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Regulation of mouse stomach development and Barx1 expression by specific microRNAs

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SUMMARY

Although microRNAs (miRNAs) are postulated to fine-tune many developmental processes, their relationships with specific targets and tissues remain largely undefined. The mesenchymal transcription factor Barx1 controls spleen and stomach morphogenesis and is required to specify stomach-specific epithelium in adjacent endoderm. *Barx1* expression is precisely regulated in space and time, with a sharp drop in stomach levels after epithelial specification. We tested the hypothesis that specific miRNAs mediate this marked decline in Barx1 levels. Depletion of the miRNA-processing enzyme Dicer in cultured stomach mesenchyme and conditional *Dicer* gene deletion in mice significantly increased Barx1 levels, disrupted stomach and intestine development and caused spleen agenesis. Computational and experimental studies identified miR-7a and miR-203 as candidate miRNAs that regulate *Barx1* and are expressed in inverse proportion to it in the fetal mouse stomach. Through specific interactions with cognate sequences in the *Barx1* 3′ untranslated region, miR-7a and miR-203 repress *Barx1* expression in stomach mesenchymal cells and its function in inducing gastric epithelium. These results indicate that miRNAs are required for proper digestive tract organogenesis and that miR-7a and miR-203 control expression of the stomach homeotic regulator Barx1.

KEY WORDS: Barx1, Stomach development, MicroRNA, Mouse

INTRODUCTION

Transient cellular interactions in embryos have lasting consequences that necessitate precise spatiotemporal control of gene expression. Multicellular organisms restrict gene expression domains in part by activating genes selectively and for defined periods in certain tissues. However, because many mRNAs are inherently stable, timed tissue-specific gene activation is insufficient to control the duration of gene expression and the decay of some transcripts might be as important in ensuring that their expression is suitably curtailed. MicroRNAs (miRNAs) represent a potent means for brisk downregulation of transcripts after their developmental requirements have lapsed. Indeed, miRNAs were first identified as regulators of *C. elegans* developmental chronology (Moss, 2007; Reinhart et al., 2000); moreover, in global analyses, transcripts show higher expression at developmental stages preceding the appearance of their cognate miRNAs and reduced expression as these miRNAs accumulate (Farh et al., 2005; Stark et al., 2005). Other studies implicate selected miRNAs in the development of vertebrate limbs (Hornstein et al., 2005), muscle (Chen et al., 2006; Cordes et al., 2009), heart (Zhao et al., 2007), neurons (Makeyev et al., 2007), blood (Li et al., 2007; Thai et al., 2007), retina (Decembrini et al., 2009) and intestine (Zeng et al., 2009). One recent study profiled miRNAs expressed in the mouse intestine and showed, through tissue-specific loss of all miRNAs, that they are necessary for epithelial barrier function (McKenna et al., 2010). More than 1000 miRNAs have been identified and some are known to sharpen spatial or temporal borders of gene expression (Stefani and Slack, 2008), but few miRNAs have well-established functions and defined target genes in tissue development and organogenesis.

Mesoderm-derived stromal tissue plays a prominent role in primitive gut tube patterning and digestive tract development (Haffen et al., 1987; Kedinger et al., 1998). The homeobox gene *Barx1* is expressed in abundance but transiently in prospective stomach mesenchyme and is required to specify this organ (Kim et al., 2005; Kim et al., 2007). *Barx1*$^{-/-}$ mouse embryos have a small, deformed stomach with a mucosal lining of the intestinal type, reflecting marked homeotic transformation (posteriorization) of the foregut. Expression of Wnt signaling antagonists is diminished in the absence of Barx1. Stomach endoderm normally shows an early burst of Wnt signaling, and Barx1-mediated attenuation of this signal seems necessary for proper gastric epithelial differentiation (Kim et al., 2005).

It is not known how *Barx1* expression is itself controlled, nor, specifically, how Barx1 levels decline in late-gestation stomach mesenchyme. We postulated that temporal regulation might occur through specific miRNAs that target *Barx1* mRNA. Here, we show that miRNAs influence mouse stomach organogenesis and the *Barx1* expression domain. We identify miR-7a and miR-203 as key miRNAs in *Barx1* regulation.

MATERIALS AND METHODS

Mice

Animals were handled according to protocols approved by an institutional committee.

miRNA binding predictions

miRNA binding was estimated as a consensus from three different prediction algorithms: miRBase (http://microrna.sanger.ac.uk/targets/v4/) uses the miRanda algorithm to predict miRNA-mRNA pairs; the miRscan (http://genes.mit.edu/mirscan/) approach allows for G:U wobbles in

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seed matches; and PicTar (http://pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi) confirmed candidates predicted by the first two algorithms.

RNA isolation and processing, quantitative analysis of mRNAs and miRNAs, and in situ hybridization

Tissue RNA was isolated using TRIzol (Invitrogen), polyadenylated using ATP and poly(A) polymerase (Ambion), and reverse-transcribed using oligo-dT adapters. miRNAs and 5.8S ribosomal RNA were reverse-primed with a universal 3′ adapter (FirstChoice RLM-RACE Kit, Ambion) and forward primers based on specific miRNA sequences (see Table S1 in the supplementary material). mRNA and miRNA levels were assessed by SYBR Green PCR in triplicate on an ABI Prism 7300 instrument (Applied Biosystems). Each experiment was repeated at least three times and PCR product sizes were verified by electrophoresis. In situ hybridization probes were generated using the *mir*Vana Probe Construction Kit (Ambion) and hybridized overnight at 58°C. Signals were detected with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (1:2000; Roche).

Plasmids

We cloned *Barx1* 3′-UTR sequences from regions complementary to miR-7a or miR-203 into the *Xba*I site of pGL3-Control (Promega) and deleted the polylinker upstream of the SV40 promoter between the *Kpn*I and *Xho*I sites (see Fig. 3A). Pre-miRNAs 7a and 203 were cloned between the *Eco*RV and *Eco*RI sites, upstream of an IRES-eGFP cassette, in pCIG (Megason and McMahon, 2002). To detect interaction between miRNAs and *Barx1* mRNA in individual cells, we replaced luciferase cDNA in pGL3-ΔKX with DsRed1, amplified by PCR from the plasmid pDsRed1-N1 (Clontech), to generate the modified reporter pGL3-ΔKX_DsRed1. We digested pCIG and its derivative pre-miRNA constructs 7a and 203 with *Sal*I and cloned these fragments into pGL3-ΔKX_DsRed1 in reverse orientation (see Fig. 3C).

Cell culture and siRNA studies

Immortalized stomach mesenchymal cells (ISMCs) were generated by infecting E12 mouse stomach mesenchyme (Kim et al., 2005) with retrovirus expressing mutant p53 (Boehm et al., 2005). Dicer1 siRNAs, prepared in vitro using the Silencer siRNA Cocktail Kit (Ambion), were introduced into immortalized cells using siPORT*Amine* (Ambion) and into fresh fetal mesenchymal cells using Lipofectamine 2000 (Invitrogen). Recombinant endoderm-mesenchyme cultures were performed as described previously (Kim et al., 2005), with transfection of the pre-miR plasmids described above.

Luciferase analysis

pGL3-Con_7a or pGL3-Con_203 were transfected transiently into ISMCs together with empty pCIG, pCIG-pmiR7a or pCIG-pmiR203 in equal mass ratios and pRL-CMV (Promega) at a 1:15 ratio, using Lipofectamine 2000. Luciferase was measured using the Dual Luciferase Reporter Assay Kit (Promega). Experiments were performed in triplicate at least three times for each reporter-pre-miRNA combination. Data are presented as normalized ratios of firefly:Renilla luciferase.

RESULTS AND DISCUSSION

Dicer1 is required for normal stomach, intestine and spleen development and regulates Barx1 expression

Barx1 mRNA is highly expressed in embryonic stomach mesenchyme, but not in intestinal mesenchyme, and the levels drop steadily after ~E12 until it is barely detectable in older embryos (Kim et al., 2005); stomach Barx1 expression is confined to the mesenchyme and mesogastrium and protein levels are also markedly reduced in late gestation (Fig. 1A). As miRNAs are known to function in post-transcriptional gene control, we postulated that miRNAs contribute to Barx1 downregulation. To test this hypothesis, we used two different strategies to deplete

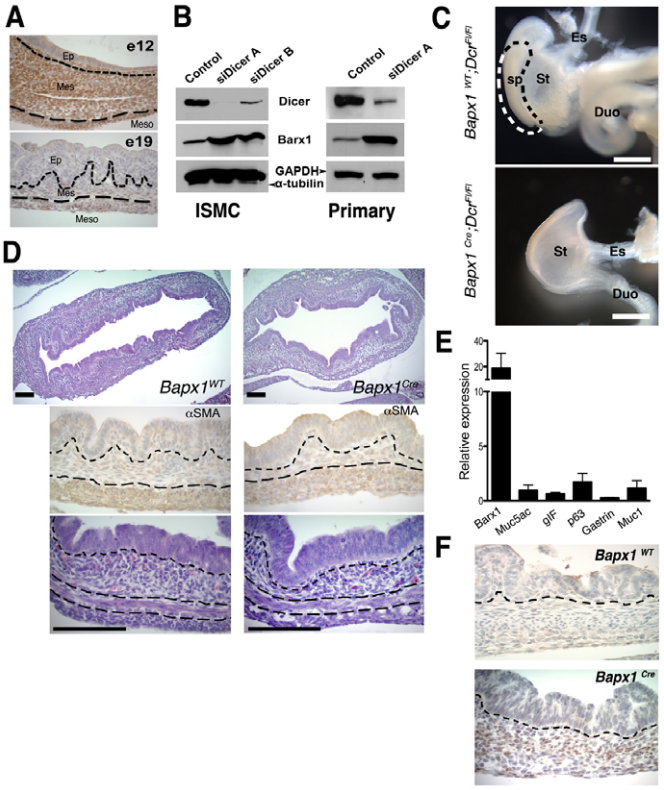

Fig. 1. Requirement of Dicer1 for normal stomach, intestine and spleen development and regulated *Barx1* expression. (**A**) Barx1 immunostaining at E12 (top) and E19 (bottom) demonstrates a substantial interim decrease in expression, raising the possibility of negative regulation by miRNAs. Epithelium (Ep) and mesenchyme (Mes) are demarcated by a dotted line and the mesothelium (Meso) by a dashed line. (**B**) Dicer and Barx1 immunoblot analysis in immortalized (ISMC, left) and freshly isolated (right) mouse fetal stomach mesenchymal cells treated with control or two mouse Dicer-specific synthetic siRNA pools: siDicerA or siDicerB. Cell extracts were prepared 48 hours after siRNA transfection. Gapdh and α-tubulin serve as loading controls. (**C**) Posterior whole-mount views of foreguts from E17.5 *Bapx1*$^{+/Cre}$;*Dicer1*$^{Fl/Fl}$ and littermate control mouse embryos, showing severe stomach dysmorphogenesis and spleen agenesis (sp, dashed outline) in the absence of Dicer. Es, esophagus; St, stomach; Duo, duodenum. (**D**) Histochemical and immunohistochemical analysis of E17.5 *Dicer1*$^{Fl/Fl}$ stomachs on *Bapx1*$^{+/+}$ (left) and *Bapx1*$^{+/Cre}$ (right) backgrounds. Hematoxylin and eosin-stained tissue sections confirm asplenia and reduced stomach size and reveal overtly intact gastric mucosa. High-magnification images are shown in the lower panels, with mesenchyme demarcated from epithelium by dotted lines and smooth muscle demarcated by α-smooth muscle actin (α-SMA) staining (top) or by dashed lines (bottom). (**E**,**F**) qRT-PCR (E) and immunohistochemistry (F) show significantly increased Barx1 expression in E17.5 *Bapx1*$^{+/Cre}$;*Dcr*$^{Fl/Fl}$ stomach, whereas molecular markers of gastric epithelial maturation are unaltered. RNA levels are represented in relation to levels measured on the *Bapx1*$^{+/+}$ background. gIF, gastric intrinsic factor (*Gif*); p63, tumour protein 63 (TP63). Epithelium and mesenchyme are separated by dotted lines (F). Error bars indicate mean + s.d. Scale bars: 0.5 mm in C; 250 μm in D.

Dicer, the RNaseIII enzyme that generates small double-stranded RNAs (Bernstein et al., 2001); Dicer-deficient cells cannot process miRNA precursors and fail to produce mature miRNAs in vitro and in vivo (Carmell and Hannon, 2004).

We first used synthetic small interfering RNAs (siRNAs) to deplete Dicer from immortalized or cultured fresh mouse fetal stomach mesenchymal cells. In both cell types, depletion of Dicer increased steady-state levels of *Barx1* mRNA (data not shown) and protein (Fig. 1B). To confirm a role for miRNAs in stomach mesenchyme in vivo, we crossed mice carrying the conditional floxed-null (Fl) *Dicer1* allele $Dcr1^{lox(ex18\text{-}20)}$ (Kanellopoulou et al., 2005) with mice that express Cre recombinase from the endogenous *Nkx3-2* (*Bapx1*) locus (Verzi et al., 2009). As the latter strain expresses Cre throughout the full thickness of the intestinal and distal stomach mesenchyme and the spleen anlage (Verzi et al., 2009), floxed alleles recombine selectively in these tissues. At embryonic day (E) 17.5, $Bapx1^{+/Cre};Dcr^{Fl/Fl}$ embryos lacked a spleen and the stomach was consistently reduced to about half the normal size (n=5), with defective morphology confined to its distal two-thirds (Fig. 1C and see Fig. S1A in the supplementary material). Mesenchymal cell mass was preserved in proportion to organ dimensions and α-smooth muscle actin immunostaining revealed overtly normal smooth muscle differentiation (Fig. 1D). Mucosal maturation was overtly unaffected; cells organized into a columnar epithelium that resembled its control counterpart in morphology (Fig. 1D) and in the expression of molecular markers (Fig. 1E). Stratified squamous epithelium in the forestomach, a region lacking Bapx1 (Cre) expression, also appeared intact but intestinal epithelial maturation was markedly aberrant, with few and structurally deficient villi (see Fig. S1B in the supplementary material). The abnormalities in the intestinal mucosa, which lacks Bapx1 (Cre) expression (Verzi et al., 2009), probably represent an indirect consequence of *Dicer1* deletion in the adjacent mesenchyme. Fully penetrant asplenia reveals a previously unappreciated requirement for miRNAs in genesis of the spleen.

Compared with littermate $Dcr^{Fl/Fl}$ controls lacking Cre expression, $Bapx1^{+/Cre};Dcr^{Fl/Fl}$ embryos showed significantly increased *Barx1* mRNA levels in the E17 stomach, where Barx1 is normally low or absent (Fig. 1E). *Barx1* mRNA levels were also elevated in the duodenum, where it is normally undetectable (see Fig. S1C in the supplementary material), but we observed no expression in the $Bapx1^{+/Cre};Dcr^{Fl/Fl}$ ileum. Immunostaining confirmed persistent Barx1 expression in the mutant stomach (Fig. 1F) and ectopic expression in the proximal intestine (see Fig. S1D in the supplementary material); similar to the normal Barx1 distribution, these aberrant signals localized in the mesenchyme. The stomach morphogenesis defects almost certainly reflect the aggregate absence of many miRNAs and not merely the significant increase in Barx1 levels. For our purposes, the key conclusion from this experiment is that depletion of miRNAs in embryonic mouse gut mesenchyme interferes with *Barx1* decay, indicating that miRNAs participate in the physiological regulation of Barx1 levels.

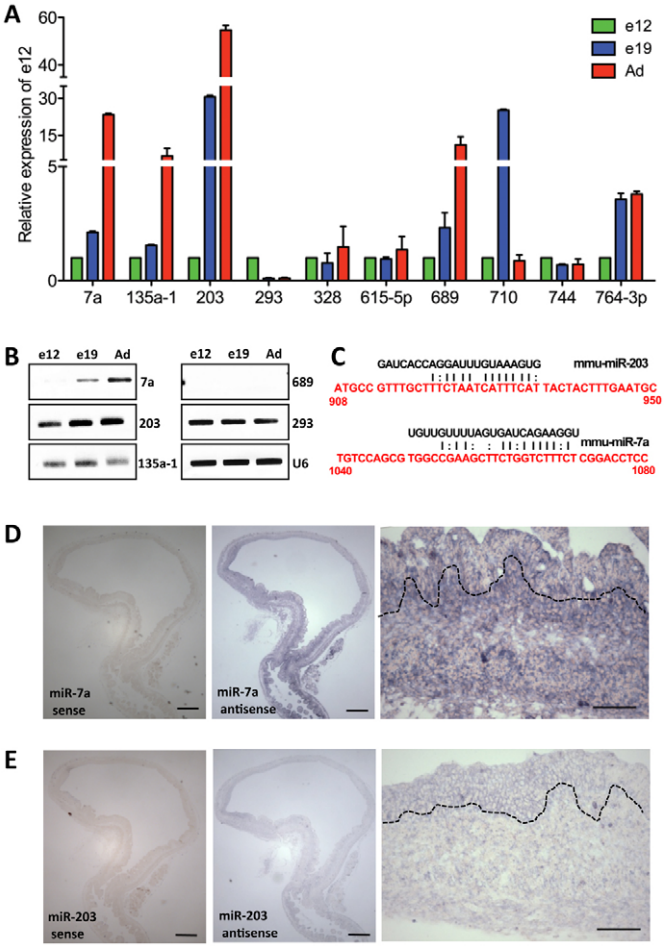

Fig. 2. Expression of candidate regulatory miRNAs in late-gestation mouse stomach. (**A**,**B**) Relative expression of candidate *Barx1*-regulating miRNAs in wild-type E12, E19 and adult (Ad) mouse stomach, assessed by qRT-PCR (A) and conventional RT-PCR (B). U6 mRNA served as a loading control. Data represent a sample (B) or mean + s.d. (A) from three independent experiments, each with single adult stomachs and at least two samples of multiple embryonic stomachs pooled at each stage. Quantitative results are represented in relation to the level of each miRNA at E12. (**C**) Sequence complementary of miR-7a and miR-203 (black) to the mouse *Barx1* 3′ untranslated region (red). Numbers correspond to nucleotide designations in *Barx1* reference clone ENSMUST00000021813 (MGI: 103124). (**D**,**E**) In situ detection of miR-7a (D) and miR-203 (E) in E19 mouse stomach. Younger embryos (data not shown) and sense riboprobes provided specificity controls. Images on the right show representative areas from the middle panels at higher magnification, with epithelium and mesenchyme separated by dotted lines. Scale bars: 1.5 mm (left and middle) and 150 μm (right).

Expression of candidate regulatory miRNAs predicted to bind the 3′-UTR of *Barx1* in late-gestation mouse stomach

To identify specific miRNAs that might target *Barx1* mRNA, we reasoned that stomach expression of candidate miRNAs would increase in late gestation, opposing the chronology of *Barx1* mRNA decay. Because miRNAs target transcripts with imperfect complementarity, any miRNA can target several mRNAs and a given transcript may pair with none, one or many miRNAs (Stefani and Slack, 2008). Using three different databases derived from pairing rules for miRNA-mRNA interaction (Lewis et al., 2003; Lim et al., 2003a) – miRBase Targets (Griffiths-Jones et al., 2008), miRscan (Lim et al., 2003b) and PicTar (Krek et al., 2005) – we identified candidate miRNAs (see Table S2 in the supplementary material) and the probability that any of these might target the *Barx1* 3′ untranslated region (UTR), where most miRNAs bind their mRNA targets (Ambros, 2004). Quantitative (q) RT-PCR analysis of the ten leading candidates (Fig. 2A) verified that the levels of miR-7a and miR-203 increased substantially between E12 and E19 and in inverse proportion to the decline in endogenous

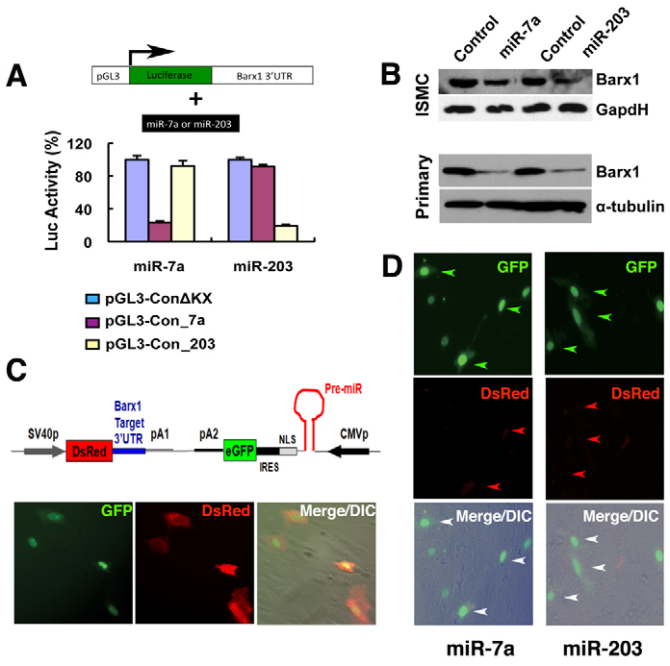

Fig. 3. Function of the candidate Barx1-targeting miRNAs 7a and 203. (**A**) The luciferase reporter and pre-miRNA constructs, and results of reporter assays in ISMCs co-transfected with a plasmid expressing firefly *luciferase* cDNA linked to the miR-7a or miR-203 recognition site in the *Barx1* 3′-UTR and a second plasmid expressing miR-7a or miR-203. Values were adjusted for plasmid uptake efficiency by co-transfecting *Renilla* luciferase and are represented in relation to a 100% value for the luciferase level without miRNA co-transfection. Reporter activity decreased specifically in the presence of cognate miRNAs. Error bars indicate mean + s.d. (**B**) Barx1 immunoblot analysis in ISMCs (top) and mouse primary fetal stomach mesenchymal cells (bottom) transfected with empty vector or with miR-7a or miR-203 expression plasmids. α-tubulin and Gapdh served as loading controls. (**C**) Plasmids for co-expression of pre-miRNAs and eGFP, by virtue of internal ribosome entry, to assess miRNA effects in individual cells. Cells transfected with a control plasmid lacking any pre-miR expressed both DsRed and eGFP. (**D**) Fluorescence imaging of ISMCs after miR-7a or miR-203 pre-miRNAs were co-expressed with *DsRed* cDNA tagged with the Barx1 3′-UTR. eGFP^{+} cells (arrows) carrying miR-7a (left) or miR-203 (right) suppressed *DsRedI* reporter gene expression.

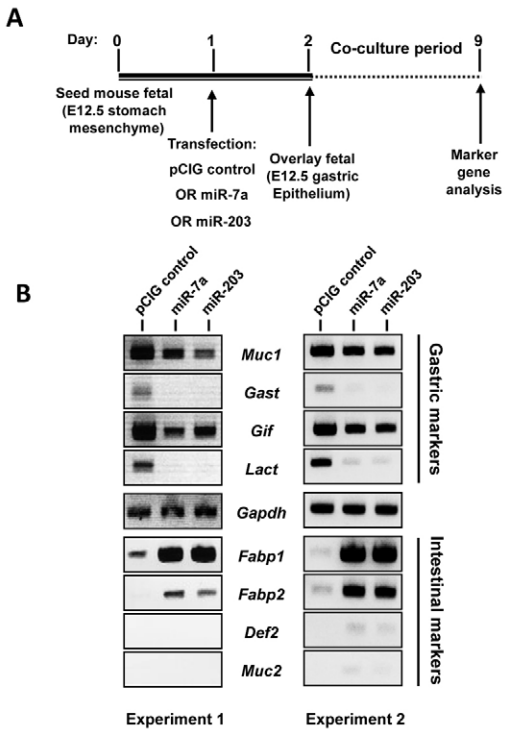

Fig. 4. miRNAs 7a and 203 abrogate Barx1 function in induction of stomach-type epithelium. Schema (**A**) and results (**B**) of mesenchyme-endoderm recombinant cultures in which pre-miR-7a, pre-miR-203 or empty pre-miR plasmid were introduced into E12 mouse primary stomach mesenchymal cells. As expected as a result of Barx1 suppression, the endoderm overlying miRNA-treated cells showed attenuated expression of the stomach-specific epithelial genes mucin 1 (*Muc1*), gastrin (*Gast*), *Gif* and lactoferrin [*Lact*; also known as lactotransferrin (*Ltf*)], and aberrant expression of intestinal markers fatty acid binding proteins 1 and 2 (*Fabp1* and *Fabp2*), defensin 2 (*Def2;* also known as *Defa2*, *Defcr-2* and alpha-defensin2) and *Muc2*, as assessed by RT-PCR. *Gapdh* mRNA provided a loading control. The left and right columns represent results from two independent experiments.

Barx1 levels. Conventional RT-PCR confirmed these trends (Fig. 2B). miR-689, miR-710 and miR-764-3p showed similar trends to miR-7a and miR-203 by sensitive qRT-PCR but their expression was too low to detect by other means (Fig. 2B and data not shown); results for miR-135a-1 were inconsistent between the two RT-PCR methods and the other candidates lacked the predicted temporal profile. As the *Barx1* 3′-UTR regions that contain miR-7a and miR-203 recognition sites are also highly conserved across species (see Fig. S2 in the supplementary material), we focused on these candidates; Fig. 2C illustrates their complementarity with the 3′-UTR of mouse *Barx1*. In situ hybridization indicated an abundance of miR-7a and miR-203 in E19 mouse stomach; miR-7a, in particular, gave stronger signals in the mesenchyme (Fig. 2D,E), where Barx1 is expressed. Thus, different computational algorithms predict *Barx1* mRNA as a target for miR-7a and miR-203, which are expressed in a tissue-specific and temporal pattern compatible with a role in Barx1 regulation.

miR-7a and miR-203 target *Barx1* and suppress its expression

To test whether miR-7a and miR-203 regulate *Barx1* mRNA levels, we appended the mouse *Barx1* 3′-UTR, carrying either the miR-7a or miR-203 recognition sequence, to a firefly *luciferase* reporter gene (pGL3-Con_7a or pGL3-Con_203; Fig. 3A). Both regions were deleted in the control construct pGL3-ConΔKX. We introduced these constructs into immortalized stomach mesenchymal cells (ISMCs) together with plasmids encoding precursor (pre-miR) forms of miR-7a or miR-203. miR-7a significantly reduced luciferase levels when co-expressed with pGL3-Con_7a, but had no effect on pGL3-ConΔKX or pGL3-Con_203. Conversely, miR-203 significantly repressed luciferase levels in conjunction with pGL3-Con_203, but not with pGL3-ConΔKX or pGL3-Con_7a (Fig. 3A). Thus, each miRNA interacts specifically with its cognate site in the *Barx1* 3′-UTR to reduce expression of a linked reporter. To test whether miR-7a

and miR-203 affect endogenous Barx1 levels in a native context, we forced pre-miR expression in ISMCs or cultured fresh E12 mouse stomach mesenchymal cells without luciferase reporter genes. Both miR-7a and miR-203 reduced Barx1 protein levels in these experiments, confirming miRNA targeting of *Barx1* (Fig. 3B).

To determine whether these miRNA effects are cell-autonomous, we assessed them at single-cell resolution by marking miRNA-expressing cells, using the CMV promoter to express pre-miRNAs and nuclear-localized eGFP from a single transcript separated by an internal ribosome entry site. The same constructs carried, in reverse orientation, an SV40 promoter-driven *DsRed* reporter gene followed by the full *Barx1* 3′-UTR (Fig. 3C). Control cells lacking exogenous miRNA (carrying an empty pre-miRNA insertion site) expressed DsRed and eGFP in the same cells (Fig. 3C). By contrast, DsRed expression was abolished in eGFP-expressing stomach mesenchymal cells that carried pre-miR-7a or pre-miR-203 (Fig. 3D). These data directly implicate miR-7a and miR-203 in regulating Barx1 expression levels through the 3′-UTR.

To verify the functional consequences of miRNA-mediated Barx1 depletion, we cultured E12 mouse stomach mesenchyme and endoderm under conditions that ordinarily favor Barx1-dependent gastric epithelial differentiation and suppress intestinal differentiation (Kim et al., 2005). We transfected the mesenchymal cells with miR-7a, miR-203 or empty pre-miR plasmids before overlaying naïve endoderm and assessed expression of stomach and intestinal epithelial markers 1 week later (Fig. 4A). Forced expression of miR-7a or miR-203 induced intestinal markers (Fig. 4B), compatible with reduced Barx1 function in the targeted mesenchymal cells. Because limited pre-miR transfection efficiency would inevitably preserve some residual Barx1 function, we expected modest effects on gastric epithelial differentiation; nevertheless, stomach epithelial marker expression was consistently reduced (Fig. 4B). These results confirm that expression of miRNAs 7a and 203 impairs Barx1 function.

Conclusions

Dynamic tissue interactions are necessary for the proper patterning, differentiation and morphogenesis of the embryonic gut (Kedinger et al., 1998; Kim et al., 2005), mandating tight control of the distribution and duration of expression of crucial factors. Barx1 is necessary for proper stomach morphogenesis and epithelial specification, acting in part through the stage-specific antagonism of nearby Wnt signaling (Kim et al., 2005; Kim et al., 2007). The onset, duration and domain of Barx1 expression are therefore important determinants of foregut development. Cis-regulatory elements and transcription factors probably drive tissue-specific *Barx1* expression but are presently unknown and likely to represent only one facet of spatiotemporal control. As an additional mechanism, the presence of distinct miRNA-complementary sequences in the short *Barx1* 3′-UTR suggests the possibility of concerted targeting by various miRNAs. Conditional Dicer depletion in vivo indicated that miRNAs are generally required for normal gut and spleen development, but more to our point, the experiment demonstrated that some miRNAs downregulate Barx1 levels in the stomach and help avoid its expression in the intestine. These results prompted the question of which miRNAs might specifically limit the duration of Barx1 expression in mouse stomach development and our studies reveal that miR-7a and miR-203 are likely to perform this function. The accumulation of these miRNAs in fetal stomach mesenchyme coincides with declining *Barx1* mRNA levels and they interact with discrete sequences in the *Barx1* 3′-UTR. miR-7a and miR-203 also repress expression of Barx1 and of linked reporter genes and repress Barx1 function in stomach mesenchymal cells. Targeted disruption of the *Mir7* and *Mir203* gene loci might formally prove the role that our study supports.

Because single miRNAs can target many transcripts with partial complementarity (Stefani and Slack, 2008), their own expression must be closely balanced with that of crucial and incidental targets. This example of Barx1 downregulation in the late embryonic mouse stomach might represent a widely used mechanism to modulate the levels and timing of genes with potent developmental activities.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

B.-M.K. and J.W. performed the research; all authors designed experiments and analyzed data; J.W. and R.A.S. wrote the manuscript.

Supplementary material

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Table S1. Primers used in qRT-PCR

Gene	Primer sequence (5′ to 3′)
miR-150	TCTCCCAACCCTTGTACCAGTG
miR-203	GTGAAATGTTTAGGACCACTAG
miR-328	CTGGCCCTCTCTGCCCTTCCGT
miR-542-3p	TGTGACAGATTGATAACTGAAA
miR-542-5p	CTCGGGGATCATCATGTCACGA
miR-667	TGACACCTGCCACCCAGCCCAAG
miR-689	CGTCCCCGCTCGGCGGGGTCC
miR-710	CCAAGTCTTGGGGAGAGTTGAG
miR-744	TGCGGGGCTAGGGCTAACAGCA
miR-763	CCAGCTGGGAAGAACCAGTGGC
miR-764-3p	AGGAGGCCATAGTGGCAACTGT
miR-764-5p	GGTGCTCACATGTCCTCCT
miR-770-3p	CGTGGGCCTGACGTGGAGCTGG
miR-7a	TGGAAGACTAGTGATTTTGTTGT
miR-99b-1	CAAGCTCGTGTCTGTGGGTCCG
miR-135a-1	TATAGGGATTGGAGCCGTGGCG
miR-293	AGTGCCGCAGAGTTTGTAGTGT
miR-330	TCTCTGGGCCTGTGTCTTAGGC
miR-615-5p	GGGGGTCCCCGGTGCTCGGATC
T adapter	GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN
Adapter rv	GCGAGCACAGAATTAATACGAC
U6F	CGCTTCGGCAGCACATATAC
U6R	TTCACGAATTTGCGTGTCAT

Table S2. Suitable complementarity of sequences in the mouse *Barx1* 3′-UTR to those of known miRNAs

miRNA	Score	Energy	Start	End	Alignment
miR-135	18.438	–32.16	362	385	GCGGUG--CCgaGguuAGGGAUAu CGCCACCGGGtgCaggTCCCTATg
miR-203	18.0042	–13.1	68	89	gAucaccAcGAUUuGUAAAGUg gTttgctTtCTAAtCATTTCAt
miR-689	17.5504	–31.51	20	39	cCuGgGgCgGCUCGCCCCUGc tGgCgCgGtCG-GCGGGGACc
miR-764-3p	17.4619	–23.62	421	441	uGUCaACggUGAuACCGGAGGa tCAGcTGatAC-cTGGCCTCCc
miR-7a	16.7304	–17.59	203	225	uguuGuUUuaguGAuCAGAAgGu tggcCgAAgcttCTgGTCTTtCt
miR-293	16.5942	–22.03	228	249	uguGAuGuuUgAgACGCCGuGA gacCTcCgcAgTgTGCGGCgCT
miR-764-3p	16.3773	–16.93	280	301	UGUcAAcgguGauaCCGGAGGa ACAcTTttatCcccGGCCTCCa
miR-615-5p	16.1604	–32.4	21	42	CuagGCucGugGCCCCUGGGgg GgcgCGgtCggCGGGGACCCag
miR-328	15.7265	–22.73	100	121	UgcCuuCccgUcucUCCCGGUC AatGcgGacaAttaAGGGCCAG
miR-689	15.2612	–25.49	340	360	ccuggGGCGgcucgCCCCUGc tacttCCGCgccttGGGGACc

The *Barx1* 3′-UTR reference sequence is aligned in red. Sequence position is numbered according to *Barx1* reference clone ENSMUST00000021813 (MGI: 103124). Complementarity appropriate for miRNA activity of each candidate is delineated in capital letters. Binding energy and miRNA score are derived from EMBL-EBI MicroCosm Target.

Barx1 3′-UTR sequence.

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1   GCGTCGCCGAGGATGCGGCTGGCGCGGTCGGCGGGGACCCAGGAGCTGGCCCTTCCGCGT 60
61  CCATGCCGTTTGCTTTCTAATCATTTCATTACTACTTTGAATGCAGACAATTAAGGGCCA 120
121 GACAAGGAAGGACACAGGCCCGGAAGCCAATCCCAGGTGTCAGCGAGCTTCTGTCCCCAG 180
181 TCTGGGAGACTTGTGTCCAGCGTGGCCGAAGCTTCTGGTCTTTCTCGGACCTCCGCAGTG 240
241 TGCGGCGCTCCACGCTCATTCACGCCCCGCTCCTTGCCCACACTTTTATCCCCGGCCTCC 300
301 AGCCGGCCTTCTGGGCCCGGACACCGGCAGGCACACACTTACTTCCGCGCCTTGGGGACC 360
361 CCGCCACCGGGTGCAGGTCCCTATGGCCCTGCCCTGCAGAGCAGAGTCGTCTCTAGCAGA 420
421 TCAGCTGATACCTGGCCTCCCCATGTCGCTGAGGCTTCT 459
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