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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> More than 20% of breast cancer patients detected by mammography are DCIS and this number keeps on increasing (1). Although DCIS is a non-invasive benign tumor, it is considered a precursor of malignant cancer. Therefore, understanding the molecular changes from normal cell to DCIS is of paramount importance and it is under intensive study. However, the exact mechanism of normal-DCIS transition is still not well understood. The purpose of this project is to identify specific microRNAs in tumor stem cells that are responsible for the formation of DCIS. The results of our microRNA array analysis for CSCs from normal and DCIS cells revealed that the expression of a series of microRNA are changed during the transition from normal to DCIS in CSCs. Particularly, we found that miR29a and miR29c function as suppressors of DCIS by blocking the expression of SREBP1, suggesting that modulation of these microRNAs in CSCs triggers the initiation of DCIS. Because the major target of miR29a and miR29c is SREBP, it is possible that this gene can be a potential candidate as a preventive drug for DCIS. Alternatively, the expression of miR29a and miR29C can be restored by a small molecule. It would be ideal if this can be accomplished with specific nutrition. However, this requires further investigation.					
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## INTRODUCTION

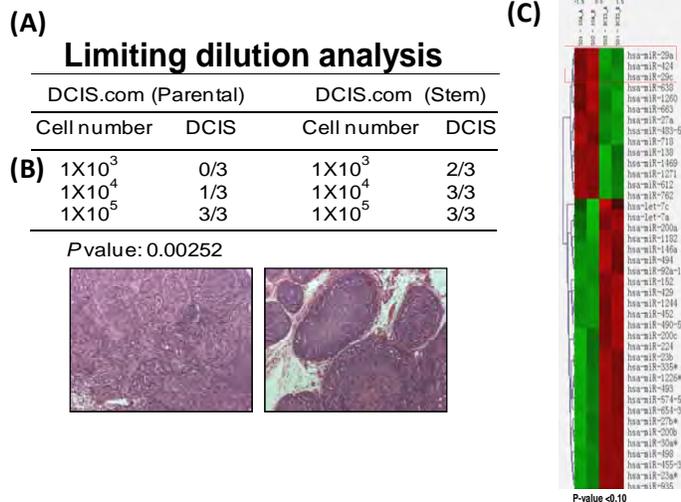
More than 20% of breast cancer patients detected by mammography are DCIS and this number keeps on increasing (1). Although DCIS is a non-invasive benign tumor, it is considered as a precursor of malignant cancer. Therefore, understanding the molecular changes from normal cell to DCIS is of paramount importance and it is under intensive study. However, the exact mechanism of normal-DCIS transition is still not well understood. MicroRNAs have been found to play active roles in tumorigenesis as both promoters and suppressors in many types of cancers (2). Therefore, it is plausible that certain microRNA species are playing critical roles in tumor stem cells during the transition of normal cell to DCIS. We propose to test our novel hypothesis that the normal/DCIS transition is triggered by specific microRNA molecules in tumor stem cells. The purpose of this project is to identify specific microRNAs in tumor stem cells that are responsible for the formation of DCIS.

## BODY

**Task 1. To isolate tumor stem cell population (24-, 44+, ESA+) from MCF10A and MCF10DCIS cell lines, and examine the expression profile of microRNA.**

- Isolate tumor stem cell population (24-, 44+, ESA+) from MCF10A and MCF10DCIS.
- Isolate RNA and examine the expression of microRNA by array analysis.
- Verify the expression by qRT-PCR.

To accomplish Task 1 (a), we cultured MCF10A and MCF10A-DCIS.com cell lines (3) followed by isolating CD24-/CD44+/ESA+ population by the magnetic bead sorting using MACS Separator (Miltenyi Biotec). CSCs from 10A-DCIS cells were tested for their tumor initiating ability by limiting dilution assay using nude mice. As shown in Fig. 1A, CD24-/CD44+/ESA+ population showed significantly higher incidence of tumor formation in the animals, indicating that these cells are CSCs. The results of our histological analysis (Fig. 1B) revealed that these tumors are all DCIS and histologically comparable to the DCIS generated by parent cells in nude mice. We then isolated small RNA from CSCs of both MCF10A and MCF10A-DCIS followed by performing a microRNA array analysis. The result of this experiment indicates that 14 microRNAs are significantly up-regulated in CSCs of MCF10A, and 27 microRNA are significantly



up-regulated in CSCs of MCF10A, and 27 microRNA are significantly

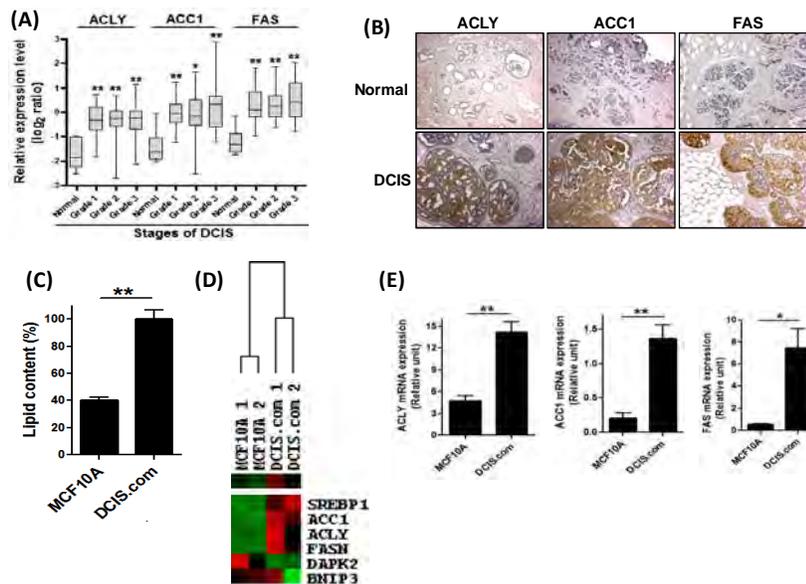
**Fig. 1. MicroRNA expression profile analysis for CSCs of DCIS.** A. A serial dilution experiment was performed to evaluate the *in vivo* tumorigenic potential of DCIS.com parental cells and DCIS.com CSCs. DCIS.com CSCs were sorted from DCIS.com cells using stem cell surface markers (CD24-/CD44+/ESA+) by MACS magnetic separation system. Both DCIS.com (parental and CSCs) cells were transplanted into the mammary fat pad of 4-week old nude mice in different cell numbers as indicated in the table. Formation of DCIS was observed every third day until 4 weeks. B. Mice were sacrificed at the endpoint and DCIS tumors were harvested and sectioned. HE staining was performed to confirm the formation of DCIS. C. MicroRNA array profiling was done in MCF10A stem-like cells and DCIS.com CSCs. The heatmap shows the expression of microRNAs in both the cell types.

up-regulated in DCIS.com (Fig. 1C). Therefore, Aim 1 was accomplished.

**Task 2. To ectopically express the microRNA in MCF10A or silence the microRNA in MCF710ADCIS, and test their ability of forming DCIS *in vitro* and *in vivo*.**

- (a) Establish cloned cell lines that ectopically express the microRNA or silencing the RNA in MCF10 and MCF10ADCIS.
- (b) Test the ability of cell lines to form DCIS in in vitro culture system as well as in an animal model.

To narrow down the list of the microRNA targets, we first analyzed a large clinical cohort database of normal and DCIS tissues from breast cancer patients. We found that many key lipogenic genes

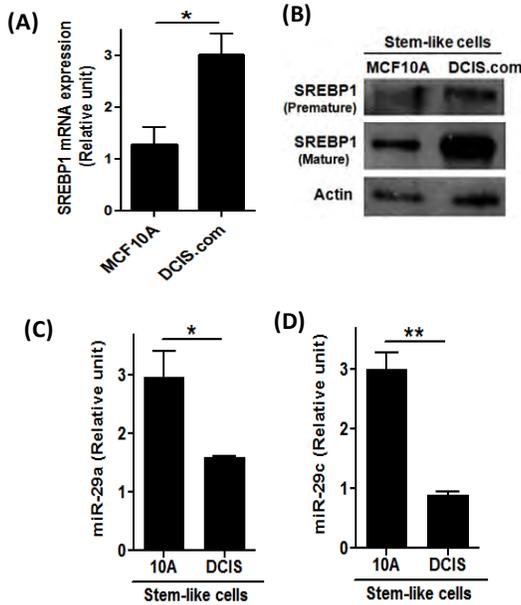


including ACLY, ACC1 and FAS are significantly up-regulated in DCIS (Fig. 2A) and this was confirmed by our immunohistochemical analysis (Fig. 2B). These results suggest that lipogenesis plays a critical role in the initial step of development of DCIS. We indeed found that DCIS.com has significantly higher amount of lipid content compared to MCF10A (Fig. 2C). This interesting observation prompted us to examine the expression profile of lipogenic genes in CSCs from both cell lines by microarray analysis. We found that these

**Fig. 2. Upregulation of lipogenic enzymes at early stage of breast tumorigenesis.** A. DCIS clinical cohort in GEO database (GSE7882) was analyzed for the expression of lipogenic genes ACLY, ACC1 and FAS. Box and whisker plots were drawn to quantitate the expression of individual genes. B. To examine the expression of lipogenic genes in DCIS, tissue sections of human DCIS clinical specimens with matched normal tissues were subjected to IHC analysis. Representative images for each antibody staining with consecutively sectioned slides are shown. C. Normal human breast epithelial cell line MCF10A and ductal carcinoma *in situ* cell line DCIS.com were cultured in 96-well plate having ultra-low attachment surface. After 48 hours, intracellular lipid content was quantified by using the AdipoRed assay. D. Microarray gene expression analysis was conducted in stem-like cells from MCF10A and DCIS.com. Expression of lipogenic genes are shown in the form of heatmap. E. MCF10A and DCIS.com cells were cultured in triplicates in 24-well plate for 48 hours. Cells were collected and RNA was extracted. qRT PCR analysis was then performed to detect the expression level of ACLY, ACC1 and FAS mRNAs.  $\beta$ -actin gene was used as an internal control.

genes (ACC1, ACLY, FAS, SREBP) are indeed highly activated in CSCs of DCIS.com (Fig. 2 D), and this was confirmed by qRT-PCR and Western blot (Fig. 2 E). These results suggest that CSCs of DCIS has significantly higher turnover of lipid synthesis. Because SREBP1 is the master regulator of lipogenesis, we also examined the expression of SREBP1 in both CSCs from MCF10A and DCIS by qRT-PCR and Western blot. We found that SREBP1 is indeed significantly up-regulated in CSCs of DCIS.com (Fig. 3 A, B). Therefore, we examined a possibility that SREBP1 is controlled by microRNAs that were identified by our microarray analysis. We then searched potential targets for

each of the 14 microRNA that were significantly down-regulated in CSCs of DCIS.com using Targetscan and found that miR29a and miR29c are possible candidates among all 14 microRNA. We found that these microRNAs were indeed significantly down-regulated in our Taqman PCR analysis (Fig. 3 C, D). Next, to see if miR29a and miR29c are able to down-regulate the expression of SREBP1, we made a 3'-UTR reporter plasmid for the SREBP1 gene followed by testing the effect of miR29a and miR29c in DCIS.com cells. As shown in Fig. 4A, both microRNAs are indeed capable of



suppressing the expression of SREBP reporter activity. The suppressive effect of these microRNAs on SREBP1 was also verified by Western blot analysis (Fig. 4B). We then established the cell line expressing miR29a or miR29c in DCIS.com. We found that cells expressing these microRNAs showed very slow growth although we were able to establish cell lines. We then examined the rate of lipid synthesis and proliferation of CSCs from these cell lines. As shown in Fig. 4C and D, the rate of lipogenesis and proliferation of CSCs in low serum medium were significantly lower compared to the control. These results strongly suggest that miR29a and miR29c are capable of suppressing proliferation of CSCs by down-regulating SREBP1 in DCIS.

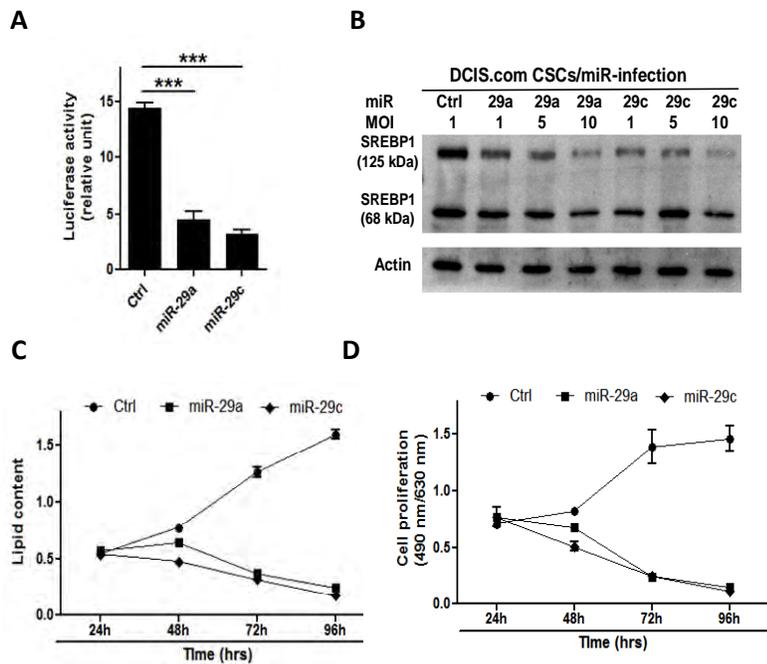
Therefore, this aim is still in progress. Although we have already exhausted the financial support, we are planning to request institutional support and complete the task in the near future.

**Fig. 3. miR29a and miR29c are down regulated in CSCs of DCIS.** **A.** Stem-like cells were cultured in triplicates and subjected to qRT PCR analysis to assess the mRNA level of SREBP1 gene. **B.** Stem-like cells were isolated from MCF10A and DCIS.com cells and Western blotting was done to detect the expression of SREBP1. **C-D.** TaqMan assay was done to validate the microarray data in the same set of cells for miR-29a and miR-29c.

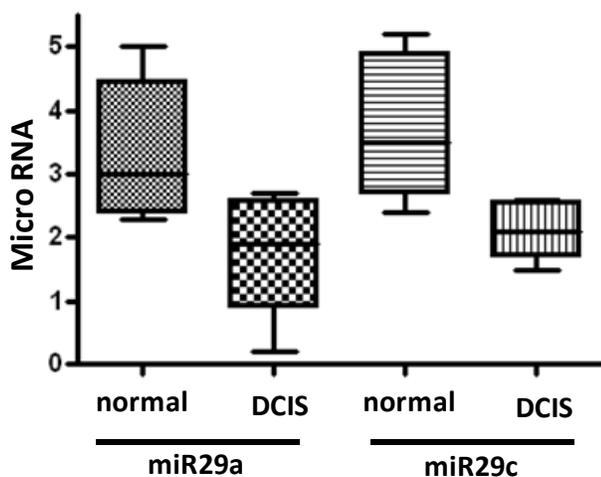
### Task 3. To examine the expression of the microRNA in DCIS of human sample

- (a) Section DCIS samples from 50 patients and perform qRT-PCR and in situ hybridization for the microRNA to verify the results of Tasks 2 and 3 in human cancer.

If we can examine the expression of microRNA using paraffin block samples, it will greatly facilitate this task. Therefore, we first explored this possibility. Paraffin block samples were sectioned and the lesion of DCIS as well as normal gland areas was micro-dissected. They were then subjected to RNA preparation using an RNA extraction kit (FFPE kit, Qiagen). However, the amount of RNA was very small and they were not suitable for a regular q-PCR. Therefore, we used Taqman PCR and found that this method indeed reproducibly detected both microRNA in normal and DCIS of paraffin block samples. Fig. 5 showed the result of 5 samples of each normal and DCIS. Although the number of samples is small, we have found clear trend of down-regulation of both microRNAs by this assay. Currently, we are collecting RNA from more samples (n=50), and we will perform the Taqman PCR. Therefore, this aim is still in progress.



**Fig. 4. miR29a and miR29c suppress lipogenesis and proliferation of CSCs.** **A.** 3'UTR-reporter gene (Luciferase) was transfected by lentivirus to DCIS.com cells and established a cell line, DCIS-3'UTR/SREBP. The cells were then infected with lentivirus carrying either miR29a or miR29c followed by luciferase assay for the reporter. **B.** DCIS.com CSCs were infected with miR-29a and miR-29c overexpressing lentiviruses with different multiplicity of infection (MOI). After 48 hours of incubation, cell lysate was prepared and Western blotting was done to detect the expression of SREBP1 (both nuclear and cytoplasmic form). **C.** DCIS.com CSCs were cultured in triplicates in 96 well ultra low attachment plate (2500cells/well in 100ul medium). The cells were then infected with miR-29a and miR29c overexpressing lentiviruses and further incubated for designated time-points. Cells were assayed for the quantification of intracellular lipid content by using AdipoRed assay kit. **D.** Similar experimental scheme was carried out as in B and MTS assay was done to measure the rate of cell proliferation.



**Fig. 5. miR29a and miR29c are down-regulated in DCIS in clinical samples.** Paraffin block samples of breast cancer patients were sectioned and DCIS and normal glands were microdissected from 5 samples each. Total RNAs were extracted from these samples and subjected to Taqman PCR analysis for miR29a and miR29c.

## KEY RESEARCH ACCOMPLISHMENTS

1. We have successfully isolated cancer stem-like cells from normal and DCIS cell lines.
2. CSCs from DCIS showed significantly higher ability of generating DCIS in nude mice.
3. microRNA array analysis for these CSCs revealed that 14 microRNA are significantly down-regulated in DCIS.com and 27 microRNA are significantly up-regulated in CSCs of DCIS.
4. Lipogenic genes were found to be significantly up-regulated in CSCs of DCIS.
5. miR29a and miR29c are found to be capable of suppressing the master gene of lipogenesis, SREBP1.
6. Ectopic expression of miR29a and miR29c significantly suppressed proliferation of CSC from DCIS.
7. We found that paraffin archive samples are suitable for examining microRNA expression using Taqman PCR.

## REPORTABLE OUTCOMES

### Peer reviewed publications

1. Puspa Pandey, Hiroshi Okuda, Megumi Gairani, Misako Watabe, Fei Xing, Aya Kobayashi, Wen Liu, Koji Fukuda, , Sudha Pai and Kounosuke Watabe. Roles of lipogenesis and microRNA in cancer stem-like cells in ductal carcinoma in situ. In preparation

### Abstract/presentation

1. Puspa R. Pandey, Hiroshi Okuda, Misako Watabe, Sudha K. Pai, Wen Liu, Aya Kobayashi, Fei Xing, Koji Fukuda , Shigeru Hirota, Tamotsu Sugai, Go Wakabayashi, Keisuke Koeda, Masahiro Kashiwaba, Kazuyuki Suzuki, Toshimi Chiba, Masaki Endo, Tomoaki Fujioka, Susumu Tanji, Yin-Yuan Mo, Deliang Cao, Andrew C. Wilber and Kounosuke Watabe. Up-regulation of lipogenic genes, SREBP-1, ACC, ACLYL and FAS, confers advantages of proliferation and survival to cancer stem-like cells in Ductal carcinoma in situ 2011 Annual meeting of American Association for Cancer Research. Orlando FL
2. Puspa Pandey, Hiroshi Okuda, Megumi Gairani, Misako Watabe, Fei Xing, Aya Kobayashi, Wen Liu, Koji Fukuda, , Sudha Pai and Kounosuke Watabe Roles of lipogenesis and microRNA in cancer stem-like cells in ductal carcinoma in situ Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope 2011 Conference Orlando, Florida

### Employment

1. Mr. Puspa Pandey (Graduate student) has been partially supported by the current grant.

## CONCLUSIONS

The tumor stem cell theory, which still remains a hypothesis, offers an attractive explanation for early stage of tumorigenesis. Although the exact origin of tumor stem cell is still not clearly defined, the transition of normal cell to pre-malignant tumor (Ductal cancer in situ or DCIS) involves many genetic and physiological changes, which likely takes place in a tumor stem cell. The results of our microRNA array analysis for CSCs from normal and DCIS cells revealed that the expression of a

series of microRNA are changed during the transition from normal to DCIS in CSCs. Particularly, we found that miR29a and miR29c function as suppressors of DCIS by blocking the expression of SREBP1, suggesting that modulation of these microRNAs in CSCs triggers the initiation of DCIS.

### **So what?**

The most important aspect of this research is whether we can develop any preventive measure against DCIS. Because the major target of miR29a and miR29c is SREBP, it is possible that this gene can be a potential candidate as the preventive drug for DCIS. Alternatively, the expression of miR29a and miR29C can be restored by a small molecule. It is ideal if this can be accomplished by taking specific nutrition. However, this requires further investigation.

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