EMT-related RNA network

regulated by non-coding transcripts

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Previous studies have shown that tumor suppressor p53 and the Wnt/EMT axis is regulated via microRNA (miRNA) -34 family. Therefore, miRNAs are regarded as the key controller for cancer development and signal transduction, as well as the key components of the genome-wide RNA network which regulates cancer development and metastasis. While Snail is directly inhibited by miRNA-34, the other EMT transcription factor,

ZEB1, is known to be regulated by miRNA-200. In this study, we identified the mechanism of non-coding RNA transcripts dependent EMT regulatory RNA network by ceRNA regulation analysis between tumor suppressor miRNAs, miRNA-34 and miRNA-200, and EMT associated markers, Snail, Zeb1. Although Snail and ZEB1 either may or may not be directly targeted by direct targets of miR-34a, miR-200a, and miR-128, they could be respectively inhibited by the miRNAs. Snail and Zeb1 transcripts expressions showed an inverse correlation with miR-34a, miR-200a, and miR-128 expression in human samples. As non-coding UTR of Snail and Zeb1 induced cellular stemness and *in vivo* carcinogenesis in nude mice, these observations indicate that EMT network could be regulated by the non-coding transcripts. Understanding of EMT-related RNA network by non-coding transcripts could provide a novel cancer therapeutic strategy by suggesting a new paradigm that redeem the previous hypothesis for genetic mutation driven cancer progression.

Key words: untranslated region, Epithelial–mesenchymal transition, RNA network, competing endogenous RNA, stemness, microRNA, miR-34, miR-20

0, miR-128, Snail, Zeb1

Abbreviations: UTR, untranslated region; EMT, epithelial-mesenchymal transition; ceRNA, competing endogenous RNA; miR, microRNA; wt, wild type

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Ⅰ. INTRODUCTION

Epithelial-mesenchymal transition (EMT), a mechanism by which epithelial cells become mobile, is known to be an important mechanism for explaining not only embryonic development but also invasive growth and metastasis of cancer cells. Recently, EMT has been recognized as an important factor in drug resistance and characteristics of cancer stem cell (Rhim, et al., 2012; Thiery, et al., 2009).

Until 10 years ago, the notion that protein abnormality caused by mutation induced cancer was predominant, but analysis of genome sequence

including TCGA showed that there was almost no mutation proving causality in most cancer patients. This suggests that there may be more important than the coding gene mutation itself (Cancer Genome Atlas, 2012). According to ENCODE analysis, the protein coding region in the entire genome was only 2%. Among the remaining 98%, studies on small non-coding RNAs in the intron and intergenic regions have been actively conducted (Kellis, et al., 2014).

MicroRNAs (miRNAs) are one of the representative small RNAs, characterized by non-coding small RNAs that do not translate into protein. Mature miRNAs, a single-stranded RNA molecule composed of 21-25 nucleotides, interact with the 5'UTR of the mRNA, a region that is not translated into a protein in the mRNA region–and the 3'UTR portion, and suppress the expression of specific gene group though mRNA degradation and translational repression (Bartel, 2004). The miRNA precursors are non-coding RNA molecules that, in mammals, give rise to mature miRNAs. The precursor miRNA stem-loop is processed in the cytoplasm of the cell, with the mature miR sequence excised from the 5' arm of the hairpin (miR-5p), and from the 3' arm of the hairpin (miR-3p) respectively. Since there are numerous miRNA binding sites in one mRNA and one miRNA targets multiple

mRNAs, identification of specific miRNA-mediated regulation of gene expression has emerged as an important challenge in cancer biology. To date, about 1,000 miRNAs have been found in humans, and they are expressed in various tissues and cells in mammals. In addition, the difference in the expression of specific miRNAs between normal and cancer cells has been reported, but little is known about the organic regulation mechanism of these miRNAs (Landgraf, et al., 2007).

In 2007, the importance of miRNAs began to be emphasized as cell proliferation, survival, and apoptosis were regulated by p53 / miR-34 networks in seven different groups (Bommer, et al., 2007; He, et al., 2007; Raver-Shapira, et al., 2007; Tarasov, et al., 2007). And in recently studies, it is identified the regulation between Wnt signaling and EMT by miR-34. That is, miR-34 tightly regulates the tumor suppressor p53 and the WNT / EMT axis associated with cancer by repressing such like EMT related proteins of Snail, Axin2 (Cha, et al., 2012; Kim, et al., 2013; Kim, et al., 2011a; Kim, et al., 2011b). In addition, it was reported that the transcriptional repressor zinc-finger E-box binding homebox 1(ZEB1) and miRNA-200 family were reciprocal repression of EMT and invasion in cancer cell (Ulrike et al., 2008; Korpal et al., 2008; Philip e al., 2008).

Exposing epithelial cells to TGF-β, as a well-known EMT initiator, promotes the loss of epithelial morphological features, the increased expression of EMT marker genes such as ZEB1 and ZEB2, and the decreased expression of miR-200 (Gill et al.,2012).

In recent days, the concept of RNA and miRNA binding has been competitive and has led to the concept that non-coding RNA transcripts act as miRNA sponge (Cazalla and Steitz, 2010; Cesana, et al., 2011; Poliseno, et al., 2010). The miRNA sponge as initially introduced on 2007 is artificial DNA construct that produces specific miRNA binding site, and the artificial RNA with miRNA binding site can absorb endogenous specific miRNA (Ebert and Sharp, 2007).

In addition, the concept of ceRNA, competing endogenous mRNA has emerged, in which a pseudogene that does not code protein expression regulates the expression of other mRNAs that compete with the same miRNA on binding site. This is a result of showing the function of pseudogene (non-coding RNA) which was not well-known before, and providing the understanding of RNA network (Poliseno, et al., 2010; Sardina, et al., 2016). Based on these results, the purpose of this study is to investigate the role of miRNA and non-coding UTR in regulation of cancer

cell metastasis signaling by co-regulation of miRNA related to EMT process, in order to identify the mechanism of structural regulation of non-coding RNAs in miRNA.

Ⅱ. MATERIALS AND METHODS

1. **miR-34a-5p, miR-200a-3p, and miR-128-3p target prediction.** miRNA sequences of various species were obtained from miRBase (http://www.mirbase.org/index.shtml). The miBridge algorithm was used to narrow down potential targets for miR-34a-5p, miR-200a-3p, and miR-128-3p (http://sitemaker.umich.edu/mibridge/home). We set the energy threshold at -13 kcal/mol using RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) based on previous study41. The identified 3'UTR interaction sites were compared with TargetScan and Pictar targets and checked for conservation using the UCSC Genome Browser (http://genome.ucsc.edu/).
2. **mRNA and miRNA microarray data analysis of clinical samples.** Publicly available gene expression data from the TCGA colorectal

adenocarcinoma (COADREAD) samples (295 cases). For an unsupervised hierarchical cluster analysis of transcript abundance for mRNA and miRNAs, Ward linkage method was used together with the Pearson distance for sample clustering.

3. **Expression profile of Snail, Zeb1 and miRNAs in Human Colon Cancer Cell Lines.** 293 cells and human colon cancer cell lines, including HCT116, HCT116 p53-/- (p53 null), and HCT116 dicer-/- (dicer null) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and penicillin/streptomycin (pen/strep) (Invitrogen, Carlsbad, CA). To determine the relative expression level of Snail, Zeb1 and miRNA transcript, these cells were isolated total RNA with Trizol (invitrogen), cDNA was synthesized using redom hexamer reverse transcription primer (Intron) and then quantitative Real-Time PCR analysis was performed.
4. **Constructs and viral transduction.** The luciferase expression construct with multiple cloning sites for UTR was used as described previously. The 3'-UTR of Snail and Zeb1 were amplified from genomic DNA of MCF-7 cells and subcloned into the BamHI and NotI

sites of pCDNA3.1-luciferase vector. The miRNA expression vectors for miR-34a, miR-200a, and miR-128 were constructed from PCR amplified product from genomic DNA of MCF-7 cells followed by subcloning into pMSCV vector (Clontech). miRNA-sponge vectors were constructed by subcloning into pCDNA3.1-mCherry vector. The target sequence were;

miR-34a-5p sponge: ACAACCAGCTAAGACACTGCCA, miR-200a-3p sponge: AAAGAGACCGGTTCACTGTGA, and miR-128-3p sponge: ACATCGTTACCAGACAGTGTTA.

5. **RT-PCR and Immunoblot analysis.** RNA was extracted with TRIzol (Invitrogen), cDNA synthesis with SuperScript III(Invitrogen), SBGR mixture(TAKARA). For quantitative analysis of mature miRNA levels, human TaqMan miRNA assay kits (Applied Biosystems) were used for reverse transcription with specific primers, and qPCR was performed with corresponding probes. To detect endogenous protein expression, total cell lysates in Triton X-100 lysis buffer were subjected to SDS-PAGE and immunoblotting. Snail and Zeb1 antibody were obtained from Cell signaling.

6. UTR reporter assays. The cells were co-transfected with each of the UTR reporter constructs (5-100 ng) and miRNA expression vectors (200-500 ng) or miRNA sponge vector. As a transfection control, 1 ng of SV40-promoter driven Renilla construct (Promega) was co-transfected with reporter vectors. Cells were lysed at 48h after transfection and the relative ratio of Renilla to firefly luciferase was measured with dual luciferase assay (Promega).
7. Morphogenesis assay. The three-dimensional culture of MCF-10A and 293cells on basement membrane was carried out. Briefly, 5 × 10^3 cells were resuspended in growth medium containing 2% Matrigel (BD Biosciences) and seeded on top of a layer of growth factor-reduced Matrigel. Allow the cells to grow in the incubator for 10 days. The acinar structures were fixed with 2% paraformaldehyde. To visualize nuclei, samples were stained with DAPI and Confocal images were acquired with an LSM510 confocal microscope.
8. Flow cytometry. Cells were washed twice in PBS supplemented with 1% bovine serum albumin (Sigma). Stained for 30 min at 4°C with anti-CD44-FITC anti body (BD Biosciences). After washing, cells

were analyzed using a FACSCalibur flow cytometer (BD Biosciences) using CellQuest Pro software at 10,000 events.

9. In vivo assay. Four-week-old female specific pathogen free (SPF) BALB/c nude mice (5 mice per group) were purchased from Nara Biotech (Seoul, Republic of Korea). HCT116 cells were made Lentivirus transduction system. Briefly, HCT116-vec, miR34a sponge, miR-200a sponge or miR-128a sponge (1 million cells per a flank) were inoculated both flank subcutaneously. After 2 weeks, primary tumor size was measured every 3-4 days using calipers. Tumor volume was calculated using the formula, V= (short diameter X short diamer X long diamer)/2. After 4 weeks, mice were killed in 7.5% CO2 chamber and tumors were harvested for immunohistochemical analysis.
10. Statistical analysis. All statistical significance of in vitro experiment was conducted with two-tailed Student's t-tests; data are expressed as means and s.d. The **denote $P < 0.01$, *$P < 0.05$. Statistical analysis of animal experiments was determined using the Mann-Whitney test. Statistical methods have not been used to pre-determine the size of the specimen.

Ⅲ. RESULTS

1. The EMT transcriptional factors, Snail and Zeb1 are synchronously regulated.

It is well known that Snail and Zeb1 is a major inducer of epithelial-mesenchymal transition (EMT). When knockdown of Snail or Zeb1, it affects to the increase of E-cadherin, as well as the correlation of them. It means that when Snail is knock-downed, the expression of Zeb1 also decreased, while the loss of Zeb1 affects to the decline of Snail (Fig. 1A). When Snail or Zeb1 were knockdown in colon cancer cells HCT116 and SW480, it also repeatedly confirmed at transcriptional level (Fig. 1B). In fact, there is a correlation between Snail and Zeb1 in human colorectal cancer tissues (Figure 1C).

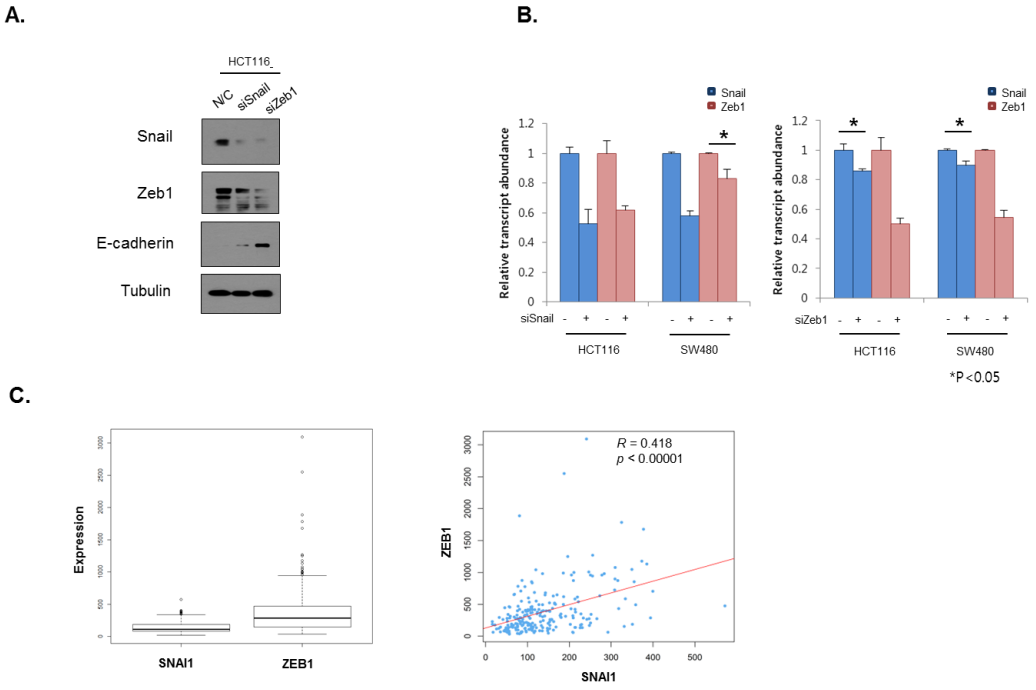


Figure 1. The synchronous expression of EMT genes, Snail and Zeb1

(A) Immunoblot analysis of Snail, Zeb1, and E-cadherin after siRNA-mediated knockdown of Snail and Zeb1 in HCT116 cell (B) Relative transcript abundance of Snail and Zeb1 under the expression of siSnail (left) and siZeb1 (right) in colon cancer cell lines. (C) Pearson correlation scatter plots between Snail and Zeb1. Results are shown as means ± s.d. from triplicate independent experiments. Statistical significances compared to control was denoted as *, P < 0.05; **, P < 0.01; ***, P < 0.001 by a two-tailed Student's t-test.

2. Both Snail and ZEB1 either may or may not be targeted by miR-34a, -200a, and -128, and miRNAs could play a role in ceRNA regulation

There are target regions of miR-34a, miR-200a, miR-128 in Snail and Zeb1 (Fig. 2A). miR-34a, miR-200a, and miR-128 affect each other's expression. To analyze the correlation between miRNAs, when miR-34a is added in HCT116 cells, both miR-200a and miR-128 are increased. And when miR-200a or miR-128 is added, the remaining two microRNAs are also increased (Fig. 2B). Reversely when measuring miRNAs in one miRNA sponge are decreased other miRNAs levels, as well (Fig. 2B). That is, there is positive regulation on miRNAs (miR-34a, miR-200a, and miR-128), as ceRNAs in the expression of miRNAs.

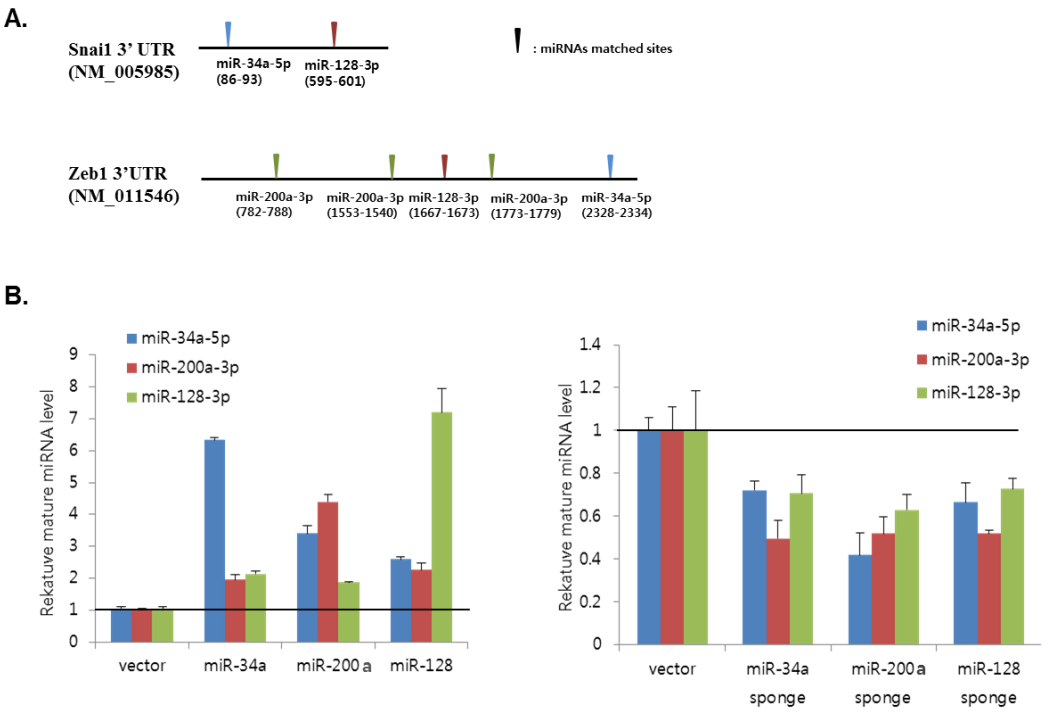


Figure 2. miRNAs could play a role in ceRNA regulation.

(A) Predicted Snail (NM_005985) and Zeb1 (NM_011546) target sites of miR-34a-5p, miR-200a-5p and miR-128-3p are indicated by the arrows on the 3' UTR. (B) The transcript level of three mature miRNA levels (miR-34a-5p, miR-200a-3p, and miR-128-3p) are measured with adding miRNAs (miR-34a, miR-200a, and miR-128) or not (vector) by qPCR. (Results are shown as means ± s.d. from triplicate independent experiments. Statistical significances compared to control was denoted as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by a two-tailed Student's t-test.)

Position 86-93 of SNAI1 3' UTR hsa-miR-34a-5p	5' ...ACCCCACAUCCUUCUCACUGCCA... 3' UGUUGGUCGAUUCUGUGACGGU
Position 595-601 of SNAI1 3' UTR hsa-miR-128-3p	5' ...AAUGUCUGAAAAGGGACUGUGAG... 3' UUUCUCUGGCCAAGUGACACU
Position 2328-2334 of ZEB1 3' UTR hsa-miR-34a-5p	5' ...CACUCUCACAUUCCUCACUGCCU... 3' UGUUGGUCGAUUCUGUGACGGU
Position 1667-1673 of ZEB1 3' UTR hsa-miR-128-3p	5' ...UUAAAUUUUGAAAUUCACUGUGU... 3' UUUCUCUGGCCAAGUGACACU
Position 782-788 of ZEB1 3' UTR hsa-miR-200a-3p	5' ...CAAAAUCAUCAGAAUCAGUGUUU... 3' UGUAGCAAUGGUCUGUCACAAU
Position 1533-1540 of ZEB1 3' UTR hsa-miR-200a-3p	5' ...UGAGAUUUGAUUUAACAGUGUUA... 3' UGUAGCAAUGGUCUGUCACAAU
Position 1773-1779 of ZEB1 3' UTR hsa-miR-200a-3p	5' ...AUUAACUUCUAUAAACAGUGUUG... 3' UGUAGCAAUGGUCUGUCACAAU

Table 1. Lists of miR-34a, miR-200a, and miR-128 match site on 3'UTR of Snail or Zeb1. The putative sequences of SNAI1 and ZEB1 targeted by respective miRNAs are marked in red.

3. Transcriptional and UTR expressions of Snail and Zeb1 are inversely correlated with the expressions of miR-34a, -200a,-128 respectively.

Snail and Zeb1 share the control mechanisms of miR-34a, miR-200a, and miR-128, respectively. When overexpression of miRNAs, Snail and Zeb1 were repressed in protein level (Fig.3A). To test in the transcriptional level, we attatched UTRs to luciferase reporter genes and by overexpressing miRNA-34a, -200a,-128. When it compared reporter activities, both the Snail and Zeb1 have reduced t luciferase activities (Fig3B). On the contrary, it's activities of both the Snail and Zeb1 were increased in the opposite directions by treatment sponge (Fig3c).

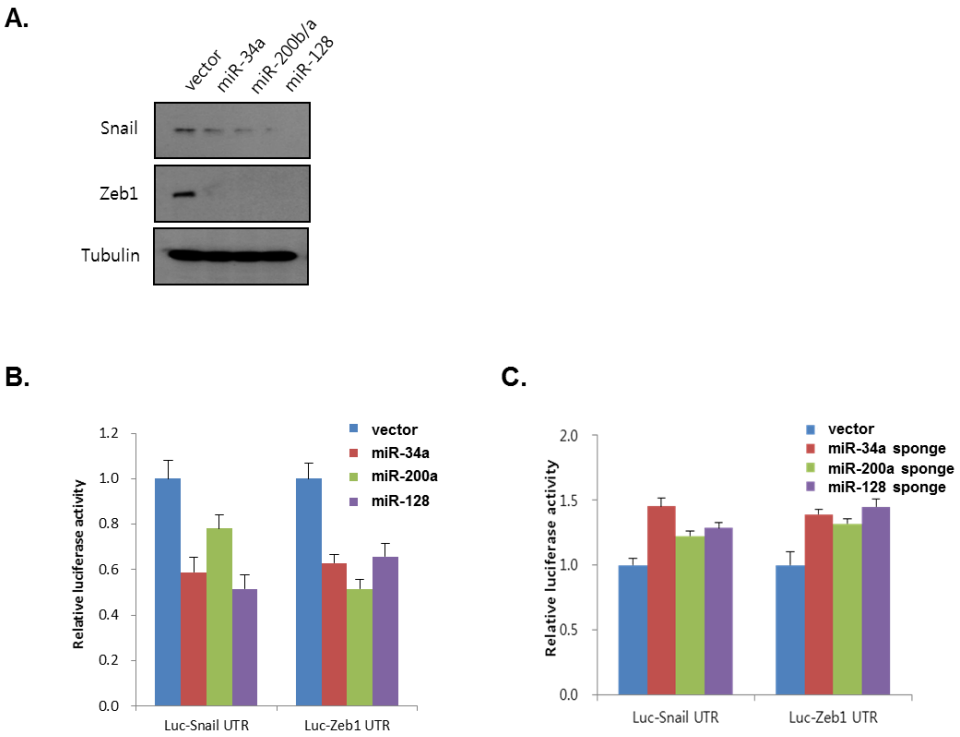
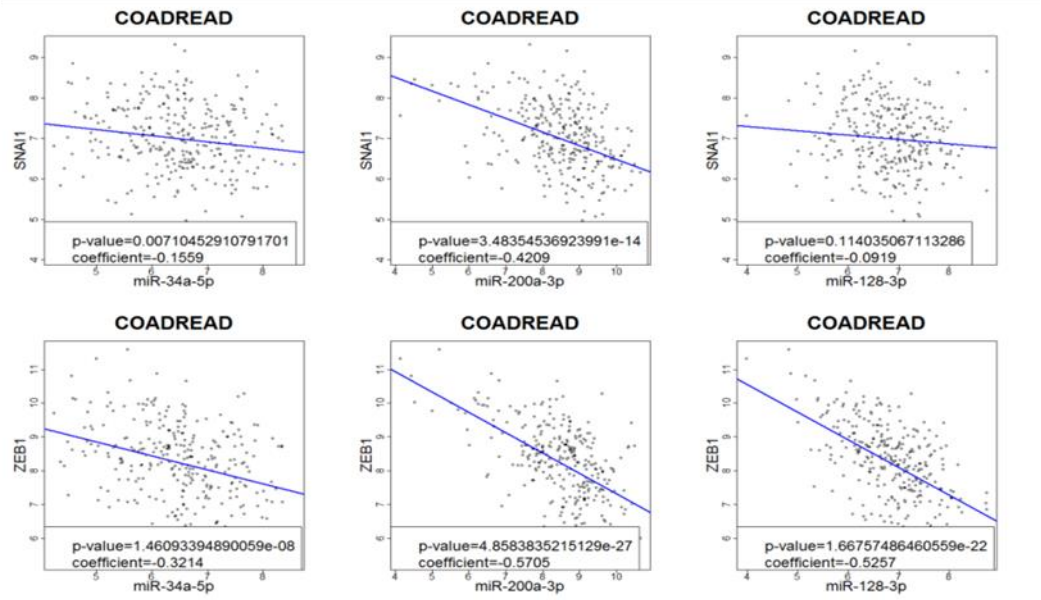


Figure 3. miR-34a,-200a, and -128 target UTR of Snail and ZEB1

(A) Immunoblot analysis of Snail and Zeb1 levels after transfection of control (−) or miR-34a, miR-200a, and miR-128 (B, C) Relative luciferase activity by miRNAs (miR-34a, miR-200a, and miR-128) against non-UTR control reporter is measured in the condition of UTR (Luc-Snail UTR or Luc-Zeb1 UTR) expressed. The luciferase reporter having UTR of Snail or Zeb1 was transfected into HCT116 cells and the relative activities of UTRs against wild type UTR were analyzed after transduction with control vector (vector) or miRNAs (miR-34a, miR-200a, or miR-128).

On Bioinformatics, miRNA and on the Snail and Zeb1 of interrelationship in colon cancer dataset analysis from the miRNA-34a, -200a, 128 manifestations of snail and opposed the manifestations of zeb1 can see each (Fig4A). Even in Microarray analysis, Snail, Zeb1 and miRNA-34a,-200a, 128 were distributed in opposing positions on heatmap. Thus, the relationship between the two reflects the inverse correlation, too.

A.



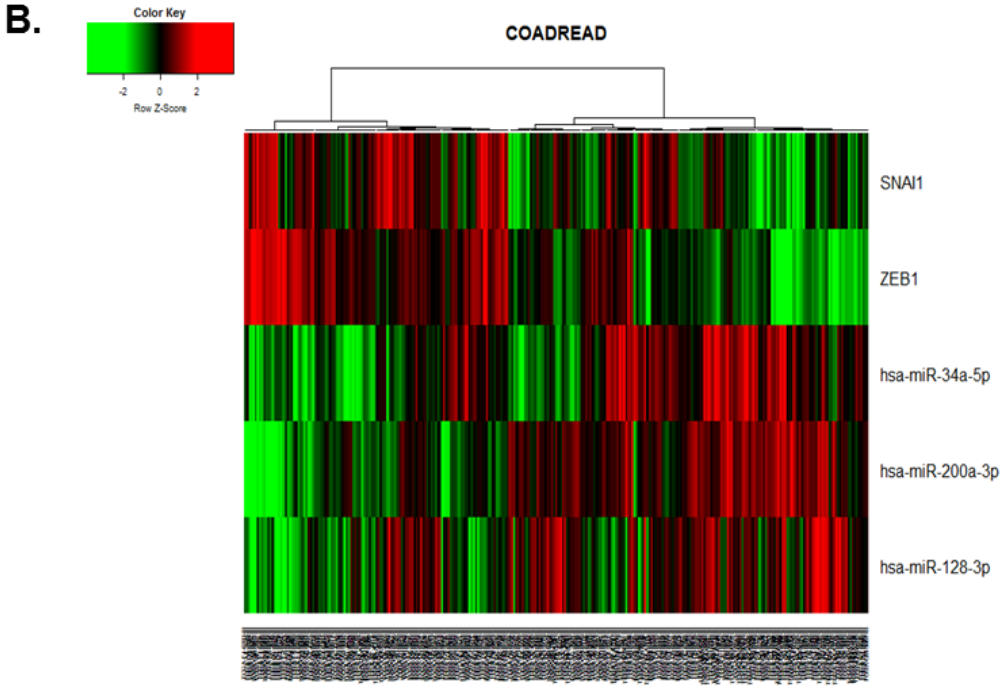


Figure 4. The relationship of miRNAs and Snail/ZEB1 expression in human colorectal cancer samples.

(A) Pearson correlation scatter plots of between Snail, ZEB1 and miR-34a, -200a, -128 in clinical colorectal cancer samples (TCGA). The p values for correlation coefficients between Snail and miR-34a, -200a, -128 were 0.007, 3.48e-14, 0.11 and coefficients between Zeb1 and miR-34a, -200a, -128 were 1.46e-08, 4.85e-27, 1.67e-22 respectively. (B) Unsupervised hierarchical clustering of a 295 TCGA colorectal adenocarcinoma (COADREAD) samples using Snail, ZEB1 and miR-34a, -200a, -128. In the

heat map, red denotes higher relative expression, whereas green indicates lower relative expression, with degree of color saturation reflecting the magnitude of the log expression signal.

But, there is a difference in the expression of Snail and Zeb1 as expressing Snail-UTR or Zeb1-UTR when Dicer is present (WT) or not (Dicer-null) in HCT116 cell. In the presence of Dicer, expression of both Snail and Zeb1 is increased by adding only Snail-UTR or Zeb1-UTR. However, there is no difference between expression of Snail and Zeb1 without Dicer. Since Dicer plays a role in processing microRNA precursors (pre-miRNAs) into mature microRNAs (miRNAs), this implies that the binding of UTR and microRNAs affects protein expressions (Fig.5A). It was confirmed that each mRNA level also changed (Fig. 5B). This is a part of the correlation shown in the past study ((Cha, et al., 2012; Kim, et al., 2011a; Kim, et al., 2011b; Lee, et al., 2009).

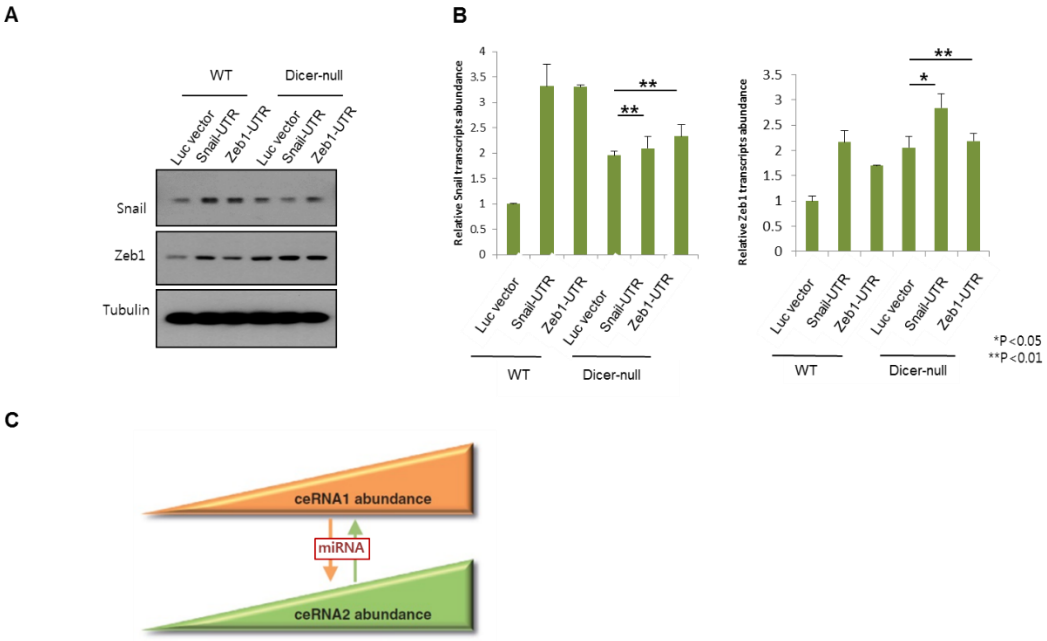


Figure 5. Non-coding UTRs can increase Snail and ZEB1 levels independently on Dicer.

(A) Immunoblot analysis of Snail and Zeb1 after transfection of UTR of Snail and Zeb1 in wt vs. Dicer−/− cells. (B) Relative transcript abundance of Snail and Zeb1 after transfection of UTR of Snail and Zeb1 in WT vs. Dicer−/− cells. (C) Schematic diagram of miRNA-mediated ceRNA regulation.

On the other hand, we looked at whether the miRNA is controlled by UTR. Both Snail and Zeb1 proteins increased when Zeb1 UTR was increased(Fig6.A), and all of miRNA-34a,-200a were reduced(Fig6B). This showed UTRs of Snail and Zeb1 acted as ceRNA, which decreased the expression of miRNA.

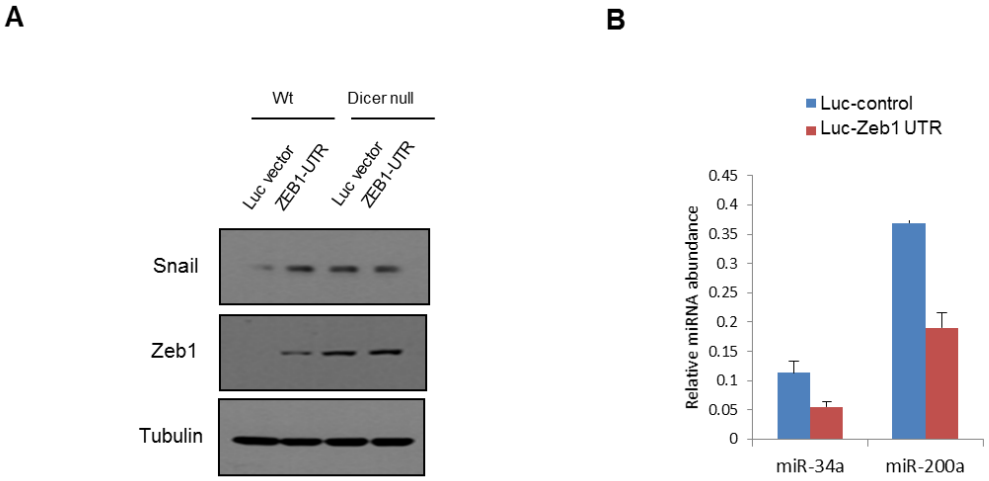


Figure 6. miRNA can be regulated by UTR of mRNAs reversely

(A) Immunoblot analysis of Snail and Zeb1 after transfection of UTR of Zeb1 in wt vs. Dicer−/− cells. (B) The relative expression levels of mature miR-34a and miR-200a were determined by TaqMan quantitative RT-PCR after transfection of the firefly luciferase expression vectors without UTR (mock) or having Zeb1 UTR.

4. Non-coding UTRs of Snail and ZEB1 can induce cancer stemness

Previous studies have shown that tumor suppressor p53 through Wnt signaling is one of the pathway regulated by miR-34(Kim, et al., 2011b). There is a clue to the EMT-related network by miRNA. Based on this fact, the observation of cancer stem cell phenotype by non-coding UTR regulation was attempted. When the Snail / Zeb1 UTR increased cells were cultured in Matrigel, the number and size of colonies increased significantly (Fig. 7A). MCF10A cells were cultured on Matrigel for 10 days, then stained with DAPI and observed under a fluorescence microscope. In the control group, luminal cell survival was observed, whereas in the cells with added Snail/Zeb1 UTR, colony became larger and no luminal morphology was observed (Fig. 7B). And then, FACS analysis showed that CD44 expression was increased in HCT116 cells overexpressed with Snail UTR and Zeb1 UTR (Fig. 7C).

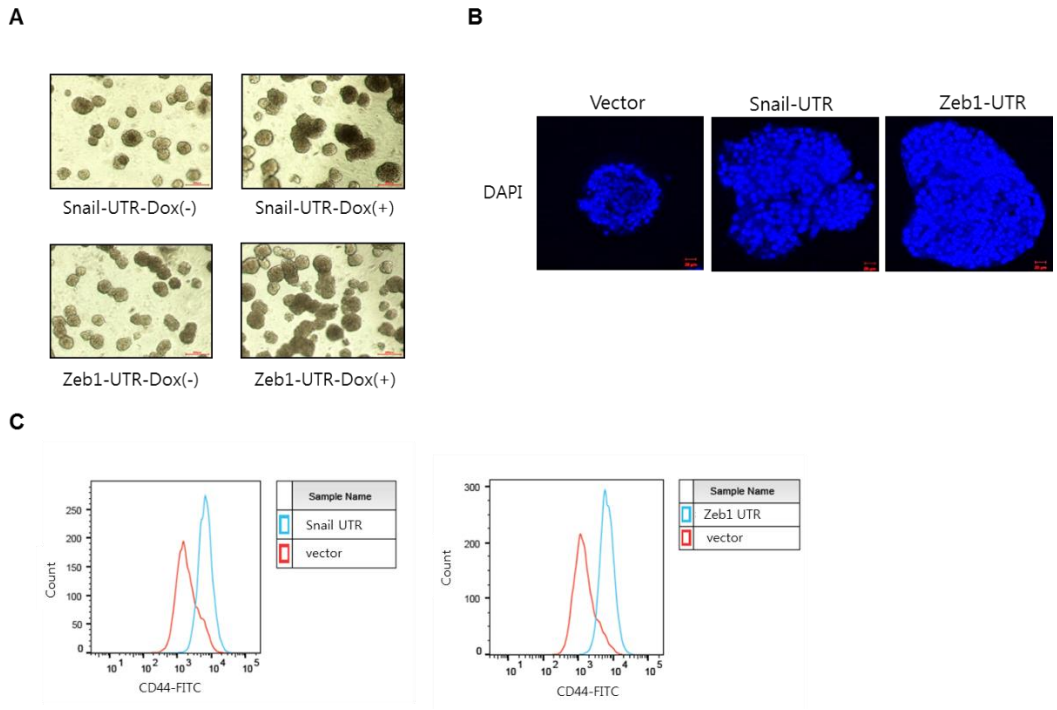


Figure 7. Non-coding UTRs of Snail and ZEB1 can induce cancer stem cell phenotype. (A, B) After MCF10A cell was cultured for 10days in Matrigel (A), it is stained with DAPI and observed by fluorescent microscope (B). (C) FACS analysis with CD44-FITC in HCT116 control (vector) or Snail-UTR expressed cell. Results are shown as means ± s.d from triplicate independent experiments. Statistical significances compared to control was denoted as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by a two-tailed Student's t-test. In vivo experiments are analyzed by Mann Whitney's analysis.

5. Non-coding UTR is functional in EMT *in vivo*

Furthermore, animal models were used to investigate whether the EMT-related functions of non-coding UTRs are suitable in vivo. The experiment is conducted by the method that miRNA or UTR-expressing cells were injected into mouse. Cells with miRNA sponges (miR-34a sponge, miR-200a sponge, and miR-128a sponge) injected into mouse, increasing tumorigenesis respectively (Fig. 8A). Cells with Snail and Zeb1 UTR-expressing injected, increased tumorigeneis respectively, also (Fig8B). Second, after the tumor formation by injecting 293 cells, which were inducible UTR by tetracycline, we obtained sample tissue by 3 times injection of doxycycline, three days ago before the sacrifice of mouse (Fig9A). Immunohistochemistry, transcript, and protein level were confirmed after injection cells with expression of Snail UTR or Zeb1 UTR into mouse (Fig. 9B). As a result, expression of Snail and Zeb1 was increased in tissue staining (Fig. 9B). In addition, we observed that when the non-coding UTR of Snail was increased, the level of transcript and protein of Zeb1 was increased by miRNA-RNA interaction through Snail UTR in vivo (Fig. 9C, D).

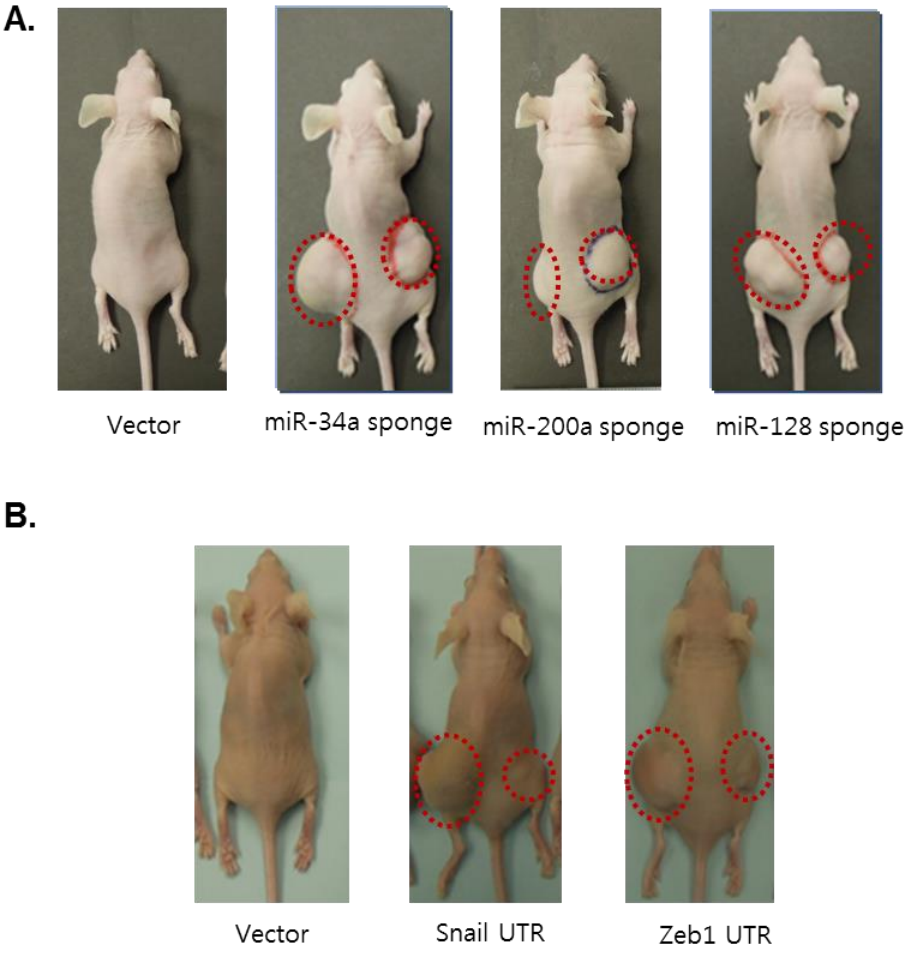


Figure 8. Non-coding RNA can induce *in vivo* tumorigenesis.

(A) $1X10^6$ cell of HCT116 transfected with control (vector), miR-34a sponge, miR-200a sponge, and miR-128a sponge were injected into mouse. Tumor growth was observed twice a week. (B) $1X10^6$ cell of HCT116 transfected without UTR (mock) or having Snail UTR and Zeb1 UTR were injected into mouse, and then growing tumor mass was observed.

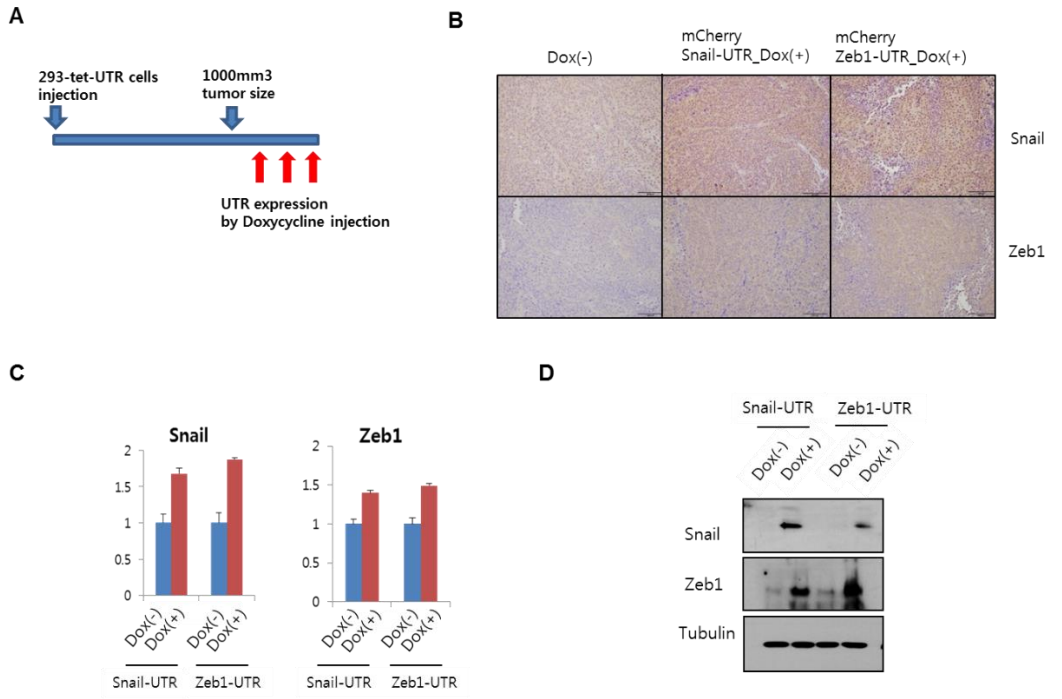


Figure 9. Non-coding UTRs act as ceRNA to regulate tumorigenesis *in vivo.*

(A) Schematic diagram of experimental design for tumorigenesis mice model with Tet-inducible Snail UTR and Zeb1 UTR cells (B) Immunohistochemical staining of Snail and Zeb1 protein levels in mouse tumor samples after overexpression of Snail UTR or Zeb1 UTR with tet-inducible system. (C, D) Relative transcript level (C) and protein level (D) of Snail and Zeb1 were measured after expression of inducible Snail-UTR or Zeb1-UTR. Results are shown as means ± s.d from triplicate independent experiments. Statistical significances compared to control was denoted as *, $P < 0.05$; **,

P < 0.01; ***, P < 0.001 by a two-tailed Student's t-test. In vivo experiments are analyzed by Mann Whitney's analysis.

IV. DISCUSSION

When talking about the p53 -known as a tumor suppressor-network by Vogelstein, it was also dominant to focus on the interaction between proteins (Vogelstein, et al., 2000). But, after human miRNA genes are frequently located at fragile sites in chromosome (Calin, et al., 2004), and p53 transactivation of the miR-34 family elucidates the p53/miR-34 tumor suppressor network, the roles of miRNA has been emphasized (Chang, et al., 2007; He, et al., 2007; Ji, et al., 2009; Merkel, et al., 2010; Raver-Shapira, et al., 2007; Tarasov, et al., 2007).

The p53/miR-34 axis can functionally regulate canonical Wnt signaling and Snail-mediated EMT program via directly targeting the UTRs of a set of genes by miR-34 (Cha, et al., 2012; Kim, et al., 2011a; Kim, et al., 2011b; Lee, et al., 2009). These findings has offered the new understandings that it is meaningless the distinction between tumor suppressor and oncogenic signaling pathways and suggested that miRNA may play highly connected “nodes” linking signaling networks (Albert, et al., 2000). Within p53-miR-34-Wnt network, transcripts of Wnt genes, including WNT1, WNT3, LRP6, AXIN2, β-catenin, LEF1 and Snail are co-regulated through ceRNA interactions (Cha, et al., 2012; Kim, et al., 2013;

Kim, et al., 2011a; Kim, et al., 2011b). Another Wnt gene, Zeb1 was also reported to have been linked to miRNA-200 family and reported cancer repression (Ulrike, et al., 2008).

In this study, it was confirmed that known transcriptional factors inducing EMT, Snail and Zeb1 can be adjusted simultaneously with the same correlation. The reason was somewhat predictable by verifying binding sites of miRNA-34a, -200a,-128, which is targeted at both the two genes concurrently. What is interesting here is the regulatory relationship between miRNA and UTRs of EMT genes, which is no directly attached. Each miRNA-34a,-200a,-128 showed the same movement in the same direction as when a certain miRNA was amplified or sponged, this could explain the possibility that although the binding site does not exist, it can control the EMT genes. This is the function of ceRNA.

The present findings revealed that ceRNA regulation of the EMT genes, Snail, ZEB1 is mediated by tumor suppressor miRNAs, miRNA-34a, miRNA-200a, and miR-128. With no direct binding between miRNAs and UTRs of EMT genes, the expression of EMT genes, Snail and ZEB1 practically repressed in cancer cell lines. In human colorectal samples, expression levels of Snail and Zeb1 transcripts and miR-34a, miR-200a, and

miR-128 showed the inverse correlation, suggesting that EMT-related ceRNA regulation is functional.

On the contrary, overexpression of Zeb1 UTR showed that by reducing the manifestation of miRNA-34a,-200a, ceRNA of the opposite function also works against the snail and zeb1 UTR. The effects of overexpression of Snail and Zeb1 UTRs were not noticeable when it blocked the production of miRNA through processing Dicer of miRNA maturation enzyme. Thus, the intervention of miRNA has been added indispensably to ceRNA network.

In the past, the emergence of stemness when cells undergo a complete EMT has been studied at a level of phenomenology (Mani et al. 2008. Morel et al. 2008), a recent study is covered in the transcription level (JollJolly et al. 2015, MK et al. 2015). EMT genes of Snail and Zeb1 related with cancer showed cancer induced cell stemness by processing UTRs. In addition, it could be observed that carcinogenesis occurred in vivo, which resulted in vitro, it means that non-coding UTR is functional in EMT network. Recent studies have reported cancer incidence in the form of ceRNA network instead of a single gene/target. The potential physiological functions of ceRNAs have been revealed in breast cancer (Zheng et al.

2015), liver cancer (Fang et al. 2012) and tamoxifen resistance (Zheng et al. 2016), and glioma (Wang, Y. & Lin, G. 2016).

As aforementioned, one miRNA can downregulate hundreds of target mRNAs, such regulation can cause a very weak gene expression in diverse cellular functions, such as cellular development, differentiation, proliferation, apoptosis and metabolism (Vandenboom et al. 2008). In reality that the focus is on the development of cancer therapeutic agent, focusing on one special target, because the RNA network allows gene expression to be adjusted in several directions, it could be another way to explain the mechanism of resistance to drugs.

Wherein miR-mRNA regulatory EMT network may provide the solutions overcoming limitations of biological and medical approach based on protein in cancer therapeutic modality.

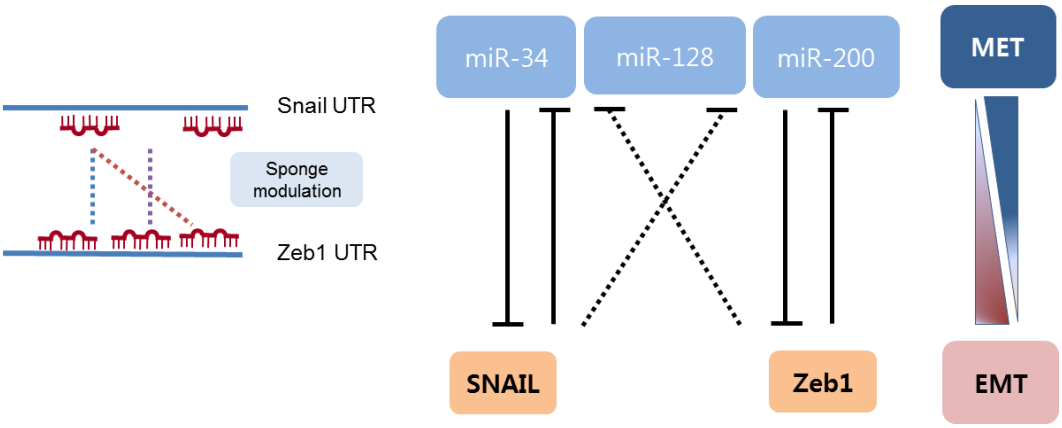


Fig. 10 Schematic diagram of EMT-related RNA Network by Non-coding Transcripts

V. CONCLUSION

This study resulted in the following conclusions:

1. The EMT transcriptional factors, Snail and Zeb1 are synchronously regulated in protein, mRNA, and human colorectal cancer samples.

2. Both Snail and ZEB1 are repressed by miR-34a, -200a, and -128.

3. Transcriptional and UTR expressions of Snail and ZEB1 are inversely correlated with the expressions of miR-34a, -200a, and -128 respectively.

4. Non-coding UTRs of EMT genes, Snail and ZEB1 can induce cancer stemness.

5. Non-coding UTR is functional in EMT *in vivo*.

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ABSTRCT (IN KOREAN)

Non-coding transcripts에 의한

EMT 조절 RNA 네트워크

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최윤희

기존 연구에서 microRNA (miRNA)-34 family에 의해 암억제자인 p53과 암 발생 신호 전달체계인 Wnt 신호전달 및 Snail/EMT axis가 조절된다고 알려짐에 따라, miRNA가 암 발생 및 신호전달을 조절하는 매우 중요한 기능을 수행하고 있고 암 발생과 전이를 조절하는 genome-wide RNA 네트워크의 핵심 요소라는 것이 밝혀졌다. Snail이 miRNA-34에 의해 직접적으로 타겟되고 억제조절됨에 비해, 또 다른 EMT 전사 조절인자 중의 하나인 ZEB1은 miRNA-200에 의해 발현이 조절된다고 알려져 있다. 본 연구는 tumor suppressor miRNAs, 즉 miRNA-34, miRNA-200 및 miR-128에 의한 EMT 관련인자들, 즉 Snail, ZEB1 간의 경쟁적

인 ceRNA 기능 조절 분석을 통해 non-coding transcripts에 의한 EMT 조절 RNA 네트워크 조절기전을 규명하는 데 목적이 있다. 본 연구는 EMT 관련 유전자인 Snail과 Zeb1의 ceRNA 조절 관계를 세포 및 인체 조직에서의 전사체(transcript) 분석을 통해 검증하고, Snail과 Zeb1의 동시조절에 있어 각각의 UTR을 타겟하는 miRNAs (miR-34a, -200a, -128)의 매개 여부를 확인하는 실험으로 구성되었다. 나아가 UTR을 과발현시켜 줄기세포적 특성(stemness) 및 종양형성 유도 동물실험을 통한 기능 검증 연구가 포함되었다. Snail과 Zeb1은 miR-34a, miR-200a, miR-128에 의한 직접적인 타겟이 되지 않는 경우에도 실제로 각각의 miRNA에 의해 억제될 수 있으며, 인체조직 상에서도 Snail과 Zeb1 transcripts의 발현은 miR-34a, miR-200a, miR-128의 발현과 역상관 관계를 보였다. 이러한 조절관계는 단백질을 coding 하지 않는 Snail과 Zeb1의 UTR 처리 만으로도 암세포는 stemness를 보이고 누드마우스에서 암형성이 유도됨을 확인함으로써 non-coding transcripts에 의한 EMT 조절이 기능적임을 확인하였다. Non-coding transcripts에 의한 EMT 조절 RNA 네트워크 조절에 대한 이해는 기존 유전자 돌연변이 중심의 암 진행 가설 보완하고 EMT 과정을 RNA 네트워크로 이해하는 새로운 패러다임 제공하여 새로운 암 치료 전략을 제시할 수 있을 것으로 기대한다.

핵심되는 말: untranslated region, Epithelial–mesenchymal transition, RNA

network, competing endogenous RNA, stemness, microRNA, miR-34, miR-200,miR-128, Snail, Zeb1

약어: UTR, untranslated region; EMT, epithelial-mesenchymal transition; ceRNA, competing endogenous RNA; miR, microRNA; wt, wild type