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MiR-495 and miR-218 regulate the expression of the Onecut transcription factors HNF-6 and OC-2

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ABSTRACT

MicroRNAs are small, non-coding RNAs that posttranscriptionally regulate gene expression mainly by binding to the 3′UTR of their target mRNAs. Recent data revealed that microRNAs have an important role in pancreas and liver development and physiology. Using cloning and microarray profiling approaches, we show that a unique repertoire of microRNAs is expressed at the onset of liver and pancreas organogenesis, and in pancreas and liver at key stages of cell fate determination. Among the microRNAs that are expressed at these stages, miR-495 and miR-218 were predicted to, respectively, target the Onecut (OC) transcription factors Hepatocyte Nuclear Factor-6 (HNF-6/OC-1) and OC-2, two important regulators of liver and pancreas development. MiR-495 and miR-218 are dynamically expressed in developing liver and pancreas, and by transient transfection, we show that they target HNF-6 and OC-2 3′UTRs. Moreover, when overexpressed in cultured cells, miR-495 and miR-218 decrease the endogenous levels of HNF-6 and OC-2 mRNA. These results indicate that the expression of regulators of liver and pancreas development is modulated by microRNAs. They also suggest a developmental role for miR-495 and miR-218.

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Introduction

The first sign of liver and pancreas development consists of the formation of tissue buds arising from the definitive endoderm. The progenitor cells of the pancreas and liver then proliferate and progressively differentiate to give rise to the pancreatic and hepatic cell lineages. A complex network of transcription factors drives the development of the pancreas and liver during embryogenesis [1,2]. In particular, we have demonstrated that the Onecut transcription factors HNF-6 and OC-2 play important roles in this process [3–14]. The analysis of knockout mice showed that HNF-6 controls the initial step of pancreas development by promoting expression of Pdx1, an essential factor for pancreas progenitor development [5]. HNF-6 and OC-2 control early pancreas morphogenesis and pancreatic duct development [9,13]. They also promote endocrine differentiation by activating the pro-endocrine gene NGN3 [4,9]. HNF-6 and OC-2 stimulate early liver expansion [12] and are required for normal differentiation of hepatoblasts to hepatocytes and cholangiocytes [3,14]. Gain-of-function studies revealed that overexpression of HNF-6 in pancreatic islets induces diabetes [15], and that overexpression in adult regenerating liver stimulates hepatocyte proliferation [16]. In addition, it has been shown that accurate levels of HNF-6 are required to determine time-specific expression of HNF-6 target genes during liver development [7,11]. Taking together, these data suggest that a precise control of the expression level of Onecut factors is necessary for acquiring or maintaining cell type-specific characters.

MicroRNAs (miRs) are short, endogenously expressed non-coding RNAs that bind to target mRNAs, mainly at their 3′UTR. By doing so, they fine-tune the expression level and repress translation of their mRNA targets. Consequently, they emerged as novel posttranscriptional regulators of gene expression. A single miR can directly target several mRNAs, and the expression of an mRNA is controlled by several miRs [17,18]. Multiple roles have been ascribed to miRs, including in embryonic development and disease [19]. By knocking-out Dicer, a key enzyme involved in miR maturation, it was shown that miRs have an important role, among others, in the development of pancreas and liver [16,20–22]. In the absence of Dicer in the pancreas, the number of endocrine progenitors and beta cells is strongly decreased [20]. Another study [21] pointed out the role of Dicer in maintaining the adult pancreatic phenotype. In the liver, hepatocyte-specific knockout of Dicer promotes hepatocarcinogenesis, and interestingly, in Dicer$^{-/-}$ adult liver, the expression of HNF-6 is significantly increased [22]. Our unpublished results also indicate that the absence of Dicer in pancreas and liver is associated with increased levels of HNF-6 and OC-

Abbreviations: HNF-6, Hepatocyte Nuclear Factor-6; OC-2, Onecut-2; miR, microRNA; 3′UTR, 3′untranslated region

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2, further suggesting that these two factors are regulated by miRs in these organs.

The aim of the present study was to identify miRs regulating Onecut factor expression. We found that two miRs, namely miR-495 and miR-218, which are expressed throughout embryonic development of the liver and pancreas, regulate Onecut factor expression by binding to target sites in the 3′UTR of HNF-6 and OC-2, respectively.

Materials and methods

An extended Materials and methods section is included in the online supplementary data.

MiR profiling. miR profiling in pancreas at embryonic day (e) 14.5 and in liver at e15.5 was performed using the miRCURY LNA™ Array (Exiqon). Tissues were dissected in cold PBS, RNA was extracted using Tripure reagent (Roche) and precipitated with 3.5 volumes of 100% ethanol for 30 min at −20 °C. RNA quality was assessed using an Agilent BioAnalyser 1200 system. Pancreas and liver RNAs were hybridized on miRCURY microarrays version 10.0 and 8.1, respectively. Exiqon carried out the labeling and hybridization steps.

MiR cloning was performed by the polyadenylation method [23]. Sequences were analyzed using the miRBase databank [24].

Reverse transcription and real-time quantitative PCR. Quantitative RT-PCR for miRs was performed as described [25,26], with minor modifications. The quantification of β-Actin, E-cadherin, N-cadherin, HNF-6, and OC-2 was as described [11].

Generation of DNA constructs. To generate miR-495 expression vector, two overlapping oligonucleotides spanning the mouse miR-495 precursor sequence were annealed and underwent short PCR amplification using Vent Polymerase (New England Biolabs). The PCR product was cloned into the pSUPER vector. To generate a scrambled miR-495 expression vector, two overlapping oligonucleotides corresponding to a modified miR-495 precursor sequence were used. The modified precursor bears the scrambled miR-495 sequence in the backbone of the mouse miR-495 precursor, and preserves the secondary structure of the latter. To generate luc-HNF-6–3′UTR reporter vector, a 1874-nucleotide-long segment of the mouse HNF-6 3′UTR was amplified by PCR from CD1 mouse tail genomic DNA and cloned into the psiCHECK vector (Promega) between the *Renilla* luciferase stop codon and the polyadenylation site. The luc-HNF-6–3′UTRmut reporter vector bearing mutations in the miR-495 target site was obtained using the QuickChange site-directed mutagenesis kit (Stratagene). To generate luc-OC-2–3′UTR reporter vectors, two segments of mouse OC-2 3′UTR were inserted into psiCHECK-1. The fragments corresponded, respectively, to the 499 and 916 nucleotides downstream from the OC-2 stop codon. The primer sequences used to obtain the different constructs are given in online supplementary data. The miR-218 target sites on OC-2 3′UTR were rendered ineffective by using the QuickChange site-directed mutagenesis kit (Stratagene).

Cell culture, transfections and luciferase assays. HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Details on the transfection protocol are given in online supplementary data. Luciferase activity was measured 24 h after transfection as described [27], using the Dual Luciferase reporter assay system (Promega) and a TD-20/20 luminometer (Promega).

BMEL cells were cultured in monolayer as described [11]. For HNF-6 mRNA quantification, 2×10^5 BMEL cells were seeded in 24-well plates and transfected 24 h later with 2 μl of LipofectAMINE and 1.6 μg pSUPER-miR-495, or pSUPER-miR-495 scrambled vector as a control. For OC-2 quantification, cells were transfected with 2 μl of LipofectAMINE and 200 nM mmu-miR-218 or control miRNA. The RNA was extracted 48 h later and RT-qPCR performed as described [11].

Isolation of pancreatic epithelial cells. Dorsal pancreata of e14.5 Pdx1-GFP embryos (kindly provided by D. Melton) were dissected in 8 mUI of RNAsin (Promega) in PBS. Up to 25 dissected pancreata were digested in 500 μl of trypsin–EDTA (Gibco) for 10 min at 37 °C and then 500 μl of DMEM containing 10% FBS were added. The cells were mechanically dissociated and the suspension was passed through a 40 μm cellulose filter (Millipore). The GFP-positive and -negative cells were then sorted using a FACSVantage cell sorter (Beckton Dickinson).

Results

Identification of microRNAs present in developing pancreas and liver

To identify miRs that target HNF-6 and OC-2 during liver and pancreas development, we first determined the miR expression

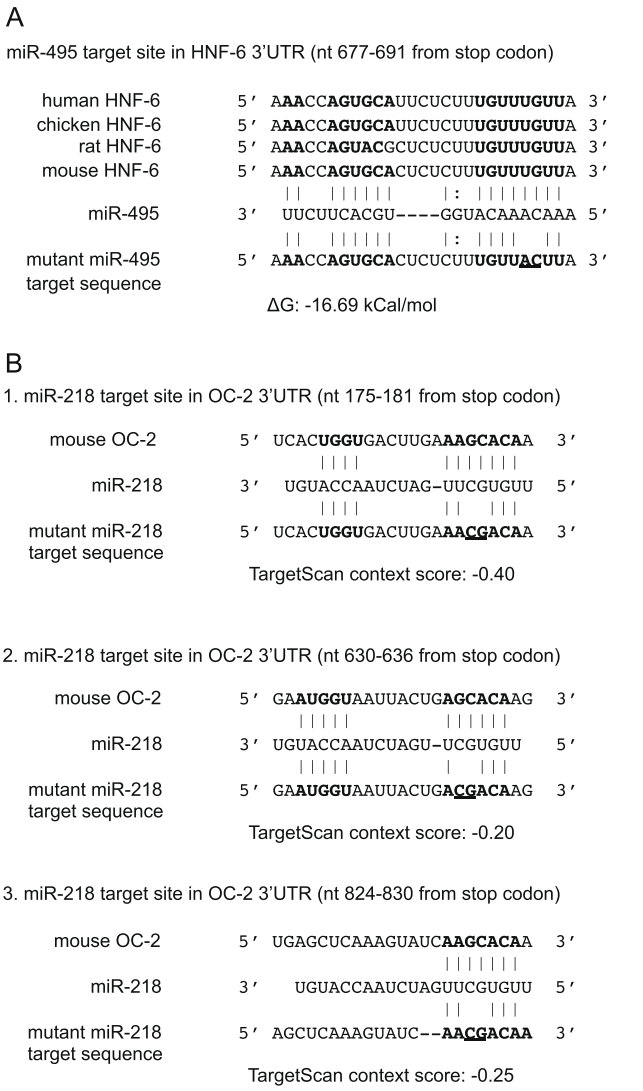

Fig. 1. Location of the target sites for miR-495 and miR-218 in the HNF-6 and OC-2 3′UTRs. One conserved miR-495 target site is present in the HNF-6 3′UTR. The seed region is conserved in the human, chicken, rat and mouse 3′UTRs. The sequence of mouse miR-495 is indicated, alongside the mutations introduced in the miR-495 target site of the mouse HNF-6 3′UTR (underlined nucleotides) for generating a mutated reporter construct (A). Location and sequence of the three miR-218 (nominated here as 1, 2 and 3) target sites in the OC-2 3′UTR; the sequence of mouse miR-218 is indicated, alongside the mutations introduced in the different target sites (underlined nucleotides) for generating mutated reporter constructs (B). For each target site, miRanda binding energy or TargetScan context score are also mentioned.

profile in embryonic pancreas and liver by a microarray approach. We found that 307 miRs were expressed in e14.5 pancreas, and 112 miRs in e15.5 liver (Supplementary Table 1). The lower number of miRs found in liver can partially be explained by a lower number of miR probes present in the version of the microarrays used, compared to the experiment with the pancreatic miRs. We next widened the miR profile to early liver and pancreas. To this end, the region containing the hepatic and pancreatic buds from an e9.5 mouse embryo was dissected, RNA was extracted and size-fractionated, and a cDNA library containing small RNA species was constructed. From 95 clones sequenced, we identified 37 different miRs, corresponding likely to the most frequently expressed sequences in the buds (Supplementary Table 1). The majority of the sequences represented already known mature miRs. One sequence was not found in the mature miR miRBase databank, although when blasted against the precursor miR catalog, it was found to match the opposite arm of miR-667 on the miR-667 precursor. Therefore, the new mature miR was designated as miR-667*.

We next used the TargetScan5.1 and miRanda prediction algorithms to ask if miRs present in developing liver and pancreas were able to target the 3′UTR of the HNF-6 and OC-2 genes. The latter were, respectively, 800 bp and 2 kb long. Candidate miRs are shown in Supplementary Table 1. We then decided to validate experimentally a miR that regulates the expression of HNF-6 and a miR regulating OC-2. For selecting these miRs, we used the following criteria: to have a conserved target site with good energy score predicted by miRanda, or a high total context score calculated by TargetScan, and to be present with a good frequency in the bud library. On this basis, miR-495, for HNF-6, and miR-218, for OC-2 were chosen. MiR-495 was found three times in the bud library and it was present in the pancreas at e14.5. Its putative target site was located 677 bases downstream of the HNF-6 stop codon, was conserved between species and showed an extensive complementarity to the HNF-6 sequence (Fig. 1A). MiR-218 had three target sites in the 3′UTR of OC-2 which are highly conserved, and had a high TargetScan total context score. Interestingly, multiple miR target sites in the 3′UTR have been shown to work cooperatively to decrease gene expression [28]. Moreover, miR-218 was cloned four times from the miR library at e9.5 and was found in the pancreas at e14.5, suggesting coexpression with its putative target OC-2. Thus we focused on miR-495 and miR-218 as potential posttranscriptional regulators of HNF-6 and OC-2, respectively.

Expression pattern of miR-495 and miR-218

We next characterized the expression profile of miR-495 and miR-218 during pancreas and liver development by using a stem-loop RT-qPCR approach. We found that miR-495 and miR-218 were detectable in e8.5 dispase-digested endodermal epithelium (Fig. 2A and B). They remained expressed at e9.5 in the pancreatic and hepatic buds emerging from the endoderm, and were found at higher concentration in embryonic pancreas and liver at e14.5 and e16.5 (Fig. 2A). In all tissues tested, miR-495 was more abundant than miR-218. In pancreas, the epithelium, but not the mesenchyme, expresses the transcription factor Pdx1, and, in transgenic Pdx1-GFP mice, GFP is expressed in the epithelium under control of Pdx1 regulatory regions. We took advantage of the epithelial expression of GFP to separate by FACS the epithelial and mesenchymal cells in e14.5 pancreata. The epithelial markers E-cadherin and miR-375 [29], were enriched in the GFP-positive cells, as expected (Fig. 2C). N-cadherin, a protein more abundant in the mesenchyme than in the epithelium, was enriched in the GFP-negative fraction (Fig. 2C). These results validated our separation procedure. We then tested miR-495 and miR-218 expression and found that miR-495 was enriched in epithelial GFP-positive cells, whereas miR-218 showed equal expression in epithelium and mesenchyme (Fig. 2C). From this set of data, we concluded that the expression pattern of miR-495 and miR-218 overlaps with that of HNF-6 and OC-2, which suggests that the two miRs may regulate the two transcription factors.

Translational repression of HNF-6 and OC-2 by miR-495 and miR-218

We then studied, by transfection experiments, if miR-495 and miR-218 can control HNF-6 and OC-2 expression. To validate the miR-495 target site, a fragment of the HNF-6 3′UTR containing the putative target site was cloned in the psi-CHECK-1 expression vector, downstream of the *firefly* luciferase stop codon. When cotransfected with synthetic miR-495, a 50% decrease in luciferase activity, relative to control, was observed (Fig. 3A). The luciferase activity was rescued when the miR-495 target site was rendered ineffective by mutating two nucleotides in the seed sequence (Fig. 3A). MiR-495 shows no inhibitory activity on psi-CHECK-1 devoid of HNF-6 3′UTR. Thus, miR-495 targets the HNF-6 3′UTR and represses gene expression via its target site.

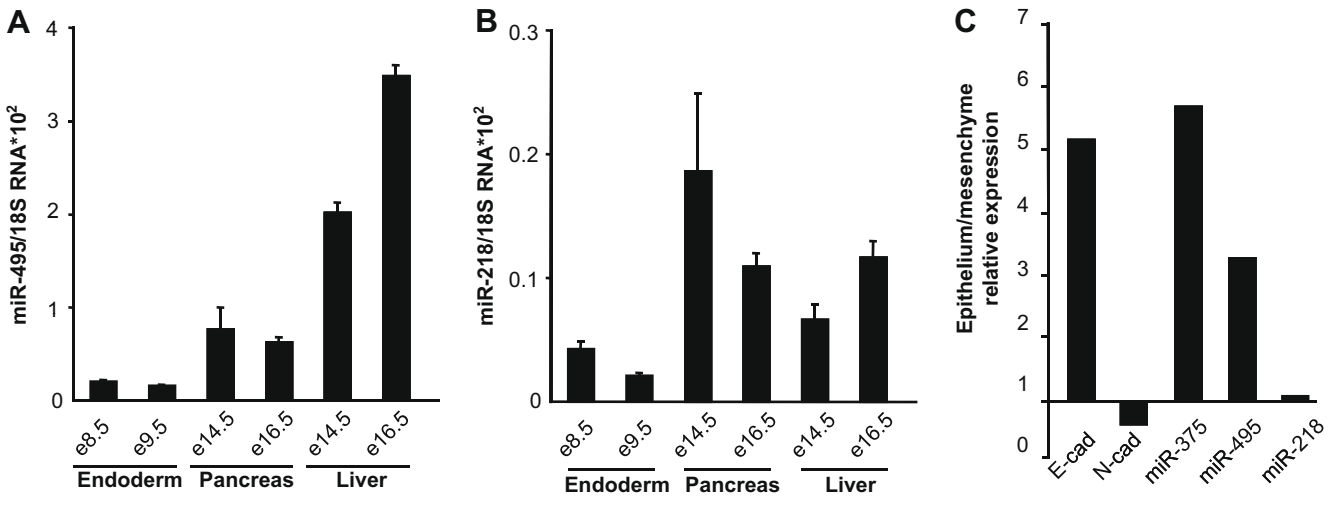

Fig. 2. Expression of miR-495 and miR-218 during pancreas and liver development. MiR-495 is expressed in the microdissected endoderm epithelium at e8.5 and e9.5, and in liver and pancreas at e14.5 and e16.5 (A). MiR-218 is expressed in the endoderm epithelium at e8.5 and e9.5 and in developing pancreas an liver at e14.5 and e15.5 (B). In the pancreas, miR-495 is preferentially expressed in the epithelium, and miR-218 is equally distributed in epithelium and mesenchyme (C).

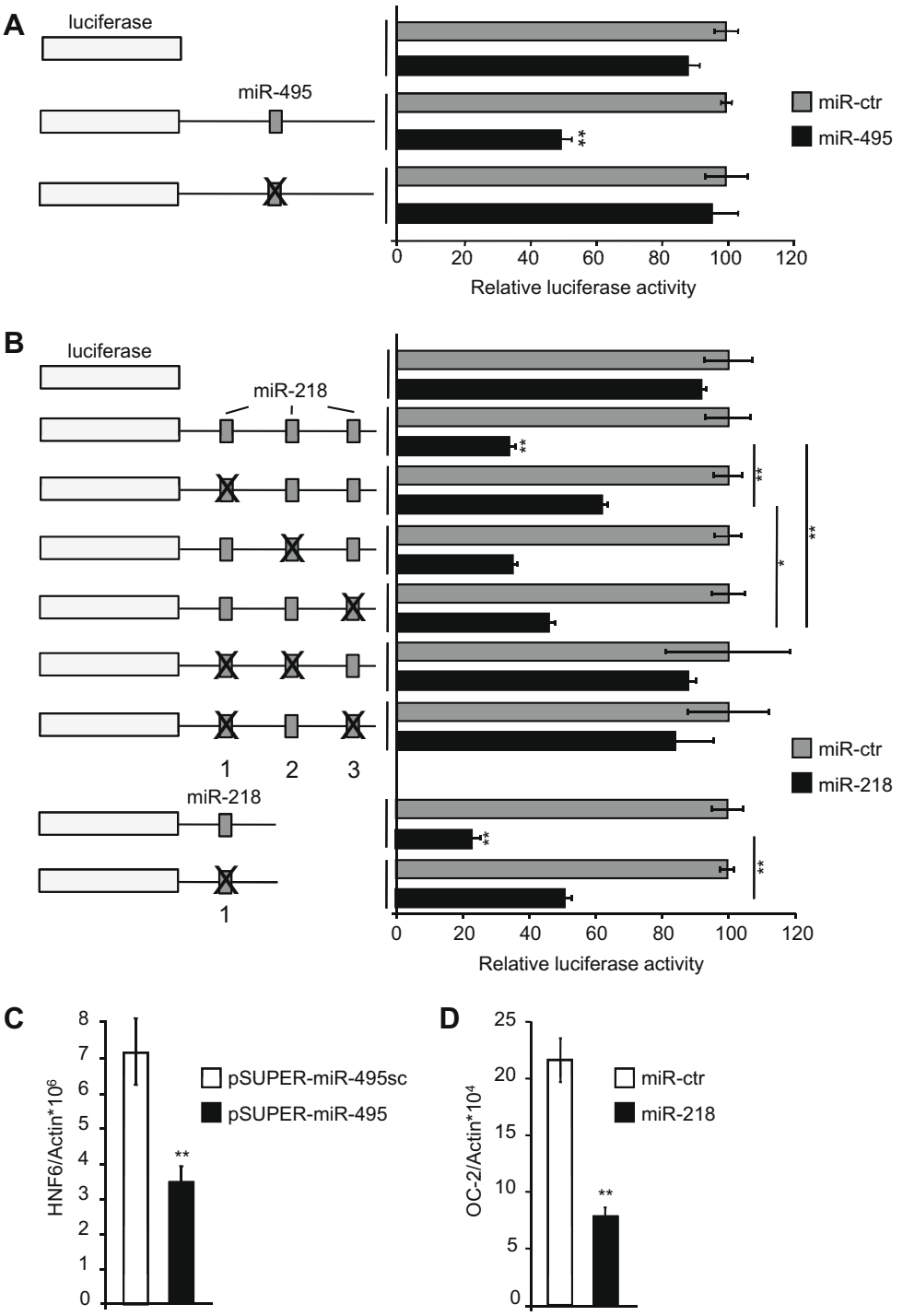

Fig. 3. Role of miR-495 and miR-218 in the control of HNF-6 and OC-2 expression. MiR-495 targets mouse HNF-6 via one target site located in the 3′UTR (A). MiR-218 targets mouse OC-2 via target sites located in the 3′UTR (B). Endogenous HNF-6 mRNA level after forced expression of miR-495 in BMEL cells (C). Reduced expression of endogenous OC-2 mRNA after transfection of synthetic miR-218 in BMEL cells (D). Single and double stars indicate $p < 0.05$ and $p < 0.01$, respectively.

We next measured by RT-qPCR the expression level of endogenous HNF-6 in BMEL cells, after overexpression of miR-495. The amount of HNF-6 mRNA was reduced by 50% in the BMEL cells transfected with a miR-495 expression vector, when compared to cells transfected with a vector coding for a scrambled version of miR-495 (Fig. 3C), further confirming the inhibitory effect of miR-495 on HNF-6 expression.

To test the functionality of the three putative mir-218 target sites located in the OC-2 3′UTR, we designed several reporter constructs containing the wild-type or mutated 3′UTR region fused to the luciferase gene. When a construct with wild-type 3′UTR was cotransfected with synthetic miR-218, a decrease in relative luciferase activity was observed, indicating that this 3′UTR region was targeted by miR-218 (Fig. 3B). We then generated constructs bearing mutations in the different sites and verified their activity in the presence of miR-218. When the first site was mutated, luciferase activity was less efficiently repressed by miR-218 as compared to the wild-type construct, indicating that the first site is functional. Mutation of the second site did not modify miR-218-induced repression of luciferase, indicating that this second site is dispens-

able for miR-218 activity. Mutations in the third site were associated with miR-218-induced repression of luciferase activity; this repression was slightly, but significantly, less efficient than in the presence of wild-type target sequence, indicating that the third site is also a functional miR-218 target site (Fig. 3B). These data suggested that the first target site is the most potent to mediate the effects of miR-218. When used alone in the 3′UTR of a luciferase construct, it mediated miR-218 inhibition (Fig. 3B lower part). Interestingly, it cooperates with sites 2 and 3: when either sites 1 + 2 or sites 1 + 3 were mutated in the 3′UTR of the OC-2 gene, miR-218 was unable to repress luciferase activity, indicating that the various target sites exert cooperative functions (Fig. 3B).

We next checked whether miR-218 was able to regulate endogenously expressed OC-2. Indeed, when overexpressed in BMEL cells, miR-218 was able to reduce by 60% the amount of endogenous OC-2 mRNA, when compared to control (Fig. 3D). These data show that miR-218 exerts a posttranscriptional control on OC-2 via multiple target sites located in the 3′UTR of OC-2.

Discussion

The Onecut factors HNF-6 and OC-2 are important regulators of pancreas and liver development. The absence of these factors leads to severe defects in pancreatic and liver cell differentiation [3–11,13,14]. Inversely, increased or ectopic expression is associated with perturbed cell homeostasis [16] or with diabetes [15]. This indicates that the concentration of HNF-6 and OC-2 has to be finely regulated, possibly by miRs. This hypothesis is supported by data showing that HNF-6 and OC-2 expression is upregulated in pancreata (A.S., F.P.L., P.J., unpublished data) and livers depleted in miRs [22]. In the present work, we identify two miRs, namely miR-495 and miR-218, which regulate the expression of HNF-6 and OC-2, respectively.

Little is known about mir-495 and mir-218. Both are present in brain [30,31] and data suggest that miR-495 regulates the expression of BDNF [30]. MiR-218 expression is upregulated in leukemia [32] and in prostate cancer [33]. In pancreas, its expression is upregulated in cancer [34] and this occurs with a parallel decrease in OC-2 expression [35], suggesting that miR-218 could play a role in this process. We also found miR-495 target sites in the 3′UTR of the Sox9 and Jagged2 genes, which like HNF-6 and OC-2, are involved in pancreas and liver development. This suggests that miR-495 could control the expression of several pancreatic and hepatic developmental regulators.

Most miRs detected at e9.5 in the liver and pancreatic buds are also found later in developing pancreas and liver. Members of the miR-302 family are a noticeable exception to this observation. These miRs are preferentially expressed in the male gonads and early embryos [36]. They are no longer found at later stages in pancreas or liver development [20,37], suggesting that they are expressed in the multipotent pancreatic and hepatic progenitors. In the pancreatic and hepatic buds, miR-17/miR-92 clusters are abundant. This is not surprising, considering that cells in these tissue buds are highly proliferative, and that these miRs are cell cycle regulators.

Taken together, the present data demonstrate that Onecut factors are direct targets of miRs during embryonic development. Further studies will be devoted to unravel the specific role of these miRs in pancreas and liver development and disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.052.

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