background [data not shown]. Together, these results suggest that DHX9 specifically associates with IGF2 P3 mRNA and that miR-483-5p greatly enhances this interaction.

While its precise functions remain to be elucidated, DHX9 has previously been reported to regulate transcription (Padmanabhan et al. 2012) as well as miRNA-dependent pathways (Robb and Rana 2007). To test the functional significance of the interaction between DHX9, miR-483-5p, and IGF2 mRNA, we knocked down expression of the helicase in MHH-ES-1 cells and analyzed the consequences on miR-483-5p-mediated induction of IGF2 mRNA. Suppression of DHX9 not only abrogated the induction of IGF2 mRNA by ectopic miR-483-5p but also reduced endogenous IGF2 mRNA levels (Fig. 4E), suggesting that it contributes to the miRNA-mediated up-regulation of IGF2. As a control for specificity, we also tested another ATP-dependent RNA helicase, DHX36, that was enriched in the miR-483-5p pull-down (Supplemental Table 4). Knockdown of the DHX36, however, did not affect miR-483-5p-mediated IGF2 mRNA induction (Fig. 4E).
miR-483-5p expression is correlated with enhanced tumorigenesis in vivo

To test whether the miR-483-5p effect on IGF2 expression has physiological consequences in modulating tumorigenicity, MHH-ES-1 cells infected with lentiviral constructs encoding miR-483-5p were inoculated subcutaneously into immunodeficient mice. MHH-ES-1 cells expressing miR-483-5p generated significantly larger tumors in a higher fraction of animals compared with cells transfected with empty vector [Fig. 5A; Supplemental Fig. 8]. IGF2 mRNA expression in tumors derived from miR-483-5p-infected cells was higher [Fig. 5B] and correlated with tumor size [Fig. 5C].

Concluding remarks

We showed that miR-483-5p, a miRNA embedded in the IGF2 gene, mediates a function in the nucleus, targeting the 5′ UTR of the major fetal IGF2 P3 transcript, leading to its up-regulation. The unexpected functional properties of miR-483-5p are particularly interesting in view of the complex IGF2 physiology, where imprinted fetal IGF2 promoters enforce high IGF2 production during rapid placental and fetal development [Moore and Haig 1991]. Interestingly, miR-483 is present only in placental mammals (Supplemental Fig. 2) despite the broad evolutionary conservation of IGF2 signaling, raising the possibility that the miRNA may have coevolved with this complex gene regulatory mechanism. In addition to this physiological role, positive feedback regulation by miR-483-5p may further contribute to increased fetal IGF2 promoter expression during tumorigenesis. More traditional 3′ UTR mRNAs targeted by miR483-5p have been reported, leading to reduced expression of some potential candidates, including ERK1, SRF, Socs3, and MeCP2 [Qiao et al. 2011; Ma et al. 2012; Wang et al. 2012; Han et al. 2013]. miR-483-5p may thus exhibit distinct nuclear and cyttoplasmic properties, both contributing to its physiological effects.

Further studies will be required to dissect the mechanisms by which miR-483-5p mediates its effect on IGF2 transcription, but the apparent requirement for RNA helicase DHX9 raises the possibility that small RNA-mediated RNA processing is involved, as proposed for splicing-coupled transcriptional silencing promoted by siRNAs [Allo et al. 2009]. The 5′ UTR of IGF2 P3 mRNA has a 72% GC content and is therefore highly structured. DHX9 may play a role in modulating this structure to facilitate immediate processing of nascent transcripts, thereby enhancing the overall transcription efficiency. Indeed, mRNA processing and transcription are tightly coupled in mammalian cells, with protein factors modu-
lating transcriptional initiation, elongation, and termina-
tion [Dahan et al. 2011]. Whether other affected miRNAs
that reside in close genomic proximity [IGF2 P2 and F3
and IGF2AS] are induced by miR-483-5p through local-
ized changes in chromosomal structure and thus facil-
itated transcription remains to be determined. Taken all
together, the self-regulation of IGF2 transcription by the
intronic and coexpressed miR-483-5p points to a novel
miRNA functional property modulating a complex de-
velopmental pathway in mammalian cells.

Material and methods

Tumor and tissue samples and cell lines

Frozen Wilms tumor, normal adult kidney, or fetal kidney tissue samples
used for miRNA screen were obtained from Children's Hospital Boston
and Brigham and Women's Hospital. All samples were collected with
institutional review board approval. Human primary tissues used to assess
the expression of IGF2 promoter-specific miRNAs and miR-483-5p were
obtained from Stratagene and Zyagen, as described in the Supplemental
Material. Cell lines were grown under standard culture conditions, as
described in the Supplemental Material.

miRNA, RNA, and protein analysis and mouse xenografts

miRNA screen, qRT–PCR, RNA stability assay, miRNA FISH, nuclear
RUN-ON, RIP, and immunoprecipitation experiments are described in the
Supplemental Material.

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