Mesenchymal miR-21 regulates branching morphogenesis in murine submandibular gland in vitro

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Abstract

Branching morphogenesis in murine submandibular glands (SMG) is regulated by growth factors, extracellular matrix (ECM) and many biological processes through interactions between the epithelium and the mesenchyme. MicroRNAs (miRNAs) are a set of small, non-protein-coding RNAs that regulate gene expression at the post-transcriptional level. We hypothesized that branching morphogenesis is partly regulated by miRNAs. Forty-four miRNAs and novel miRNA candidates were detected in SMG at embryonic day 13 by a cloning method combined with Argonaute-2 immunoprecipitation. MicroRNA21 (miR-21) expression in the mesenchyme was up-regulated and accelerated by epidermal growth factor, which is known to enhance branching morphogenesis in vitro. Down-regulation of miR-21 in the mesenchyme by locked nucleic acids was associated with a decrease in the number of epithelial buds. Relative quantification of candidates for target genes of miR-21 indicated that two messenger RNAs (for Reck and Pdcd4) were down-regulated in the mesenchyme, where miR-21 expression levels were up-regulated. These results suggest that branching morphogenesis is regulated by miR-21 through gene expression related to ECM degradation in the mesenchyme.

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Introduction

MicroRNAs (miRNAs) are 19–24 nt endogenous RNA products of non-coding genes and are present in all multicellular organisms (Bartel, 2009; Kim et al., 2009). It is known that miRNAs triggering RNA silencing are generated from endogenous hairpin-shaped transcripts and are loaded onto a member of the Argonaute (Ago) family of proteins (Kim et al., 2009). RNA silencing by miRNAs occurs through cleavage of RNA, degradation of messenger RNAs (mRNAs) or suppression of mRNA translation (Bartel, 2005; Kim et al., 2009). Most miRNAs show tissue-specific or developmental stage-specific expression and are involved in cell differentiation and developmental transitions. It is known that miRNAs recognize target mRNAs based on seed region, and bind to partially complementary sites, usually in the 3′ untranslated region of mRNAs (Bartel, 2009). It is estimated that, on average, each miRNA can target 200 different transcripts (Lewis et al., 2005). Since there is just partial complementarity between miRNAs and their targets in animal cells, the identification of specific target genes for a given miRNA remains a major challenge in our understanding of miRNA function (Bartel, 2009). Recently, it was reported that target mRNAs tended to be present at low expression levels in domains expressing the cognate miRNAs (Farh et al., 2005; Lim et al., 2005; Sood et al., 2006; Stark et al., 2005). This resulted in quantitatively complementary expression patterns between miRNAs and their targets, leading to postulation of a “mutual exclusion model” of miRNA regulation of gene expression (Bushati and Cohen 2007; Stark et al., 2005).

The fetal murine submandibular salivary gland (SMG) is a well analyzed model system for studying organogenesis, including branching morphogenesis. Branching morphogenesis is a basic developmental process in the formation of many organs, such as exocrine glands, the lung and the kidney. In addition, this process is known to be dependent on reciprocal epitheliomesenchymal interactions (Bernfield et al., 1984; Birchmeier and Birchmeier, 1993; Hogan, 1999; Lu and Werb, 2008; Metzger and Krasnow, 1999) and in part on the ErbB family of tyrosine kinase receptors and some of the ligands that activate them (Jaskoll and Melnick, 1999; Kashimata and Gresik, 1997; Koyama et al., 2008; Larsen et al., 2003; Miyazaki et al., 2004; Morita and Nogawa, 1999; Nogawa and Takahashi, 1991; Umeda et al., 2001). Epidermal growth factor (EGF) is one of the ligands for the ErbB family, and it has been shown to stimulate branching morphogenesis (Kashimata and Gresik, 1997; Nogawa and Takahashi, 1991).

There has been only one report regarding expression of miRNAs in the SMG (Jevnaker and Osmundsen, 2008). That study was conducted using microarrays only on embryonic day 15.5 (E15.5) SMGs and on glands from postnatal days 0, 5 and 25. However, it is on E12.5–13.5 that the epithelium begins branching morphogenesis and forms approximately 4–5 buds (e.g. Tucker, 2007). These buds continue branching, resulting in a highly branched gland by E14.5. However,
the miRNA profile has not been studied at this critical stage for branching morphogenesis (i.e. E13).

For this study, we hypothesized that miRNAs can act as important regulators of developmental events leading to branching morphogenesis. We first analyzed miRNA expression in E13 SMG using a cloning method to obtain an expression profile. Then we focused on one miRNA, miR-21, which was up-regulated during SMG culture, by analysis using both EGF treatment and RNA interference (RNAi) methods. This report shows that miR-21 expression in the mesenchyme regulates branching morphogenesis in vitro.

Materials and methods

Culture of SMG rudiments

Pregnant, time-mated ICR mice were obtained from SLC, Ltd. (Shizuoka, Japan). SMG rudiments were isolated from fetuses on the 13th day of gestation (E13, taking the day of discovery of the vaginal plug as day 0). Dissection of SMG rudiments was performed in DMEM:F12 serum-free medium with GlutaMAX (GIBCO) by using fine tweezers under a dissection microscope. For EGF treatment (20 ng ml⁻¹), E13 SMG rudiments were incubated in 24-well plates for 0, 6, 12 and 24 h in 5% CO₂:95% air and 80% humidity at 37 °C in DMEM:F12 serum-free medium with GlutaMAX that contained 100 units ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 150 μg ml⁻¹ vitamin C and 50 μg ml⁻¹ transferrin (Steinberg et al., 2005).

Transfection of artificial small RNA

For miR-21 inhibition and enhancement, SMG rudiments were cultured for 48 h in dishes coated with Matrigel. In brief, a growth factor-reduced Matrigel (BD Biosciences) was diluted to 5% with the medium described above, without antibiotics. Glass-bottom dishes (IWAKI) were coated with the 5% Matrigel and incubated for 1 h at 37 °C in a humidified 5% CO₂:95% air atmosphere for polymerization. Then SMG rudiments were placed in these dishes with a small amount of medium (Kadoya and Yamashina, 2010). After the first 24 h of culture, the medium was changed to the appropriate medium for each experimental condition.

For miR-21 inhibition, mirCURY LNA microRNA Power Inhibitor for miR-21 (LNA-21) or Negative Control A (LNA-cont) (Exiqon), and for enhancement, mirIDIAN microRNA Mimic for miR-21 (MIM-21) or Negative Control (MIM-control) (Thermo Scientific), were added to the medium at 20 nM in the presence of N-TER Nanoparticle siRNA Transfection System (SIGMA). After 24 h the medium was replaced with medium containing artificial RNA and transfection reagent. As controls, SMG rudiments were treated with transfection reagent only (without artificial RNA, i.e. RNA(−−)) to compare with SMG rudiments cultured with only medium (without any RNA or transfection reagent). The numbers of branches formed were compared by a one-tailed t-test to evaluate the inducing effects of the artificial RNA and transfection reagents.

To test for uptake of artificial RNAs, single-stranded RNA (ssRNA), Cy3-labeled Anti-miR Negative Control #1 (Ambion) and double-stranded RNA (dsRNA), Silencer Cy3-labeled Negative Control siRNA #1 (Ambion) were added to culture medium at 20 nM as described above. After culture for 24 h, the SMG rudiments were washed three times with medium to remove residual artificial RNA and observed by microscopy (Leica).

Separation of epithelium and mesenchyme

The epithelium and mesenchyme were separated according to the methods of Carraro et al. (2009). In brief, SMG rudiments from E13 embryos were cultured for 24 h with and without EGF and then treated with Dispase (5000 units ml⁻¹) (Godo Shusei) for 5 min at 4 °C. The SMG rudiments were then transferred to DMEM:F12 serum-free medium with GlutaMAX (GIBCO) containing 10% fetal bovine serum (Invitrogen) to block the enzymatic reaction. Epithelium and mesenchyme were separated in serum-free medium by using fine tweezers under a microscope (Shimadzu). The separated samples were immediately transferred to RNAlater (Ambion). RNase-free phosphate-buffered saline was added to the RNAlater at 1:1, and the samples were recovered by centrifugation.

RNA extraction and quantification by real-time RT-PCR

Total RNA was extracted using TRIzol (Invitrogen) in accordance with the manufacturer's instructions. The total RNA concentration and RNA integrity were assessed using the RNA 6000 Pico LabChip Series II Assay (Agilent Technologies) with an Agilent 2100 bioanalyzer (Agilent Technologies). The RNA integrity number of all samples in this report ranged from 8.5 to 10.0. The small RNA concentration was assessed with an Agilent Small RNA kit (Agilent Technologies) for real-time RT-PCR of miRNAs.

Cloning analysis

For cloning analysis, the mature miRNA fraction was extracted by immunoprecipitation using Ago 2 antibody of a microRNA Isolation kit, Mouse Ago2 (Wako), according to the manufacturer’s instructions. Small RNA fractions including miRNAs were confirmed using an Agilent Small RNA kit (Agilent Technologies). Subsequently, for cloning, both 3’-biotinylated linker and 5’ linker RNAs were ligated to miRNAs, followed by the reverse transcription reaction and polymerase chain reaction (PCR) using a small RNA Cloning Kit (TaKaRa). PCR was performed for 15 cycles. The PCR product was run on a 15% nondenatured polyacrylamide gel at a constant current of 40 mA for 120 min. A band of approximately 65 bp (i.e. the product with a miRNA between linkers) was cut out of the gel. The gel fragment was crushed, and the PCR product was eluted using a Biomasher (Wako). The methods for cloning and elution were described in the manufacturer's instructions for the microRNA Cloning Kit Wako (Wako). A second PCR of 10 cycles was performed on a 1 µl aliquot of the eluted solution (Watanabe et al., 2007). The second PCR product was obtained as described above. A single fragment, approximately 65 bp, was cut out of the gel and cloned with a TOPO-TA cloning kit (Invitrogen). Sequencing analysis for white colonies was carried out, and the sequences between linkers were analyzed to identify miRNAs, as described below.

Computational analysis

To identify miRNAs, retrieved sequences were searched in the miRBase (Release 16.0; http://microrna.sanger.ac.uk/) (Ambros et al., 2003; Griffiths-Jones, 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008). If sequences showed no match to any registered miRNAs, further analyses were performed to determine whether the sequences were novel miRNA candidates. The genomic positions in the mouse and the corresponding sequences in each genome for various animals were identified by using the University of California, Santa Cruz (UCSC) web site (http://genome.ucsc.edu/). Putative secondary structures were predicted using two RNA fold programs, mfold (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-formLgi) (Zuker, 2003) and CentroidFold (http://www.ncrna.org/centroidfold/) (Hamada et al., 2009). Unknown small RNAs can be registered as novel miRNAs at miRBase.

Real-time reverse transcription-PCR for miRNA quantification

Reverse transcription of miRNAs was carried out using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, 1997). Real-time reverse transcription-PCR (RT-PCR) assays were performed...
on an iCycler iQ Detection System (Bio-Rad Laboratories). Taqman microRNA assays (Applied Biosystems, 1997) were used for relative quantification of miRNAs (i.e. miR-199b-199a-3p, miR-125b-5p, miR-214, and miR-21). The expression levels of these miRNAs were normalized to snRNA202, and determined by the comparative Ct method (Applied Biosystems User Bulletin). Real-time RT-PCR was performed under the following conditions: 95 °C for 10 min and 40 cycles of 95 °C for 10 s and 60 °C for 1 min. Assays were performed using a total sample volume of 20 μl per RT reaction, including 6 μl of complementary DNA (cDNA) derived from 10 ng of small RNA.

Real-time RT-PCR for messenger RNA quantification

Total RNAs from SMG rudiments were treated with DNase I (Invitrogen) and subjected to the reverse-transcription reaction by superscript II (Invitrogen) with random hexamers to generate cDNA. Real-time RT-PCR with SYBR green assay was performed in a total volume of 25 μl per RT reaction, including 1 μl of cDNA derived from 50 ng of total RNA. Assays were performed under the following conditions: 95 °C for 5 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min using iQ SYBR Green Supermix (Bio-Rad Laboratories). The melting curve data were collected by increasing the temperature from 60 °C to 95 °C after 2 cycles in increments of 0.5 °C.

Specific primers for messenger RNAs (miRNAs), serine peptidase inhibitor (Maspin; NM_009257), nuclear factor I/B (Reck; NM_011050), reversion-inducing-cysteine-rich protein with kazal motifs (Spry2; NM_011897) and ribosomal protein S29 (Rps29; NM_009093), were designed for SYBR green assay with DNASIS Pro version 2.08 (HITACHI) and subjected to the reverse-transcription reaction by superscript II (Invitrogen) with random hexamers to generate cDNA. Real-time RT-PCR with SYBR green assay was performed in a total volume of 25 μl per RT reaction, including 1 μl of cDNA derived from 50 ng of total RNA. Assays were performed under the following conditions: 95 °C for 5 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min using iQ SYBR Green Supermix (Bio-Rad Laboratories). The melting curve data were collected by increasing the temperature from 60 °C to 95 °C after 2 cycles in increments of 0.5 °C.

Results and discussion

Relative spatial localization of miRNAs

Three miRNAs, miR-199b/199a-3p, miR-125b-5p and miR-214, showed relatively high cloning frequencies, indicating that their expression levels were high in E13 SMGs. The spatial relative localization of the three miRNAs was investigated using epithelium and mesenchyme separated from E13 SMGs as described in Materials and methods (Fig. 2A). For each tissue, the expression levels of the above three miRNAs were analyzed by real-time RT-PCR (Fig. 2B). The spatial expressions of all three miRNAs were higher in the mesenchyme than in the epithelium, suggesting that miRNAs with relatively high expression levels are distributed mainly in the mesenchyme of E13 SMGs.

Novel miRNA candidates

We obtained four clones which did not match any sequences in miBase Release 16.0 (Table 1). These small RNA sequences were compared with the nucleotide database by NCBI Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The secondary structures of the four sequences were predicted by two programs, mfold (Zuker, 2003) and CentroidFold (Hamada et al., 2009). Only one hairpin structure met the criteria for miRNAs using the two programs (Fig. 1A). This clone sequence, SMG-sRNA-1 (AB597295), was encoded on the 3’ untranslated region (genomic context: 19;6904407−6994403[−]) of Rps6Ka4 (AF074714). The regions of the mature sequence and hairpin structure were mostly phylogenetically conserved (Fig. 1B). The alterations in the seed region between rodents and anthropoids suggest that the target miRNAs are different among these species (Fig. 1C). Taken together, we identified this clone sequence, SMG-sRNA-1, as a novel miRNA (named as mmu-mir-5046 by miRBase). On the other hand, three of four sequences, registered as AB597296−AB597298, did not form a significant hairpin structure. However, considering that those three sequences were obtained by Ag2I immunoprecipitation, they are probably functional small RNA like endogenous short interfering RNAs (Siomi and Siomi, 2009), which suggests that they were related to RNA silencing in SMG.

Table 1

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Number of clones</th>
</tr>
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<tbody>
<tr>
<td>miR-199b/199a-3p</td>
<td>27</td>
</tr>
<tr>
<td>miR-125b-5p</td>
<td>6</td>
</tr>
<tr>
<td>miR-214</td>
<td>4</td>
</tr>
<tr>
<td>miR-16, miR-19b, miR-503, miR-687, let-7a</td>
<td>3</td>
</tr>
<tr>
<td>miR-17, miR-23a, miR-26a, miR-30c, miR-99a, miR-126-3p, miR-199a-5p, miR-200b, miR-214*, miR-335-5p</td>
<td>2</td>
</tr>
<tr>
<td>let-7e, let-7g, let-7i, miR-21, miR-22, miR-29b, miR-106b*, miR-107, miR-125a-5p, miR-127, miR-154, miR-181a-2*, miR-199b*, miR-200c, miR-205, miR-301b, miR-322*, miR-379, miR-411, miR-411*, miR-431, miR-466c-5p, miR-484, miR-500, miR-532-3p, miR-652</td>
<td>1</td>
</tr>
</tbody>
</table>

Novel miRNA candidates

This study was approved by the Ethics Committee of Asahi University School of Dentistry (No. 07−004).

Results and discussion

MiRNAs in native E13 SMG

Cloning analysis revealed the presence of various miRNAs expressed in native SMGs at E13 (Table 1), including 44 miRNAs composed of 98 clones, and four previously unidentified small RNAs that had no match in the miRNA database (miRBase Release 16.0). The number of sequence reads for a particular miRNA is known to be proportional to its molecular abundance (Landgraf et al., 2007). Analysis revealed that miR-199b-199a-3p appeared most frequently among the total miRNAs in this clone library. miR-199b and miR-199a-3p are identical in mature sequence, and in this study they are referred to as miR-199b-199a-3p. Eighteen miRNAs were detected as multiple clones (Table 1). The relative cloning frequencies of these miRNAs probably reflect their respective levels of expression. Therefore, it is possible that the expression levels of these miRNAs are higher than the expression levels of the 26 miRNAs detected as single clones (Table 1).
Relative quantification of miR-21 in EGF stimulation

miR-21 was detected as just one clone in the clone library derived from SMG at E13 (Table 1). miR-21 is perfectly conserved in mammals and is one of the most studied miRNAs (Krichevsky and Gabriely, 2009). This miR-21 has been especially associated with cancer, because up-regulation of its expression was found in various tumors (Krichevsky and Gabriely, 2009). In addition, it was reported that there was a relationship between regulation of miR-21 expression and EGF signaling (Fujita et al., 2008; Seike et al., 2009). Therefore, we hypothesized that EGF treatment of native E13 SMG leads to alteration of the miR-21 expression level, followed by enhancement of branching morphogenesis. To test this hypothesis, relative quantification of the miR-21 expression level was performed by real-time RT-PCR in various animal species. Nucleotides differing from those of mice are shown in white. Corresponding sequences, except for mice, were extracted from each genome at the University of California, Santa Cruz [web site (http://genome.ucsc.edu/)]. Human, Homo sapiens; orangutan, Pongo pygmaeus abelli; mouse, Mus musculus; rat, Rattus norvegicus; dog, Canis lupus familiaris; horse, Equus caballus.

Fig. 1. Novel miRNA discovered in a submandibular gland at embryonic day 13. (A) Putative secondary structure of the novel miRNA. Two RNA fold programs, mfold (upper) and CentroidFold (lower), were used to predict the secondary structure. (B) The putative secondary structure is phylogenetically conserved. (C) Alignments of sequences corresponding to the novel miRNA are shown for various animal species. Nucleotides differing from those of mice are shown in white. Corresponding sequences, except for mice, were extracted from each genome at the University of California, Santa Cruz [web site (http://genome.ucsc.edu/)]. Human, Homo sapiens; orangutan, Pongo pygmaeus abelli; mouse, Mus musculus; rat, Rattus norvegicus; dog, Canis lupus familiaris; horse, Equus caballus.

Branching morphogenesis in SMGs after miR-21 up/down regulation

RNAi methods were applied to E13 SMG cultures to evaluate the role of miR-21 in branching morphogenesis by counting the number of end buds (Fig. 5). First, for inhibition of miR-21, preliminary tests of uptake revealed that Cy3-labeled ssRNAs were distributed mainly in the mesenchyme (Figs. 5B and D). The signal intensity was weak, but positive transfection was distinguishable from autofluorescence by SMG (Fig. 5F). Next, E13 SMGs were transfected with locked nucleic acids (LNA), LNA-21 and LNA-cont, respectively. In total, transfection was carried out for 28 SMGs as matched pairs: 14 rudiments for LNA-21 and 14 rudiments for LNA-cont, respectively (Fig. 6A). After culture for 48 h, the structure of SMG became more complex than that at 24 h because of a large number of end buds, which made accurate counting difficult. Therefore, the number of epithelial buds at 24 h was counted to evaluate the influence of LNA-21 transfection on branching morphogenesis. After 24-h culture, there was a significant decrease in the bud number of SMGs transfected with LNA-21 (P<0.05) (Fig. 7). As a control, SMGs were treated with transfection reagent only (RNAi−); Fig. 6B). The number of buds after 24 h of culture was compared with SMG cultured in plain medium (without any RNA or transfection reagent), but there was no significant difference between the results (P=0.42–0.05; data not shown). Taken together, these results indicate that up-regulation of miR-21 in the mesenchyme leads to a decrease in the number of epithelial buds.

Preliminary tests of uptake revealed that Cy3-labeled dsRNAs were distributed in both the epithelium and mesenchyme (Fig. 8). Interestingly, their positive signals were quite different in intensity and distribution from those of ssRNAs, suggesting two possibilities: there was a difference in the uptake system between ssRNAs and dsRNAs, or the half-life of dsRNAs in the epithelium was longer than that of ssRNAs. Next, for enhancement of functional miR-21, SMGs at E13 were transfected with MIM-21 and MIM-cont. In total, transfection was carried out for 24 SMGs as matched pairs: 12 rudiments for MIM-21 and 12 rudiments for MIM-cont, respectively. After culture for 24 h, the number of epithelial buds was counted to evaluate the influence of MIM-21 transfection on branching morphogenesis. However, there was no significant difference in bud number between SMGs transfected with MIM-21 and MIM-cont (P=0.46–0.05; data not shown). There is a possibility that MIM-21 was transfected into not only the mesenchyme but also the epithelium (Fig. 8). Considering that up-regulation of miR-21 occurred only in the mesenchyme (Fig. 4B),
enhancement of functional miR-21 in the epithelium might exert no significant effect on the number of end buds. This mechanism remains to be elucidated.

Prediction of target mRNAs of miR-21

miR-21 in the mesenchyme of SMG rudiments regulates branching morphogenesis. In order to understand this miRNA function in the mesenchyme, target mRNAs need to be characterized. However, identification of specific target mRNAs remains a major challenge in miRNA research (Bartel, 2009; Krichevsky and Gabriely, 2009). Based on a mutual exclusion model, up-regulation of a miRNA leads to down-regulation of its target mRNAs, and vice versa. Therefore, in the SMG mesenchyme, an increase in the miR-21 expression level would lead to a decrease in its target mRNAs’ expression levels. Accordingly, relative quantification of target miRNA candidates was performed by real-time RT-PCR. Earlier reports experimentally validated that five mRNAs (i.e. Reck, Pdcd4, Maspin, Nfib and Spry2) were targeted by miR-21 in different cellular contexts (Frankel et al., 2008; Fujita et al., 2008; Gabriely et al., 2008; Sayed et al., 2008; Zhu et al., 2008). Since we were unable to design an experiment in which MIM-21 as dsRNAs was transfected into only the mesenchyme to enhance miR-21 function (Fig. 8), the expression levels of five target mRNAs were quantified in both the epithelium and mesenchyme after 24-h culture with and without EGF treatment (Fig. 9).

The results showed that the expression levels of two genes, Reck and Pdcd4, were lower in the mesenchyme separated from SMG that had been treated with EGF compared with no EGF treatment. Because their expression levels correlated inversely with the miR-21 level, repression of Reck and Pdcd4 was probably due to interference by miR-21. One of the five genes, Maspin, was deleted from the following analysis, because it was undetectable in SMG samples (data not shown). Interestingly, although the two remaining mRNAs, Nfib and Spry2, were also shown to be target genes, their expression levels did not seem to be in accordance with the mutual exclusion model, which suggests that miR-21’s regulation of its target mRNAs depends on different cellular contexts (Krichevsky and Gabriely, 2009).

RECK is a membrane-anchored inhibitor of metalloproteinases (MMPs), and reduction or inactivation of MMPs’ expression seems to be critical for the invasiveness and metastasis of various cancers (Krichevsky and Gabriely, 2009). For example, RECK inhibited MMP-9 secretion and activity and attenuated metastasis of lung cancer cells (Chang et al., 2008). MMPs are a group of peptidases involved in degradation of the extracellular matrix (ECM) and in many other biological processes (Holmbeck et al., 1999; Hotary et al., 2000; Page-McCaw et al., 2007; Rebustini et al., 2009; Sternlicht and Werb, 2001). It has been suggested that degradation of ECM leads to enhancement of branching morphogenesis because ECM in the mesenchyme is remodeled during SMG development, which makes epithelial cells invade, cleave and grow properly (Bernfield et al., 1984; Hogan, 1999; Hotary et al., 2000; Patel et al., 2006; Umeda et al., 2001). In SMG, most MMPs are known to be expressed in the mesenchyme, but MMP2, MMP14 and MMP15 are expressed in the epithelium (Steinberg et al., 2005; Rebustini et al., 2009). It is known that epithelial MMP2

Fig. 2. Relative expression levels of miRNAs (i.e. miR-199b/199a-3p, miR-125b-5p and miR-214) in the epithelium versus the mesenchyme of E13 SMG, showing their relative spatial localization. (A) SMG rudiments at E13 were separated into two tissues, epithelium and mesenchyme, by dispase treatment. (B) Each miRNA expression level was normalized to snoRNA202. Data are the mean±SE for three independent experiments. *, P<0.01, one-tailed t-test. RNA integrity number (RIN) = 8.9–9.5. Scale bar = 500 μm.

Fig. 3. Up-regulation of endogenous miR-21 expression level in SMG by epidermal growth factor (EGF) treatment. SMG rudiments isolated from a mouse at E13 were cultured for 0 h, 6 h, 12 h and 24 h with and without EGF (i.e. EGF(+) and EGF(−), respectively; EGF 20 ng ml−1). Expression levels were analyzed by real-time RT-PCR and normalized to snoRNA202. Data are the mean±SE for three independent experiments. *, P<0.01, one-tailed t-test. RIN = 8.5–9.3.
influences collagen IV metabolism and epithelial proliferation during branching morphogenesis (Rebustini et al., 2009). In this study, mesenchymal MMPs including MMP9, appeared to be regulated by RECK, because most MMPs and Reck were distributed mainly in the mesenchyme (Fig. 9; Steinberg et al., 2005). Therefore, inhibition of Reck by increased miR-21 expression in the mesenchyme might...
result in degradation of ECM by MMPs, leading to enhancement of branching morphogenesis.

PDCD4 was initially discovered as a protein encoded by a gene that is up-regulated in apoptosis, and it was later characterized as a potent tumor suppressor (Krichevsky and Gabriely, 2009). It was reported that PDCD4 binds to and inhibits the translation initiation factor, eukaryotic initiation factor 4a, thereby impacting on protein translation (Göke et al., 2002; Yang et al., 2003). In addition, PDCD4 was found to inhibit transcription factors, such as AP-1-mediated trans-activation (Yang et al., 2001). Interestingly, AP-1 has been implicated as a direct regulator of MMP activity (Angel et al., 1987). Putative AP-1 binding sites have been identified on several MMP promoters, including MMP9 (Benbow and Brinckerhoff, 1997). Furthermore, AP-1 is one of the major downstream effectors for mitogen-activated protein kinase (MAPK), and it is known that EGF activates the MAPK pathway (Kashimata and Gresik, 1997; Kajanne et al., 2007; Prenzel et al., 2001; Yarden and Sliwkowski, 2001; Whitmarsh and Davis, 1996). Recent studies reported that miR-21 promoter has a binding site for AP-1, and that AP-1 triggers the transcription of primary miR-21 (Fujita et al., 2008; Krichevsky and Gabriely, 2009). It was thus reasonable that EGF treatment of SMG enhanced the miR-21 expression level (Figs. 3 and 4). Therefore, in the mesenchyme of SMG, double-positive feedback may occur according to the following steps: 1) endogenous miR-21 inhibits PDCD4, a protein that blocks trans-activation of AP-1; 2) activated AP-1 binds to the transcription sites of the promoters for miR-21 and MMPs, respectively; and 3) elevated miR-21 expression promotes the AP-1 activation through PDCD4 inhibition. Hence, inhibition of PDCD4 by miR-21 expression in the mesenchyme would result in ECM degradation by MMPs activated by AP-1.

In Fig. 9, EGF treatment of mesenchyme led to a fold reduction in the expression levels of the genes, Reck and Pdcd4, targeted by miR-21. The decrease in the expression levels of each target gene was relatively modest. However, both genes are related to the regulation of MMPs activities, as described above. The findings suggest that the phenotype was sufficiently induced by additive changes in the expression levels of Reck and Pdcd4, even though each individual change was relatively modest.

Additionally, based on a mutual exclusion model, we hypothesized that knock-down of miR-21 using LNA would lead to up-regulation of the target genes, Reck and Pdcd4, in the mesenchyme of SMG. As expected, the expression levels of both target genes tended to increase in the mesenchyme treated with LNA-21. Both the Reck and Pdcd4 expression levels showed 1.4-fold up-regulation, with an 88% decrease in the bud number, compared with mesenchyme treated
The expression levels of four target mRNA candidates, then separated into two tissues, epithelium and mesenchyme, by dispase treatment. analyzed by real-time RT-PCR and normalized to three independent experiments. *, 

**Fig. 8.** Cy3-labeled control double-stranded RNA (dsRNA) was shown to be distributed in both the epithelium and mesenchyme of SMG rudiments. E13 SMG rudiments were cultured for 24 h in the presence of N-TER Nanoparticle siRNA Transfection System (SIGMA). (A) Phase-contrast images show living glands. (B) Fluorescent microscopy images show that Cy3-labeled control dsRNAs (20 nM) were transfected into the SMG and distributed in both the epithelium and mesenchyme. Scale bar = 100 μm.

with LNA-cont. These data support our conclusion that miR-21 in the mesenchyme of SMG targets two genes, Reck and Pdc4.

Taken together, although the detailed molecular links between miR-21 and its target mRNAs remain to be identified, miR-21 expression in the mesenchyme leads to enhancement of branching morphogenesis through mesenchymal ECM degradation by MMPs that are activated as a result of inhibition of at least two miRNAs, Reck and Pdc4.

**Conclusion**

We inventoried miRNAs expressed in SMG at E13 and showed that miR-21 was involved in branching morphogenesis. miR-21 showed increased expression in the mesenchyme of SMG, and it regulated branching morphogenesis by inhibition of at least two genes (Reck and Pdc4) expressed in the mesenchyme. To date, numerous studies focused mainly on an isolated epithelium to investigate branching morphogenesis in SMG (e.g. Rebustini et al., 2009; Onodera et al., 2010). However, a recent study of SMG development demonstrated that parasympathetic innervation in the mesenchyme maintained epithelial progenitor cells through the activation of EGF receptor signaling (Knox et al., 2010). To further elucidate the effects of interaction between the epithelium and mesenchyme on branching morphogenesis in SMG, the role of the mesenchyme needs to be investigated in future studies.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.01.030.

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