PROCEEDING

Detection of EGF-dependent microRNAs of the fetal mouse submandibular gland at embryonic day 13

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Abstract : Fetal murine submandibular salivary gland (SMG) is known as a model to study organogenesis including branching morphogenesis, which is a basic developmental process for formation of a wide variety of arborized organs. Branching morphogenesis is under the control of a complex network of regulatory proteins, such as the ErbB family of tyrosine kinase receptors, activated by members of the epidermal growth factor (EGF) family of ligands. Recent reports identify critical roles for micro RNAs (miRNAs) on many developmental processes through regulation of gene expression. We hypothesize that miRNAs regulating branching morphogenesis are expressed in fetal murine SMG and that expression of the miRNAs associated with branching morphogenesis is modulated in part by EGF. Using cloning methods, we obtained the expression profiles on miRNAs derived from fetal murine SMG under three different conditions : (1) native E13 SMGs (freshly isolated), (2) E13 SMGs cultured for 24 hours with no added EGF (controls), or (3) cultured with EGF. There were 44 known miRNAs and four novel miRNAs candidates in native SMG at E13. Comparing the three profiles revealed that several miRNAs were expressed specifically at each condition. These results suggested that these miRNAs were associated with regulating organogenesis, possibly including branching morphogenesis. J. Med. Invest. 56 Suppl. : 250-252, December, 2009

Keywords **:** *microRNA, EGF, salivary gland, expression profile*

INTRODUCTION

MicroRNAs (miRNAs) are 19-24 nt endogenous RNA products of non-coding genes, present in all multicellular organisms (1, 2). It is known that miRNAs triggering RNA silencing are loaded onto a member of the Argonaute (Ago) family of proteins (2). MiRNAs have the potential to cleave, degrade or suppress translation of messenger RNAs (mRNAs) transcribed from thousands of different genes. Most miRNAs show tissue-specific or developmental stage-specific expression and are involved in cell differentiation and developmental transitions. MiRNAs are known to recognize and bind to partially complementary sites usually in 3' untranslated regions of mRNAs (2). It is estimated that, on average, a miRNA can target 200 different transcripts (3).

The fetal murine submandibular salivary grand (SMG) is a useful model to study organogenesis, including differentiation, proliferation, epithelial-mesenchymal interaction, and branching morphogenesis, a basic developmental process for formation of a wide variety of arborized organs. There is only one report on expression of miRNA in the SMG (4) ; this study was conducted by microarray on

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the murine SMGs (E15.5, P0, P5 and P25). However, it is at E12.5-13.5 that the epithelium begins branching morphogenesis and forms approximately 4-5 buds (5). These buds continue branching, producing a highly branched gland by E14.5. Thus, the miRNAs profile has not been studied at this critical stage for branching morphogenesis, *i.e.*, at E13.

Branching morphogenesis of the fetal murine SMG is known to depend in part on the ErbB family of tyrosine kinase receptors and some of the ligands activate them (6-13). ErbB 1, 2 and 3 have been localized in the fetal glands, and EGF, HB-EGF and NRG-1 have each been shown to stimulate branching morphogenesis. Messenger RNA levels of these three ligands vary significantly during fetal development of the SMG.

We hypothesize that growth factor activation of the ErbB receptors modulates expression of miRNAs that can act as regulators of developmental events for branching morphogenesis. In these ongoing studies, we begin our analysis of miRNA expression induced by EGF in the SMG at E13, with future plans to extend the analysis to the effects by HB-EGF and NRG-1.

MATERIALS AND METHODS

In this work, detection of miRNAs from the fetal murine SMG was carried out using a combination of Ago2-immunoprecipitation and sequencing. SMGs were sampled from fetal mice at E13. A total of 106 rudiments, freshly isolated (referred to as "native"), were pooled and homogenized. Additionally, a total of 292 rudiments were sampled as matched pairs for each condition, 24 hours culture with or without EGF stimulation. The miRNA fractions were extracted by immunoprecipitation using Ago2 antibody. Small RNA fractions including miRNAs were confirmed by an Agilent 2100 Bioanalyzer. Subsequently, reverse transcription reaction, cloning, and sequencing analysis were carried out. To identify miRNAs, retrieved sequences were searched in the miRBase (Release 13.0 ; http : //microrna.sanger.ac.uk, ref. 14-17). If sequences showed no match with any registered miRNAs, further analyses were performed to determine whether the sequences were novel miRNA candidates using the Mfold program (http : //frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-forml.cgi, ref. 18).

The study was approved by the Ethical Committee of Asahi University School of Dentistry (No.07-004).

RESULTS AND DISCUSSION

MicroRNAs on native E13 SMGs

Cloning analysis of 102 clones revealed the presence of various miRNAs expressed in native SMGs at E13 (Fig. 1). The 44 miRNAs composed of 98 clones and four previously unidentified small RNAs, not matched to miRNA database (miRBase Release 13.0), were detected. Analysis revealed that the miRNA, mmu-miR-199a, appeared frequently of total miRNAs in this clone library. Additionally, four novel miRNA candidates were also detected.

These data on the freshly isolated SMG from E13 fetuses provided a baseline for comparison of changes resulting from culture for 24 hours with or without EGF. These changes in expression of specific miRNAs may be related to branching morphogenesis.

MicroRNAs on E13 SMGs with conditioned stimuli

After 24 hours cultivation, with or without EGF stimulation, 48 and 54 miRNAs were detected respectively (Fig. 1). Without EGF stimulation, just cultivation in medium for 24 hours, 29 new miRNAs were detected compared with native E13 SMGs (Fig. 1). Although it is possible that stress had some influence on the outcome of these experiments, these miRNAs showed a time-dependent expression pattern. In contrast, 19 miRNAs were unchanged (Fig. 1), suggested that they were house-keeping genes.

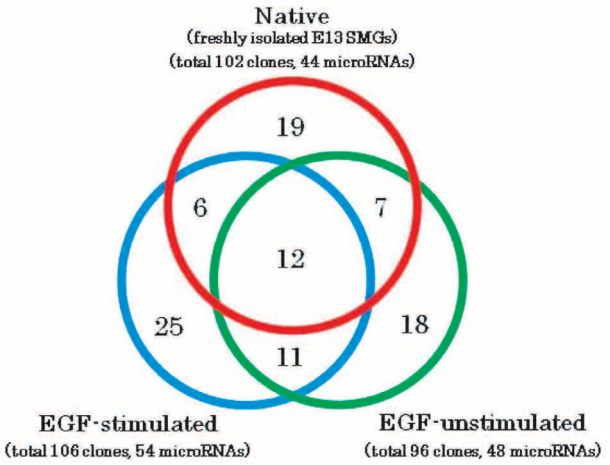

Fig. 1 The distribution of detected microRNAs between EGF-stimulated, unstimulated and native E13 SMG.

With EGF stimulation, 25 new miRNAs were

detected (Fig. 1). Eight of these were expressed at relatively high levels, determined by relative cloning frequencies (data not shown). These miRNAs were observed only in the presence of EGF, implying that EGF induced the expression of these miRNAs in E13 SMG.

We find that the expression profile of miRNAs is time- and EGF-dependent and changes in complex patterns, consistent with our hypothesis. These results indicate that the expression of several miRNAs in E13 SMGs are under regulation by EGF and suggest that some of miRNAs impact biological processes involving branching morphogenesis.

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