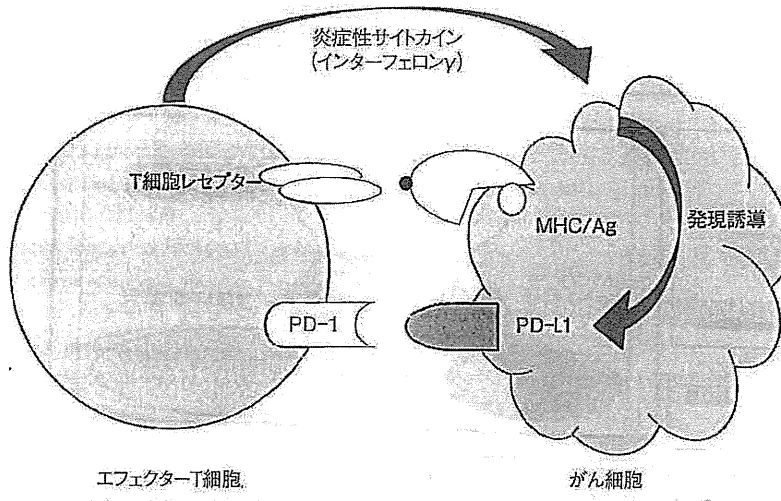

図4 PD-1/PD-L1 チェックポイントによるがん細胞の免疫抑制

た。さらに，CTLA-4 と PD-1 抗体の併用や抗癌剤との併用，対象癌種の拡大にて immune checkpoint を標的とする治療開発は急速に発展すると考えられる。

おわりに

膵癌遠隔転移症例を対象に FOLFIRINOX 療法が無作為化比較試験でゲムシタビン塩酸塩に対し有意に生存期間を延長することが報告され[4]，Nanoparticle albumin-bound paclitaxel (nab-paclitaxel) も今後の有望な薬剤となってくると考えられる。FOLFIRINOX 療法はゲムシタビン塩酸塩を含まない regimen であり，臨床の現場ではその効果に期待がもたれるが，feasibility の検証結果，とくに好中球減少などの有害事象の検討が待たれるところである。しかし，膵癌は診断時に栄養状態不良な症例や高齢者も多く含まれるため，このような toxic regimen が非適応の症例も多数存在し，副作用の軽微な治療法の開発も非常に重要である。ペプチドワクチン療法は副作用の少ない治療法であり，今後，有効なペプチドワクチンの同定だけでなく，quality assessment や quality control が必要である。また，T 細胞反応，Treg・myeloid-derived suppressor 細胞・NK 細胞・樹状細胞の解析，腫瘍関連抗原に対する抗体などが検討されているが[33,34]，免疫療法において有効性を示唆する biomarker の更なる開発が必要である。また，ペプチド投与により有効に強力な CTL が誘導できるワクチンアジュバントの開発も必要である。腫瘍周辺の微小環境は免疫療法の場としては有利な場ではないが，OK-432 と NY-ESO-1 タンパク質ワクチンと併用することで，Treg 存在下でも CTL が活性化可能[35]である。CpG-ODN は TLR-9 agonist であるが，CpG は樹状細胞の活性化を介して大量の type-1 インターフェロン産生を誘導し，自然免疫とともに獲得免疫を活性化することでペプチドワクチンの効果増強が得られる可能性が報告された[30]。さらに，LY6K および TTK 由来のペプチドワクチン療法に CpG-B を併用する第Ⅰ相臨床試験において，CpG-ODN の癌ワクチンにおけるアジュバントとして有用であった[36]。

2010 年に免疫療法治療薬としては sipuleucel-T (Provenge) が，世界初の前立腺癌に有効な癌ワクチン療法として，2011 年には免疫チェックポイント阻害薬である抗 CTLA-4 抗体を FDA が認可した。PD-1 (Programmed Cell Death-1) といった腫瘍免疫において抑制的に働く補助刺激分子を標的とした薬剤も[37]免疫療法においては画期的なものとなるかもしれない。一方，膵癌に対するペプチドワクチンの分野では承認されたものはいまだないのが現状であり，ペプチドワクチンに代表される免疫療法薬が開発されるよう治験などの質の高い臨床試験の推進が必要である。

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The detection of gastric cancer cells in intraoperative peritoneal lavage using the reverse transcription–loop-mediated isothermal amplification method

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ARTICLE INFO

Article history:
Received 27 April 2012
Received in revised form
29 December 2012
Accepted 3 January 2013
Available online 25 January 2013

Keywords:
Gastric cancer
RT–LAMP
Peritoneal lavage

ABSTRACT

Introduction: To detect a small number of malignant cells, we used a highly sensitive detection system that measures the expression levels of cytokeratin (CK) 19 messenger RNA by reverse transcription–loop-mediated isothermal amplification (RT–LAMP).

Materials and methods: We evaluated the clinical relevance of our novel diagnostic method with an RT–LAMP assay using CK19 as a target gene for the detection of free cancer cells in peritoneal lavage and assessed the clinical significance of the molecular diagnosis by survival analysis and frequency of recurrence, with a median follow-up period of 39 mo. We observed 52 patients with gastric cancer who underwent gastrectomy, bypass operation, and exploratory laparotomy.

Results: Those 52 patients, who were subjected to both RT–LAMP and cytologic examination, were divided into the following three groups: (1) patients positive by cytology and RT–LAMP (CY+/LAMP+) ($n = 9$), (2) patients positive by LAMP and negative by cytology (CY–/LAMP+) ($n = 12$), and (3) patients negative by both cytology and LAMP (CY–/LAMP–) ($n = 31$). All patients with simultaneous peritoneal dissemination and positive cytology were positive on RT–LAMP. The results of RT–LAMP were statistically significant for recurrence by univariate analysis ($P < 0.005$). Cytology-positive cases had a very poor prognosis, and RT–LAMP-positive cases had a worse prognosis than RT–LAMP-negative cases.

Conclusions: Our findings suggest that CK19 RT–LAMP would be useful as an intraoperative diagnostic modality to detect patients with a high risk of recurrence even after clinically curative surgery, who thus require proper adjuvant therapy.

1. Introduction

Peritoneal carcinomatosis is the most frequent pattern of recurrence in patients with gastric cancer [1,2]. The prognosis of patients with advanced gastric cancer invading the gastric serosa is very poor even after curative resection, mainly because of the high incidence of peritoneal recurrence [3]. Recurrence with this pattern is most likely caused by the presence of free cancer cells in the abdominal cavity exfoliated from the serosal surfaces of the primary gastric tumor [4]. Therefore, detection of such micrometastatic cells in the peritoneal cavity is likely to be a useful tool in the selection of

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0022-4804/$ – see front matter © 2014 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.jss.2013.01.001

intra or postoperative chemotherapy and for predicting the outcome of such therapy in these cases [5]. In this regard, cytologic examination of lavage fluid obtained at the time of surgery is a conventional method for detecting free cancer cells in peritoneal space. However, the sensitivity of this assay has been reported to be relatively low, ranging from 19% to 30% in gastric cancer invading the serosa [6–9]. As a result, some patients with negative cytology have nevertheless developed peritoneal recurrence. Therefore, there is an urgent need for more sensitive methods to detect micrometastasis in the peritoneal cavity. To detect small numbers of malignant cells among the cytologically negative cases, we developed a highly sensitive detection system that measures the expression levels of cytokeratin (CK) 19 messenger RNA (mRNA) by reverse transcription–loop-mediated isothermal amplification (RT–LAMP). The RT–LAMP method is a new method of gene amplification, the efficacy of which has been reported [10,11]. The reaction is accelerated by the use of two additional loop primers (called loops F and B) [11]. The LAMP method can be conducted simultaneously with reverse transcription from mRNA (RT–LAMP) [10–14]. There are several practical advantages to the RT–LAMP technique: it requires only simple reaction procedures, the compact and inexpensive incubator or turbidimeter equipment costs <$5000, and <1 h is needed to obtain the final results [11–15]. Application of the LAMP technique has been reported for breast and lung cancers [16–18]. This technique might be one of the most promising candidates for analyzing the genetic features of samples obtained during surgery.

CK proteins of the intermediate filaments of epithelial cells have been used as specific markers for tumor cells of epithelial origin [19,20]. In the present study, we evaluated the clinical relevance of a new diagnostic method using an RT–LAMP assay with CK19 as the target gene for the detection of free cancer cells in the peritoneal lavage and assessed the clinical significance of the molecular diagnosis by survival analysis and frequency of recurrence.

2. Materials and methods

2.1. *Cell lines*

A sensitivity assay for detecting a gastric cancer cell line was performed. The human gastric cancer cell line MKN-45, obtained from the Riken Cell Bank (Institute of Physical and Chemical Research, Saitama, Japan), was incubated in RPMI-1640 medium containing 10% fetal calf serum (Invitrogen, Carlsbad, CA) at 37°C in 5% CO_2.

2.2. *Patients*

Between May 2007 and November 2008, we observed 52 patients (35 males and 17 females; mean age, 67.5 ± 2.8 y) with gastric cancer who underwent gastrectomy (n = 45), bypass operation (n = 2), and exploratory laparotomy (n = 7) for histologically proven gastric cancer at the Department of Surgery, Nagasaki University. Written informed consent for participation in this study was obtained from all the patients. All were followed up for a median of 39 mo (range, 6–51 mo) or until death. The primary tumor was resected in 45 of the 52 patients (five patients had peritoneal dissemination but underwent resection of their primary tumor because of the stenosis and bleeding caused by primary tumor as a palliative treatment) but was unresectable in seven patients because of peritoneal dissemination and positive cytology. These seven patients underwent a bypass operation or exploratory laparotomy. The resected specimens were histologically examined by hematoxylin and eosin staining according to the general rules of the Japanese Classification of Gastric Carcinoma [21]. Clinicopathologic features of the patients are shown in Table 1.

2.3. *RT–LAMP reaction*

LAMP primers targeting the CK19 complementary DNA were designed based on a past report [22] (Fig. 1). To quantify and prove the integrity of isolated RNA, we also performed RT–LAMP for β-actin.

The RT–LAMP method was carried out on 25 μL of the total reaction mixture with a Loopamp RNA amplification kit (Eiken Chemical Co, Tokyo, Japan) containing 40 pmol each of the forward and backward inner primers, 5 pmol each of the outer primers F3 and B3, 20 pmol each of the loop primers loops F and B, 35 pmol of dNTPs, 20 μL of Betamine, 0.5 μM Tris–HCL (pH 8.8), 0.25 μM KCL, 0.25 μM (NH4)SO4, 0.2 μM MgSO4, 0.2% Tween 20, 1.0 μL of Enzyme Mix (Bst DNA polymerase and

Table 1 – Clinicopathologic factors were determined according to the Japanese classification of gastric carcinoma.

	Number of patients
Total cases	52
Age (y)	67.5 ± 2.8
Sex: male/female	35/17
Depth of tumor invasion	
M	11
SM	13
MP	6
SS	9
SE and SI	13
Lymph node metastasis	
N0	22
N1	13
N2	13
N3	4
Peritoneal metastasis	
Absent	40
Present	12
Cytology	
Negative	43
Positive	9
Stages	
IA	18
IB	7
II	8
III	4
IV	15

M = mucosa; SM = submucosa; MP = muscularis propria; SS = subserosa; SE = serosa exposed; SI = serosa infiltrating.

F3 :
CCTCCTACCTGGCAAGGT
B3 : ATGCGCAGAGCCTGTC
FIP (F1c+F2):TAGTGGCTGTAGTCGCGG
GAAACGGCGAGCTAGAGGAGAA
BIP (B1c+B2):CGGGACAAGATTCTTGGTGCCAAAGT
CATCTGCAGCCAGACG
loopF : AGGCCCCTGCTTCTGGTAC
loopB : CCTGCAGATCGACAATGCC

Fig. 1 – Primer design for the detection of CK19 mRNA by RT–LAMP.

avian myeloblastosis virus reverse transcriptase), and 5 μL of RNA at a constant temperature of 63.5°C for 60 min. Temperature control for the LAMP reaction and turbidity measurement was achieved using a turbidimeter (LA-200; Teramecs Co, Kyoto, Japan) especially developed for DNA analysis by LAMP reaction.

2.4. *Sensitivity evaluation of RT–LAMP on CK19 mRNA detection*

A fundamental experiment was performed to determine the sensitivity of the RT–LAMP method for detecting gastric cancer cells in peripheral blood mononuclear cells (PBMCs). Peripheral venous blood was obtained from healthy volunteers. Gastric cancer MKN-45 cells were serially diluted from 1×10^6 cells to one cell per 1×10^7 PBMCs. The mRNA was extracted from each cell fraction, and RT–LAMP for CK19 mRNA was performed.

2.5. *Preparation of peritoneal floating cells*

At the beginning of the operation, before the manipulation of the tumor, 400 mL of physiological saline was introduced into the upper abdominal cavity and recovered after being gently stirred. Part of the peritoneal lavage fluid was subjected to conventional cytology after standard Papanicolaou staining, and the remaining fluid was immediately centrifuged at 2000 rpm for 10 min. The pellets of lavaged fluid were rinsed with phosphate-buffered saline, dissolved in RNAlater solution (Ambion, Austin, TX), and stored at −80°C until use.

2.6. *mRNA extraction*

The mRNA was extracted using the Dynabeads mRNA DIRECT kit (Veritas, Tokyo, Japan) according to the manufacturer's instructions. Briefly, collected cells were lysed by the lysis or binding buffer, and the released mRNA and its poly (A) residue were hybridized with oligo (dT) conjugated with Dynabeads; then, this complex was immobilized onto the surface of magnetic beads. Contaminating components were washed away by repeated steps of separation and resuspension in washing buffer. Finally, the purified mRNA was eluted from the particles.

2.7. *RT–LAMP on mRNA derived from peritoneal lavage*

Twenty-five microliters of reaction mixture was applied to each reaction. Positive control primers (β-actin) and a negative control mixture (PBMC-derived sample) were used for all reactions.

2.8. *Statistical analysis*

All statistical analyses were performed using StatView 5.0 software (SAS Institute, Cary, NC). The chi-square test was used to evaluate the correlation between positive results and clinicopathologic factors, and the univariate analysis was used to evaluate the results of RT–LAMP and recurrence. Survival curves were calculated using the Kaplan–Meier method. The survival curves were compared using the log-rank test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. *Evaluation of the sensitivity of RT–LAMP for identifying gastric carcinoma cell line*

As few as 10^0 MKN-45 cells in 10^7 normal PBMCs were detectable with the RT–LAMP procedures targeting CK19–mRNA using extracted mRNA of cell mixtures lysate (Fig. 2).

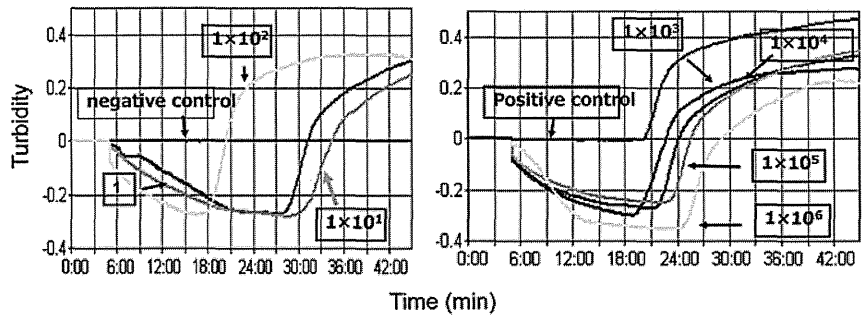

Fig. 2 – Sensitivity analysis of the RT–LAMP method. As few as 10^0 MKN-45 cells in 10^7 normal PBMCs were detectable with the RT–LAMP procedures targeting CK19 mRNA using extracted mRNA of the cell mixture lysate.

3.2. *Detection of cancer cells in intraoperative peritoneal lavage*

The mRNA of β-actin, a housekeeping gene, was detected in all the present samples. RT–LAMP reaction diagnosis significantly correlated with lymph node metastasis, depth of invasion, lymphatic invasion, and vessel invasion (Table 2). The total of 52 patients who were subjected to both RT–LAMP and cytologic examination were divided into the following three groups: (1) patients positive by cytology and RT–LAMP (CY+/LAMP+) ($n = 9$), (2) patients positive by LAMP and negative by cytology (CY−/LAMP+) ($n = 12$), and (3) patients negative by both cytology and LAMP (CY−/LAMP−) ($n = 31$). The stage of each groups was showed on Table 2. There were no patients negative by LAMP and positive by cytology; all patients with simultaneous peritoneal dissemination and positive cytology were positive on RT–LAMP. Simultaneous peritoneal dissemination at surgery or staging laparoscopy was detected in 12 patients (nine in the group CY+/LAMP+ and three in the group CY−/LAMP+). No patients with negative RT–LAMP and negative CY status had recurrence after surgery. The results of RT–LAMP were statistically significant for recurrence by univariate analysis ($P < 0.005$). Figure 3 shows the overall survival (Fig. 3A), recurrence-free survival (Fig. 3B), and peritoneal recurrence–free survival (Fig. 3C) curves for the three patient groups subjected to both RT–LAMP and cytologic examination. Figure 3 shows that cytology-positive cases had a very poor prognosis and that cases with negative cytology but positive RT–LAMP had a worse prognosis than that of the cases who were RT–LAMP negative.

Table 2 – Correlation between RT–LAMP diagnosis of peritoneal lavage and clinicopathologic parameters and the stage of each groups.

	Expression of CK19 mRNA (RT–LAMP)		P-value
	Positive	Negative	
Number of patients	21	31	
Sex			
Male	14	21	NS
Female	7	10	
Age (y)	62.45	67.8	NS
Depth of tumor invasion			
M	0	11	
SM	0	13	
MP	3	3	0.00001
SS	6	3	
SE and SI	12	1	
Lymph node metastasis			
−	3	23	0.00002
+	18	8	
Lymphatic invasion			
−	1	16	0.0091
+	11	15	
Vessel invasion			
−	1	18	0.0033
+	11	13	

M = mucosa; SM = submucosa; MP = muscularis propria; SS = subserosa; SE = serosa exposed; SI = serosa infiltrating.
CY+/LAMP+ ($n = 9$): stage IV, $n = 9$.
CY−/LAMP+ ($n = 12$): stage II, $n = 4$; stage III, $n = 3$; and stage IV, $n = 5$.
CY−/LAMP− ($n = 31$): stage IA, $n = 19$; stage IB, $n = 6$; stage II, $n = 4$; and stage III, $n = 2$.

4. Discussion

Although the standard surgical lymphadenectomy for gastric cancer has been established and applied, patients with advanced stages of this cancer continue to face a poor prognosis. A previous study suggested that the presence of free cancer cells in peritoneal lavage was responsible for the formation of micrometastasis and subsequent extensive dissemination [23,24]. Cytologic examination aimed at the detection of these cells, therefore, has been generally accepted as the golden criterion for the prediction of peritoneal recurrence, and this procedure has been incorporated in the Japanese staging system for gastric cancer [21]. However, peritoneal recurrence sometimes occurs in patients with negative cytology, which indicates the lack of sensitivity of conventional cytologic examination for the prediction of peritoneal recurrence. We believe that it is difficult to confirm peritoneal metastasis from cytology because of its low sensitivity. Jung *et al.* [25] reported that even in patients with clinically diagnosed carcinomatosis with ascites, only 54% of patients were positive for cytology.

Thus, a more sensitive assay for the detection of peritoneal micrometastasis is required. New, simple, and rapid molecular techniques for the detection of target genes have been developed in recent years, and one of these is the LAMP reaction. This reaction is a novel approach to the DNA amplification of target sequences, providing high sensitivity, specificity, and rapidity under isothermal conditions. The LAMP reaction relies on autocycling strand displacement DNA synthesis that is performed with a DNA polymerase with high-strand displacement activity and four specific primers recognizing six independent sequences. Specifically, it synthesizes a large amount of amplification products, comprising a mixture of stem–loop DNA and cauliflower-like structures with multiple loops (except for the loops that are hybridized by the inner primer) and prime strand displacement DNA. When the target DNA is amplified by LAMP reaction, a white precipitate derived from magnesium pyrophosphate (a byproduct of LAMP reaction) is observed [10]; thus, the LAMP method does not require special reagents or electrophoresis to detect the amplified DNA. Amplification of the targeted gene is detectable in real-time fashion by an increase of the turbidity of the solution.

CK19 has been shown to be widely expressed by cancer cells of epithelial origin but not by lymphoid or hematopoietic cells [26]. Among some 20 different isotypes, CK19 and CK20 are expressed more selectively by mucosal epithelial cells than are the others [27]. It has been reported that CK19 is superior to CK20 in detecting circulating cancer cells using reverse transcription–polymerase chain reaction (RT–PCR) in peripheral blood from patients with gastric cancer [28]. Thus, this molecule may be a suitable general marker of

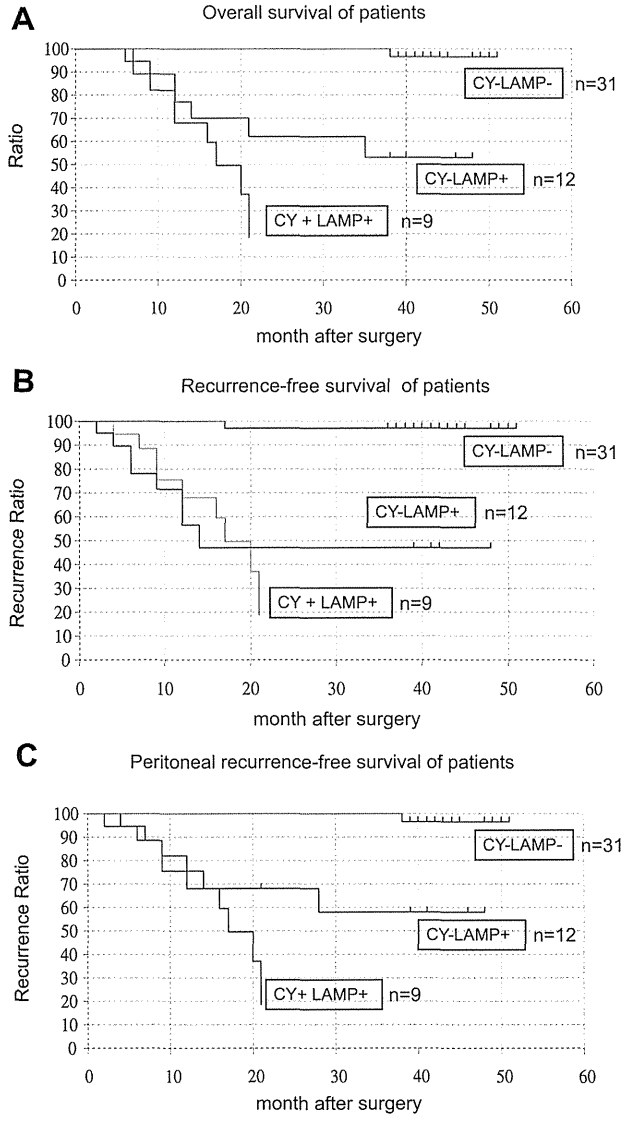

Fig. 3 – (A) Overall survival curves of patients stratified according to the results of cytology and RT–LAMP. Significant differences between the CY–LAMP– and CY–LAMP+ groups were found ($P < 0.05$). (B) Recurrence-free survival curves of patients stratified according to the results of cytology and RT–LAMP. Significant differences between the CY–LAMP– and CY–LAMP+ groups were found ($P < 0.05$). (C) Peritoneal recurrence–free survival curves of patients stratified according to the results of cytology and RT–LAMP. Significant differences between the CY–LAMP– and CY–LAMP+ groups were found ($P < 0.05$).

micrometastasis in peritoneal lavage of patients with gastric cancer. LAMP primers were generated to detect the CK19 sequence, and the performance of the RT–LAMP reaction to detect gastric cancer cells was tested. Only cells of epithelial origin were detected by this technique, with sensitivity to concentrations as low as one cell per 10^7 normal PBMCs. CK expression was observed in the RT–LAMP reaction, presumably because of its high specificity, which requires the recognition of six independent sequences within the target molecule [22].

The development of a rapid technique for detecting cancer cells in peritoneal lavage of patients with gastric cancer using the RT–LAMP reaction is herein described. Among the patients with negative cytology, those with a positive RT–LAMP reaction had a poorer prognosis than those with negative RT–LAMP reaction results. In three patients, laparotomy was performed preceding the staging laparoscopy to verify negative cytology and the lack of obvious peritoneal dissemination. However, peritoneal dissemination was found after open exploration, and further surgery was canceled. For all these patients, the results of the RT–LAMP reaction were positive. As these results indicated, even when cancer cells are not found by cytology, the RT–LAMP method could be used to determine the necessity of surgery. Our results demonstrated significant correlations between the CK19 findings and depth of cancer invasion, the presence and extent of lymph node metastasis, and the vessel and lymphatic invasion. In this sense, CK19 expressed by free cancer cells in peritoneal lavage could be a candidate molecular marker indicating high invasive potential and aggressive behavior in gastric cancers. It is remarkable that the results of the RT–LAMP method correlated not only with the peritoneal recurrences but also with other fashions without tumor exposure to the serosa. Minor diffusion of tumor cells might occur during the migration through the lymphatic vessels [29–31], and further evaluation would be needed around the origin.

The benefit of adjuvant chemotherapies in solid tumors is known to be related to the amount of remnant tumor burden. Therefore, patients with positive RT–LAMP without macroscopic peritoneal dissemination seem good candidates for a cure through appropriate adjuvant therapy. Kodera *et al.* [32] reported that postoperative S-1 monotherapy could make no difference in survival between patients with visible peritoneal deposits and patients with only positive cytology. To improve the prognosis, it is crucial to identify high-risk patients at a much earlier phase of peritoneal dissemination. As molecular approaches such as RT–LAMP and RT–PCR analysis have the potential to do just that, adjuvant therapy could eliminate remnant cancer cells detected only by molecular diagnosis. Limitations include a small sample size.

The RT–LAMP technique could be performed as an alternative to an intraoperative cytologic examination. Because of its high sensitivity and rapidity, this method could provide an opportunity to perform a reliable tailor-made surgery for gastric cancers as a common procedure in general hospitals. However, the present study has some limitations. First, this study included a too small sample size to assess whether RT–LAMP positivity could be independent prognostic biomarker in cytology-negative patients. Second, this molecular-based method is known to be concerned with a high false-positive rate. Kodera *et al.* [33] reported that when the cutoff value was set at 0.1 for CEA mRNA, the false-positive rate of CEA RT–PCR exceeded 10%. To confirm the efficacy of the RT–LAMP method, additional efforts are necessary to assess the outcome of the treatment of patients with RT–LAMP-positive peritoneal lavage findings in larger sample size.

5. Conclusion

Our findings suggest that CK19 RT–LAMP would be useful as an intraoperative diagnostic modality to detect potential high-risk patients who may develop recurrence even after clinically curative surgery and guide choices about proper adjuvant therapy.

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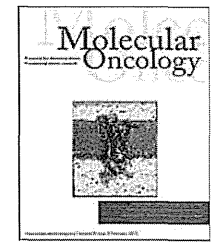

Enhanced expression of retinoic acid receptor alpha (*RARA*) induces epithelial-to-mesenchymal transition and disruption of mammary acinar structures

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ARTICLE INFO

Article history:
Received 29 May 2014
Received in revised form
15 September 2014
Accepted 15 September 2014
Available online 22 September 2014

Keywords:
3D culture
Gene amplification
Breast cancer
EMT
ERBB2
RARA

ABSTRACT

The early steps of mammary tumorigenesis include loss of epithelial cell polarity, escape from anoikis, and acquisition of proliferative capacity. The genes responsible for these processes are predicted to be early diagnostic markers or new therapeutic targets. Here we tested 51 genes coamplified with *ERBB2* in the 17q12–21 amplicon for these tumorigenic activities using an MCF10A 3D culture-based screening system. We found that overexpression of retinoic acid receptor α (RARA) disrupted normal acinar structure and induced epithelial-to-mesenchymal transition (EMT). The mRNA levels of known EMT-inducing factors, including *SLUG*, *FOXC2*, *ZEB1*, and *ZEB2*, were significantly increased upon *RARA* overexpression. Knockdown of *ZEB1* suppressed the RARA-mediated EMT phenotype. These results suggest that overexpression of RARA enhances malignant transformation during mammary tumorigenesis.

Abbreviations: Dox, doxycycline; RARα, retinoic acid receptor alpha; ERBB2, v-erbB-2 avian erythroblastic leukemia viral oncogene homolog B2; ZEB, zinc finger E-box-binding homeobox; FOXC2, forkhead box protein C2; RXR, retinoid X receptor; TGFB, transforming growth factor beta; EMT, epithelial-to-mesenchymal transition; DCIS, ductal carcinoma *in situ*; IBC, invasive breast cancer; MOI, multiplicity of infection; TRE, tetracycline responsive element; LBD, ligand-binding domain; RARE, retinoic acid response element; ER, estrogen receptor; ATRA, all-trans retinoic acid; BCSC, breast cancer stem cell; MaSC, mammary stem cell.

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http://dx.doi.org/10.1016/j.molonc.2014.09.005
1574-7891/

1. Introduction

The majority of breast cancers originate from the epithelial cell layers and progress through a continuum of changes to malignancy. Ductal carcinoma *in situ* (DCIS) is an early premalignant stage of breast cancer progression and is recognized as the proliferation of neoplastic epithelial cells within the duct, surrounded by myoepithelial cells and an intact basement membrane. DCIS is the most common type of non-invasive breast cancer in women, yet has the potential to progress towards malignant invasive breast cancer (IBC) and subsequently metastatic cancer (Espina and Liotta, 2011).

Three-dimensional (3D) culture of mammary epithelial cells embedded in Matrigel is an *in vitro* culture system for understanding the biological processes and signaling pathways that lead to the disruption of epithelial architecture at the early stages of mammary tumorigenesis *in vivo* (Debnath and Brugge, 2005; Vargo-Gogola and Rosen, 2007; Yamada and Cukierman, 2007). Matrigel is rich in basement membrane proteins, such as type IV collagen, laminin, and heparan sulfate proteoglycan (Kleinman et al., 1982). When cultured in Matrigel, MCF10A, a spontaneously immortalized but non-transformed human breast epithelial cell line, forms 3D acinar structures characterized by hollow lumens surrounded by polarized and growth-arrested luminal epithelial cells. These structures arise through changes in many biological processes including proliferation, cell polarity, apoptosis, and cell cycle distribution, and resemble mammary acini *in vivo*, which constitute the multiple lobular units at the end of the mammary ducts (Vargo-Gogola and Rosen, 2007; Debnath et al., 2003).

The amplification and overexpression of *ERBB2* in breast cancer is correlated with poor prognosis due to an increased rate of metastasis and chemotherapy resistance. In general, this gene is overexpressed in 50–60% of DCIS (Lu et al., 2009). Muthuswamy et al. (2001) showed that activation of ERBB2 in 3D-cultured MCF10A cells resulted in aberrant multi-acinar structures with filled lumens, similar to *ERBB2*-overexpressing DCIS *in vivo*. These cells, however, lacked an invasive phenotype, inconsistent with high metastatic rates of clinical ERBB2-positive breast cancer, suggesting the existence of other cofactors necessary for malignant progression. Previous studies have shown coexpression of genes such as *TGFB*, *RHOG*, and *FOS* with *ERBB2* induced invasive behaviors in the MCF10A 3D culture system (Seton-Rogers et al., 2004; Witt et al., 2006). However, the clinical significance of these observations remains to be evaluated.

ERBB2 gene is not amplified alone, but is coamplified together with adjacent genes on the same chromosomal segment (Bieche et al., 1996; Kauraniemi et al., 2001). Given that breast tumors with gene amplification on the distal side of the chromosome 17q12–21 containing *KRT20* and *KRT19* are more aggressive than tumors with amplification restricted to the small region adjacent to ERBB2 (Lamy et al., 2011; Jacot et al., 2013), it is likely that the other genes localized in the same amplicon as *ERBB2* play a significant role in breast cancer progression.

Here, we established an MCF10A 3D culture-based screening system to identify genes that disrupt acini formation with or without oncogenic *ERBB2 (ERBB2VE)* expression. We found that overexpression of retinoic acid receptor alpha gene (*RARA*) in the 17q12–21 amplicon induces both the collapse of luminal morphology and an invasive phenotype. We also found that *RARA* upregulated EMT-inducing transcription factors such as *SLUG*, *FOXC2*, *ZEB1*, and *ZEB2*, and TGF-β–SMAD signaling-activating factors including TGFBR1, TGFBR2, TGFB2, and SMAD3. Of these genes, ZEB1 in particular was identified as an essential gene in *RARA*-induced EMT.

Our studies propose a model in which overexpression of *RARA* resulting from gene amplification contributes to the progression of mammary epithelial cell transformation towards more malignant invasive phenotypes through stimulation of the EMT interactome.

2. Materials and methods

2.1. Cell culture

MCF10A cells were purchased from the American Type Culture Collection (ATCC). Cells were maintained in growth medium consisting of DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Invitrogen), 20 ng/mL human epidermal growth factor (EGF) (BD), 0.5 μg/mL hydrocortisone (Sigma), 100 ng/mL cholera toxin (List Biological Laboratories), 10 μg/mL insulin (Sigma), 100 μg/mL streptomycin (Meiji-Seika), and 100 U/mL penicillin (Meiji-Seika).

2.2. Generation of MCF10A cell clones

A DNA fragment of rtTA-Advanced (Clontech) was ligated into an EF1α promoter-driven plasmid followed by the insertion of IRES-neo. MCF10A cells were transfected with the resultant plasmid pEF-rtTA-Advanced-IRES-neo by calcium phosphate transfection and selected with 800 μg/mL G418 (MCF10A/Tet-on). For generating clones inducibly-expressing $ERBB2^{V659E}$, the MCF10A/Tet-on clones were cotransfected with two vectors, a puromycin-resistant gene expression vector and a Tet-responsive (Clontech) *ERBB2VE* expression vector (pTRE-Tight-*ERBB2VE*) (1:25 ratio), and selected with 0.5 μg/mL puromycin (MCF10A/Tet-on/TRE-*ERBB2VE*). The MCF10A/Tet-on and MCF10A/Tet-on/TRE-*ERBB2VE* clones were then transfected with a vector encoding a murine ecotropic retroviral receptor (pLenti6/Ubc/mSlc7a1, Addgene) and selected with 15 μg/mL blasticidin (MCF10A/Tet-on/Eco and MCF10A/Tet-on/TRE-*ERBB2VE*/Eco, respectively). During serial cloning steps, we selected MCF10A clones with the ability to form hollow acini-like structures as most suitable for 3D culture-based screening.

2.3. Viral infection

The construction of the pMXs retroviral vectors and retroviral packaging was performed as previously described (Saito et al., 2012). Lentiviral packaging was performed by cotransfection of 293T cells with HIV-based vectors including an expression construct and the pPACK packaging plasmids (System

Biosciences). MCF10A cells were seeded at a density of 2.0×10^5 cells in each well of a 12-well plate, followed by infection with a 1:1 mixture of diluted viruses and growth medium the next day. After 24 h for retroviruses, or 16–20 h for lentiviruses, the viral supernatant was replaced with growth medium for an additional 24 h period following which the cells were moved to new dishes.

2.4. *Three-dimensional morphogenesis assay*

Forty microliters of Matrigel (growth factor reduced, BD) was added to each well of 48-well plates and then the plates were incubated at 37 °C to allow solidification of the Matrigel. MCF10A cells were trypsinized and resuspended in assay medium (DMEM/F12 supplemented with 2% horse serum, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin, 100 μg/mL streptomycin, and 100 U/mL penicillin). Five thousand cells were seeded onto the surface of the solidified Matrigel in a mixture of 400 μL of assay medium containing 5 ng/mL EGF and 2% Matrigel. On day 4, the assay medium supplemented with 5 ng/mL EGF and 2% Matrigel was replaced, and from day 7 the liquid medium was replaced with fresh assay medium containing 1 ng/mL EGF and 2% Matrigel every 4 days. For assays with type I collagen, Matrigel was mixed with a bovine collagen solution (PureCol, Advanced BioMatrix) at a final concentration of 50% Matrigel and 1.2 mg/mL collagen I, and 50 μl of the mixture was used to coat 48-well plates. Before mixing, the collagen I-PureCol was neutralized by addition of 10 × PBS and 100 mM NaOH (10×), and the pH was adjusted to 7.2–7.6. Cells were seeded on the underlayer using the same procedure described above.

2.5. *Antibodies*

For immunoblotting, the following antibodies were used. α-Tubulin (DM1A), Sigma–Aldrich (T9026); FLAG M2, Sigma; Neu (ERBB2), Santa Cruz (sc-284); E-cadherin, BD (610182); N-cadherin, BD (610921); Vimentin (D21H3), Cell Signaling (#5741S); RARα, Cell Signaling (#2554S); Akt1 (2H10), Cell Signaling (#2967); phospho-Akt (Ser473), Cell Signaling (#9271); Smad2, Cell Signaling (#5339S); phospho-Smad2, Cell Signaling (#3108S); HRP-linked sheep anti-mouse IgG, GE healthcare (NA931); HRP-linked donkey anti-rabbit IgG, GE healthcare (NA934).

2.6. *Dual-luciferase reporter assay*

MCF10A cells were co-transfected with pRL-SV40 (Promega), 2 × DR5-tk/pGL3, which has two retinoic acid response elements, 2 × DR5 (kindly provided by Dr. Shigeaki Kato), and pMXs-RARA or pMXs-*rara*Δ*408-416* by calcium phosphate transfection; total DNA was equalized by the addition of empty vector. The day after transfection, extracts were harvested and assayed with the dual-luciferase reporter kit (Promega).

2.7. *Quantitative real-time PCR*

Total RNA was extracted using Chomczynski's method (Chomczynski and Sacchi, 1987) or ISOGEN (NIPPON GENE) and incubated with reverse transcriptase SuperScript III (Invitrogen) using random hexamers to obtain cDNA. Real-time PCR was performed by the StepOnePlus real-time PCR system (Applied Biosystems) using Taqman gene expression assays (Applied Biosystems) and Taqman gene expression master mix (Applied Biosystems). Quantification of relative mRNA expression levels was normalized to 18S ribosomal RNA. The following Taqman gene expression assays were used. *SNAIL*, Hs00195591_m1; *SLUG*, Hs00950344_m1; *FOXC2*, Hs00270951_s1; *ZEB1*, Hs00232783_m1; *ZEB2*, Hs00207691_m1; *TGFBR1*, Hs00610320_m1; *TGFBR2*, Hs00234253_m1; *TGFB1*, Hs00998133_m1; *TGFB2*, Hs00234244_m1; *SMAD3*, Hs00969210_m; *18S*, Hs99999901_s1.

2.8. *Knockdown viral vectors*

H1 promoter-driven *ZEB1* knockdown vectors were constructed from an HIV-based SIN lentiviral vector (System biosciences), into which a puromycin resistance marker was introduced. An H1 promoter-driven *ZEB2* knockdown vector was constructed from the pMXs retroviral vector bearing blasticidin resistance marker. Pairs of oligonucleotide sequences encoding shRNA are listed in Table S1.

3. Results

3.1. *Overexpression of* RARA *gene disrupted hollow lumen structures of 3D-cultured MCF10A mammary epithelial cells*

To identify genes cooperating with *ERBB2* in the early stages of breast cancer tumorigenesis under microenvironments mimicking that of the mammary gland, we first established an MCF10A clone suitable for 3D culture screening. To evaluate the capability of the clone to induce disruption of 3D structures, and to enable screening of genes cooperating with *ERBB2*, we introduced an oncogenic *ERBB2* expression unit under the control of a tetracycline-responsive DNA element (MCF10A/Tet-on/TRE-ERBB2VE). We used a full-length *ERBB2* mutant, $ERBB2^{V659E}$, which forms homo- and heterodimers with other ERBB receptor family members and whose tyrosine kinase activity is constitutively activated (Yarden and Sliwkowski, 2001; Baselga and Swain, 2009). During the serial cloning steps, we chose the MCF10A clones most suitable for screening. When embedded in Matrigel, the MCF10A clone was required to show normal development of acinus structures without *ERBB2VE* induction (Figure S1B, Dox.(–) panel). Normal acinus had well-ordered spheroid structures and its inner area seemed to have a lower cell density. Then, the induction of *ERBB2VE* (Figure S1B, Dox.(+) panel) had to induce clear phenotypic changes including distorted luminal structures with high density of inner cells, "multi-acinar" morphology. To efficiently introduce target genes into human cells, we established an MCF10A clone expressing an ecotropic retroviral receptor (mSlc7a1) (MCF10A/Tet-on/TRE-ERBB2VE/Eco, Figure S1A).

We selected the 52 genes (51 genes plus wild type *ERBB2*) in the 17q12–21 amplicon and divided them into 10 subgroups consisting of five to six genes (Table S2). Each subgroup was introduced into the MCF10A/Tet-on/TRE-ERBB2VE/Eco cells

and cultured with or without doxycycline to examine the morphological changes induced by genes cooperating with *ERBB2VE* (Figure 1A, lower panels) or acting alone (upper panels) in the same cells, respectively. Albeit in the absence of *ERBB2VE* (Dox(–)), subgroup #3 showed remarkable filled luminal structures with larger spherical bodies (approximately 200 μm in diameter) compared with the mock control (less than approximately 120 μm) and #10 showed aggregation (Figure 1B, S2). Subgroup #6 enhanced the aggregation by day 15 when *ERBB2VE* expression was induced at day 4 (Figure 1B, S2). We subsequently tested each of the 16 individual genes from these three subgroups, and found that the *RARA* gene from subgroup #3 caused similar aberrant structures in both the absence and presence of *ERBB2VE* (Figure 1C), as seen in the subgroup #3-infected cells. We did not detect any remarkable transforming activity in candidate genes included in both subgroup #6 and #10 using the method of evaluating the function of those alone (Supplementary file 2).

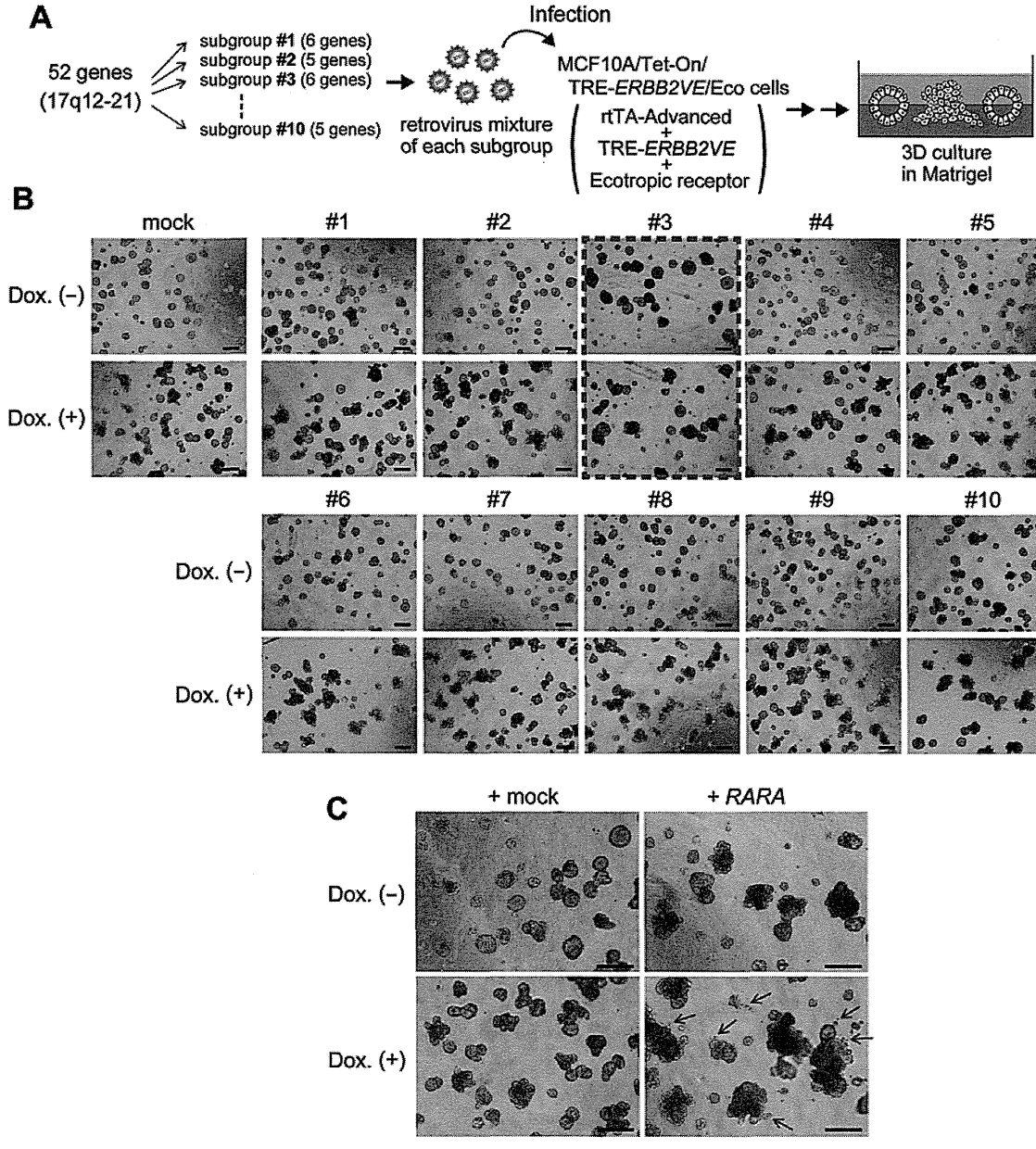

Figure 1 – MCF10A 3D culture-based screening for genes coamplified with *ERBB2* and identification of *RARA* as a gene inducing filled-lumen structures. (A) Overview of the screening system. Fifty-two genes on the 17q12–21 amplicon including wild type *ERBB2* were selected as screening targets. We divided full-length cDNAs corresponding to those genes into ten subgroups and introduced them into MCF10A/Tet-on/TRE-*ERBB2VE*/Eco cells using retroviruses. The cells were embedded in Matrigel and allowed to proliferate. (B) Selection of transformed gene subgroups using a 3D culture system. Cells were infected with retrovirus mixture for each subgroup, embedded in Matrigel and cultured for 15 days. In the bottom panels, *ERBB2VE* was induced with Dox at day 4. Scale bars represent 200 μm. Magnified pictures are provided in Figure S2. (C) Activity of *RARA* in both *ERBB2VE* (–) and (+) backgrounds. MCF10A/Tet-on/TRE-*ERBB2VE*/Eco cells were transfected with mock (left panels) or *RARA* (right panels) retroviruses and were cultured on Matrigel for 14 days. In the right panels, *ERBB2VE* was induced by the addition of Dox at day 4. Arrows indicate raptured structures. Scale bars represent 200 μm.

3.2. *Overexpression of* RARA *induced invasive transformation in Matrigel-containing collagen I cultures*

Coexpression of *RARA* and *ERBB2VE* led to the formation of larger cell clusters with a slightly protrusive outgrowth (Figure 1C), raising the possibility that RARA conferred migratory or invasive activities to MCF10A cells. To test this hypothesis, we cultured RARA-expressing cells in collagen I-containing Matrigel, as previously performed, to assess the invasiveness of MCF10A cells (Seton-Rogers et al., 2004). Intriguingly, we noticed that the MCF10A cells overexpressing RARA displayed a lattice-like network of invasive projections in the Matrigel-collagen I mixture, while those expressing ERBB2VE alone did not (Figure 2A). This response of RARA-expressing cells was dependent on the multiplicity of infection (MOI) of retroviruses, whereas control virus did not induce such response (Figure 2B and C). In addition, coexpression of both RARA and ERBB2VE resulted in thicker cords containing multiple acini-like structures (Figure 2A). These results suggest that RARA is a potent invasion-associated gene that is qualitatively different from ERRBB2VE, which is an inducer of acinar expansion without invasion into the surrounding matrix.

RARα, which is encoded by RARA, is a nuclear receptor for retinoic acid and functions as a transcriptional regulator for genes involving multicellular development, differentiation, and apoptosis. In the absence of ligand or in the presence of antagonists, target genes are silenced due to the binding of co-repressors (CoRs) to RARα. Binding of RARα agonists to the ligand binding pocket induces allosteric changes in the ligand binding domain (LBD), leading to the movement of the C-terminal helix H12, which then generates a novel interaction surface for coactivators (CoAs) (Bastien and Rochette-Egly, 2004; Altucci and Gronemeyer, 2001). In a previous study, it was demonstrated that deletion of H12 increases CoR-binding and results in the repression of gene expression (Farboud et al., 2003). To examine the role of the transcriptional activity of RARα for the invasive protrusion phenotype, we constructed a RARα deletion mutant (RARαΔ408–416) lacking the H12 motif. In a retinoic acid response element (RARE)-Luc reporter assay, the wild type RARα enhanced luciferase activity in a dose-dependent manner whereas the RARαΔ408–416 mutant rather suppressed it (Figure S3A). When we introduced retrovirus for the mutant at the same MOI as the wild type, MCF10A cells expressing RARαΔ408–416 did not form protrusions in the Matrigel and collagen I culture (Figure S3B). Thus, it is likely that the transcriptional activation by RARα, not the transcriptional repression in cooperation with CoR, is required for the invasive phenotype in cells. The dominant negative activity of RARαΔ408–416 likely resulted from an antagonistic role against intrinsically activated endogenous RAR proteins, which is insufficient to cause transformation.

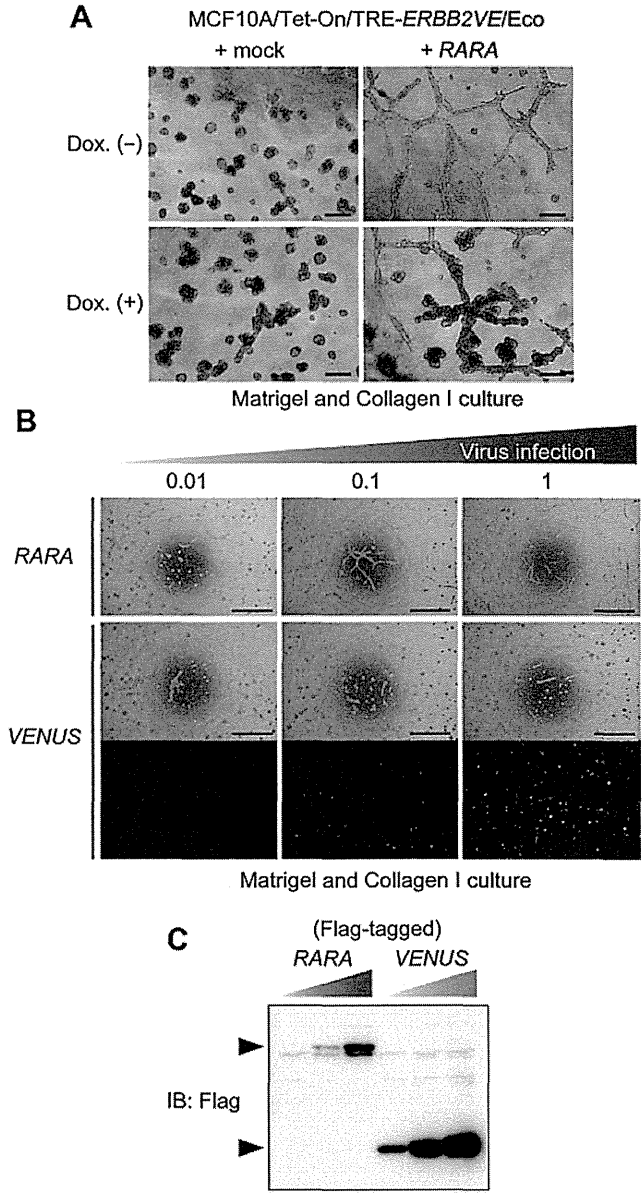

Figure 2 – *RARA*-induced invasive transformation in Matrigel containing collagen I in a manner dependent on its expression levels. (A) Identification of an invasive phenotype induced by *RARA* in Matrigel and collagen I culture. MCF10A/Tet-on/TRE-*ERBB2VE*/Eco cells overexpressing *RARA* were cultured in a 1:1 mixture of Matrigel and collagen I for 11 days. *ERBB2VE* was induced by the addition of Dox at day 4. Scale bars represent 200 μm. (B) Detection of dose-dependent *RARA* activity. MCF10A/Tet-on/TRE-*ERBB2VE*/Eco cells were infected with retroviruses containing *RARA* or *Venus* with Flag epitope tag at the indicated MOIs. Cells were grown in a 1:2 mixture of Matrigel and Collagen I for 4 days in the absence of Dox. Scale bars represent 1 mm. (C) MOIs-related expression levels. Protein levels in the cells used in (B) were analyzed by immunoblotting using the antibody against Flag. The upper band indicates the expression of RARα, and the lower band indicates that of Venus.

3.3. RARA *induced EMT-related gene signatures*

In 2D cultures, we observed that MCF10A cells overexpressing RARA displayed a fibroblast-like morphology, whereas control mock-infected cells grew in well-ordered epithelial clusters (Figure 3A), suggesting that RARA acts as an EMT-inducing factor. To address this hypothesis, we examined the expression of epithelial and mesenchymal markers by immunoblotting. In MCF10A cells expressing RARA, the epithelial marker E-cadherin was downregulated, whereas mesenchymal

markers N-cadherin and vimentin were upregulated (Figure 3B). These changes in E-cadherin and N-cadherin expression were totally abrogated by the mutant *rara*Δ408–416 (Figure S3C), suggesting that *RARA* induces EMT in MCF10A cells via RARα-mediated transcriptional activation. In contrast, *ERBB2VE* did not affect the epithelial morphology in monolayer cultures nor the expression of EMT markers (Figure 3B). N-cadherin upregulation by *RARA* was also seen in MDA-MB-361 breast cancer cells (Figure S3D).

To examine whether the expression levels of EMT-related genes were regulated by *RARA*, we performed real-time PCR analysis. *RARA*-overexpressing MCF10A cells exhibited approximately 4-, 2.5-, and 12-fold increases at day 4 in the mRNA levels of the *SLUG*, *FOXC2*, and *ZEB* gene families, respectively, compared with control MCF10A cells. Also, the mRNA levels of *TGFBR1*, *TGFBR2*, *TGFB2*, and *SMAD3* increased by 4-, 2.5-, 7-, and 2.5-fold, respectively, at day 4 in *RARA*-overexpressing MCF10A cells (Figure 3C). Time-course experiments revealed that changes in E-cadherin and N-cadherin occurred after 24 h of *RARA* induction (Figure S4), suggesting that transcriptional cascades involving EMT-inducing factors are activated during this time period. These results suggest that RARα activates the EMT signaling program before inducing a protrusive behavior in 3D cultures with collagen I.

Because *RARA* induced upregulation of key factors involved in the TGF-β/SMAD signaling pathway (Figure 3C), we hypothesized that *RARA* activated this pathway and then induced invasion in 3D cultures as well as EMT. Therefore, we examined whether the inhibition of the TGF-β/SMAD pathway suppressed phenotypic changes in *RARA*-expressing MCF10A cells. Optimal concentration of an inhibitor of the TGF-β receptor type I, SB-431542 (Figure S5A), partially suppressed changes in the expression levels of EMT markers (Figure S5B) and protrusion (Figure S5C). These results suggest that the TGF-β signaling pathway is partially responsible for EMT and invasion caused by *RARA*. We subsequently examined whether the activation of the TGF-β pathway was sufficient to induce such changes. Stimulation of parental MCF10A cells with TGF-β1 led to both slight downregulation of E-cadherin and upregulation of N-cadherin (Figure S5D). Also, modest effects on the phenotype were observed in 3D cultures using Matrigel and collagen I (Figure S5E), consistent with the previous result showing that the expression of TGF-β1 without the activation of ERBB2 hardly displayed any invasive activity (Seton-Rogers et al., 2004). These results suggest

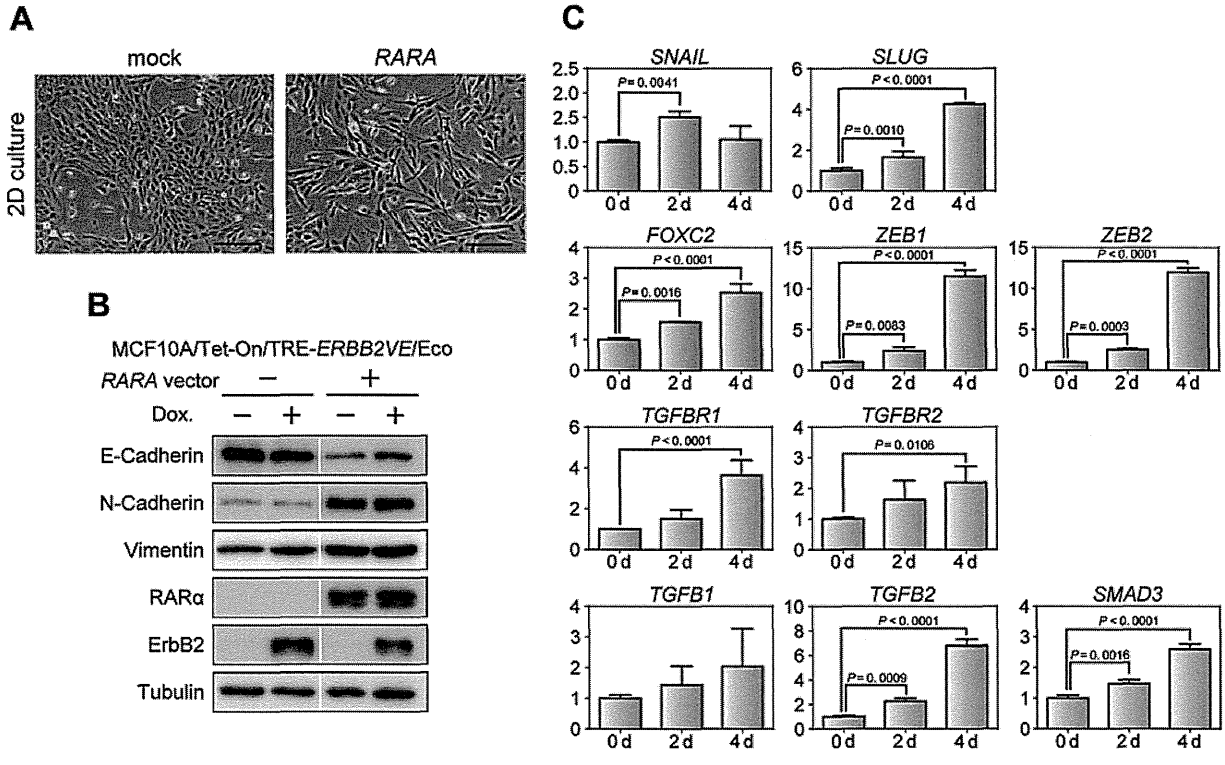

Figure 3 – Induction of EMT-like morphological changes and upregulation of EMT-related genes by *RARA* overexpression. (A) Mesenchymal-like transition induced by *RARA*. MCF10A/Tet-on/TRE-*ERBB2VE*/Eco cells overexpressing the indicated genes exhibited different morphologies in 2D culture. Scale bars represent 200 μm. (B) Expression analyses for EMT markers. The expression levels of the indicated proteins were analyzed by immunoblotting. Tubulin was used as a loading control. To evaluate effects of *ERBB2VE*, Dox-treated samples for 2 days (Dox (+) lanes) were simultaneously evaluated, but revealed to be negligible compared with the effects by *RARA*. White line, trimmed margin of unrelated sample lanes. (C) Quantitative analyses of mRNA changes induced by *RARA*. The mRNA expression levels of the indicated genes were measured by real-time PCR. MCF10A/Tet-on cells were infected with lentiviruses for TRE-*RARA* (MCF10A/Tet-on/TRE-*RARA*), and RNA was extracted at the indicated days after Dox addition. Real-time PCR values were normalized to the internal control, 18S ribosomal RNA. The *y*-axis depicts the fold-change in each normalized mRNA compared with Dox (−) cells (0 d). The represented data are shown as mean ± s.d.. Dunnett's multiple comparisons test were performed to assess statistical significance.

that TGF-β1 alone is not sufficient to induce an invasive phenotype in MCF10A cells.

3.4. *ZEB1 knockdown in RARA-expressing MCF10A cells inhibited EMT and protrusion*

To further investigate the mechanism of EMT induced by *RARA*, we established MCF10A cells that express *RARA* under the control of a tetracycline-responsive DNA element. We examined the effects of silencing of *ZEB1* and *ZEB2* using short hairpin RNAs (shRNAs) in the cells because these genes exhibited the most marked increase in expression levels among the known key EMT-inducing transcription factors (Figure 3C). We obtained two shRNAs (Figure 4A) and one shRNA (Figure 4B) that efficiently suppressed *ZEB1* and *ZEB2* mRNAs induced by *RARA*, respectively. Western blot analysis of EMT markers revealed that reduction of *ZEB1* inhibited *RARA*-induced EMT, whereas that of *ZEB2* did not (Figure 4C). Furthermore, we observed *ZEB1* knockdown inhibited the protrusive phenotype in Matrigel and collagen I cultures caused by *RARA* (Figure 4D). These results indicate that *ZEB1* is required for the *RARA*-mediated EMT and invasive phenotype.

4. Discussion

ERBB2 amplification is a so-called "driver" mutation (Stratton et al., 2009) that confers growth advantages on cancer cells. However, accumulating evidence suggests that breast cancer development probably results from the involvement of some other genes coamplified with *ERBB2* in the 17q12–21 amplicon, not through *ERBB2* acting alone. For example, previous studies identified that *STARD3* and *C35* localized within the amplicon expressed with *ERBB2*, and contributed to upregulated proliferation, decreased cell death, cell cycle progression (Kao and Pollack, 2006), and malignancy of breast cancer cells (Katz et al., 2010). We recently reported that *GRB7*, which is also located in the 17q12–21 amplicon, transforms NIH3T3 cells due to enhancement of ERBB2-mediated signaling pathways (Saito et al., 2012). In this report, we examined the genes in the amplicon for their ability to transform 3D-cultured MCF10A, and identified *RARA*, which disrupted acinar structures (Figure 1C) and induced EMT (Figure 3A and B). In contrast to the synergetic transforming activity of *ERBB2* and *GRB7* (Saito et al., 2012), *RARA* independently induced an invasive phenotype (Figure 2A). These studies indicate that the 17q12–21 amplicon harbors a surprising number of genes that play functional roles in the development and progression of breast cancers.

RARA amplification in parallel with *ERBB2* in breast tumors was first reported by Keith et al. (1993) and is observed in 26.7% of *ERBB2*-amplified breast cancer patients. Such simultaneous amplification is statistically associated with malignant lesions, such as lymph node invasion (Lamy et al., 2011), suggesting that *RARA* could have the potential to efficiently promote progression to malignancy. Furthermore, Peng et al. (2004) described that RARα expression was enhanced in premalignant MCF10AT and malignant MCF10CA1a cells at the protein level, and suggested the association between upregulation of RARα and malignant transformation of MCF10A cells.

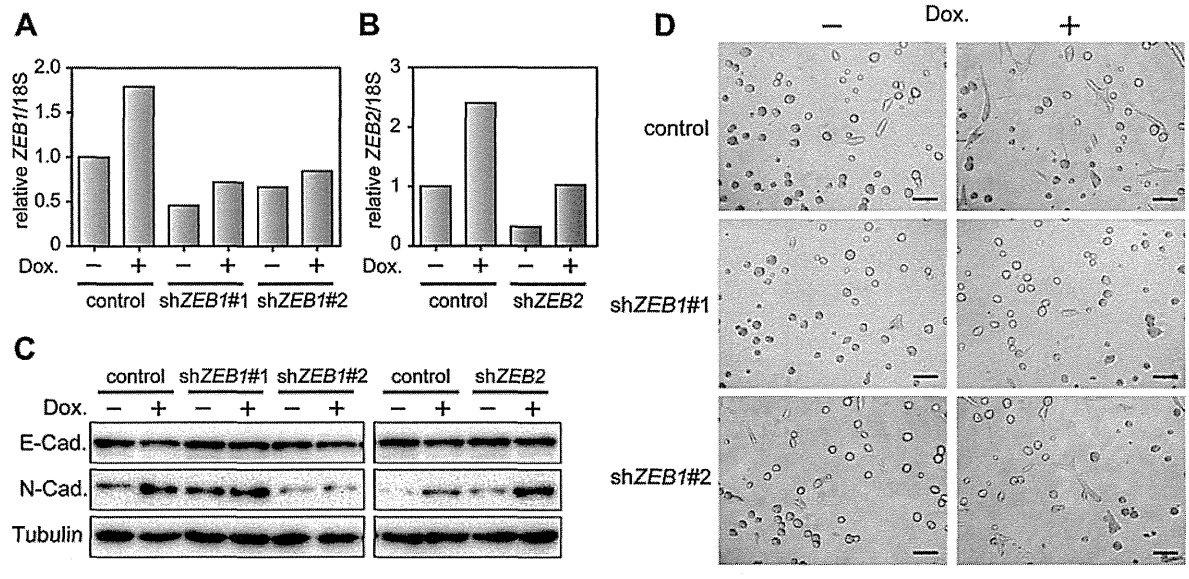

Figure 4 – Essential roles of ZEB1 in *RARA*-induced invasive phenotype. (A, B) Assessment of knockdown effects of shRNAs targeting *ZEB1* and *ZEB2*. MCF10A/Tet-on/TRE-*RARA* cells infected with indicated shRNA viral vectors were cultured in the absence or presence of Dox for 3 days. *ZEB1* and *ZEB2* mRNA was analyzed by real-time PCR analysis. The data were normalized by the amount of 18S rRNA and shown as a relative value compared with the control shRNA-infected cells without Dox. Representative results of one of two independent experiments are shown. (C) Inhibition of *RARA*-induced typical EMT marker transition by *ZEB1* knockdown. Protein extracts from the *ZEB1*- or *ZEB2*-knockdown cells were analyzed by immunoblotting. These cells were prepared as shown in (A) and (B), and cultured in Dox for 4 days with one passage. Tubulin was used as a loading control. (D) Inhibition of an *RARA*-induced protrusive phenotype in Matrigel and collagen I cultures by *ZEB1* knockdown. The *ZEB1*-knockdown cells were prepared as shown in (A), and cultured in Dox for 2 days in 2D cultures and for 4 additional days in 3D cultures. Invasive protrusions of these cells in Matrigel and collagen I cultures were observed on day 4.

Our results that *RARA* overexpression contributes to the phenotypic change of non-malignant cells to invasiveness support these hypotheses for the first time. We propose that *RARA* expression levels could be an important indicator to evaluate breast cancer malignancy.

Classically, RAR family members form heterodimers with retinoid X receptor (RXR) family members. Their ligand, retinoic acid (RA), activates these heterodimers, consequently leading to differentiation, apoptosis, and cell cycle arrest through transcriptional activation (Bastien and Rochette-Egly, 2004; Altucci and Gronemeyer, 2001). Consistent with these observations, a recent study revealed that $ERBB2^{+}$/$RARA^{+}$ breast cancer cells undergo apoptosis by the combination of a specific ERBB2 inhibitor, trastuzumab and a pan-RAR ligand, all-trans retinoic acid (ATRA) (Paroni et al., 2012). Here we demonstrated that overexpression of RARα accompanied by transcriptional activation induced large acinar structures presumably filled with surviving inner cells (Figure 1C and S3A). These results are apparently opposed to the effect of ATRA. It is known that ATRA differentially regulates all α, β and γ members of RARs. A previous report has shown that ATRA suppresses RARα expression in premalignant MCF10AT and malignant MCF10CA1a cells, while it suppresses RARγ expression only in MCF10CA1a cells (Peng et al., 2004). Also, it is known that distinct RXR-RAR isotype combinations lead to different expression patterns of RA-responsive genes (Chiba et al., 1997). Thus, we assume that the individual role of each RAR subtype is different and that phenotypes of breast cancer cells caused by treatment with ATRA represent effects on not only RARα but also RARβ and γ. Further analyses are needed to contribute to the understanding of the whole regulatory role of RARs.

In addition to typical heterodimer formation, another mechanism of RARα-induced cell-fate decisions could be considered. Previous studies implicated that RARα is an essential component of the estrogen receptor (ER) transcription complex and regulates the expression of estrogen-target genes, leading to cell proliferation in ER-positive cell lines (Ross-Innes et al., 2010; Toma et al., 1998). Activation of the classical RARα pathways could deplete RARα from the ER complex (Ross-Innes et al., 2010). Thus, any shift between the classical and non-classical RARα-pathways is assumed to influence cell fate. Previous evidence showed that intracellular lipid binding proteins, CRABP-II and FABP5, delivered RA from the cytosol to each particular nuclear receptor RARα and PPARβ/γ, respectively (Schug et al., 2007). It can be considered that dominant expression of FABP5 abolishes CRABP-II-mediated import of RA to RARα-RXR heterodimers, activating the non-classical pathway, while that of CRABP-II stimulates the classical pathway. Thus, the relative expression levels between those intracellular lipid binding proteins might be important to determine which classical or non-classical pathway RARα preferentially contributes to. Taken together, whether RARα promotes or suppresses tumor progression relies on several cellular contexts including the expression levels of ligands, nuclear hormone receptors and intracellular lipid-binding proteins. Efforts to identify RARα interaction targets in MCF10A cells will provide a molecular basis for the positive transforming activity observed in this study.

We observed that overexpression of *RARA* in MCF10A cells induced EMT (Figure 3A and B). The mechanisms inducing EMT are quite complex because a variety of signal transduction cascades such as the TGF-β, Wnt, Notch, EGF, FGF, and HIF pathways are involved (Polyak and Weinberg, 2009; Yang and Weinberg, 2008; Thiery et al., 2009). Which pathway is employed is dependent on the tissue, and even in the same tissue, on the particular cellular context. Our study suggests that MCF10A cells utilize, in part, the TGF-β pathway in the context of *RARA* overexpression (Figure S5). Repression of E-cadherin is an established hallmark of EMT, which is directly or indirectly mediated by EMT-inducing transcription factors including SNAIL, TWIST, SLUG, ZEB1, and ZEB2 (reviewed in Polyak and Weinberg (2009); Yang and Weinberg (2008); Thiery et al. (2009)). There is also extensive crosstalk among the factors that make up the "EMT interactome". In this network, a single EMT-inducing transcription factor affects the expression of other EMT-inducing transcription factors (Taube et al., 2010). It is likely that the EMT-interactome in mammary epithelial MCF10A cell lines is activated within 24 h after induction of *RARA* expression, as shown in Figure S4. During this period, RARα may activate the transcription of several EMT-related genes and subsequently regulate other EMT inducers, coordinately contributing to execution of the EMT program. Our results that ZEB1-depleted cells did not induce EMT phenotypes (Figure 4) strongly suggest that ZEB1 is a key factor for the RARα-activated EMT interactome. We also found that *RARA* overexpression upregulates *TGFB2* (Figure 3C), raising the possibility that RARα is involved in autocrine TGF-β/ZEB/miR-200 signaling (Burk et al., 2008; Gregory et al., 2011).

Accumulating evidence suggests that cells undergoing EMT acquire stem cell-like properties (Mani et al., 2008). RARα may also contribute to these changes. A recent report showed that mammary epithelium obtained from RARα1 knockout mice contained a greater percentage of progenitors but fewer mammary stem cells (MaSCs) compared with wild type, suggesting that RARα plays an important role in the maintenance of MaSCs, and affects the mammary epithelial hierarchy (Cohn et al., 2010). Furthermore, another report has shown that ZEB1 is required for the initial acquisition of both $CD44^{hi}$ expression, which is a marker of breast cancer stem cells (BCSCs), and the stem-like activity of $CD44^{hi}$ cells (Chaffer et al., 2013). In pancreatic cancer cells, ZEB1 maintains stem-like properties via downregulation of miR-183, 200c, and 203, which suppress the expression of stem cell factors such as BMI1, SOX2, and KLF4 (Wellner et al., 2009). Future experiments are required to investigate the potential role of the RARα–ZEB1 axis as a promoting factor of MaSCs and BCSCs.

5. Conclusion

We established an MCF10A 3D culture-based screening system and performed a screen of genes coamplified with *ERBB2* in the 17q12–21 amplicon. As a result, we revealed that *RARA* induced the malformation of acinar structures in Matrigel and facilitated an invasive phenotype in Matrigel/collagen I 3D culture. Furthermore, *RARA* upregulated the

transcription of EMT-related genes including ZEB1, which was a key mediator of EMT in RARA-expressing MCF10A cells. Our results suggest that overexpression of RARA is a potentially important factor for breast cancer malignancy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We would like to acknowledge the secretarial assistance of Kumiko Semba. This research was partially supported by Japan Society for the Promotion of Science KAKENHI 23241064 and a grant for Translational Research Program: Achievement of personalized medicine and acceleration of anti-cancer drug development by using gene expression analysis technology from the New Energy and Industrial Technology Development Organization (NEDO).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.09.005.

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Article

TRANSPARENT PROCESS

THE EMBO JOURNAL

MiR-133 promotes cardiac reprogramming by directly repressing Snai1 and silencing fibroblast signatures

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Abstract

Fibroblasts can be directly reprogrammed into cardiomyocyte-like cells (iCMs) by overexpression of cardiac transcription factors or microRNAs. However, induction of functional cardiomyocytes is inefficient, and molecular mechanisms of direct reprogramming remain undefined. Here, we demonstrate that addition of miR-133a (miR-133) to Gata4, Mef2c, and Tbx5 (GMT) or GMT plus Mesp1 and Myocd improved cardiac reprogramming from mouse or human fibroblasts by directly repressing Snai1, a master regulator of epithelial-to-mesenchymal transition. MiR-133 overexpression with GMT generated sevenfold more beating iCMs from mouse embryonic fibroblasts and shortened the duration to induce beating cells from 30 to 10 days, compared to GMT alone. Snai1 knockdown suppressed fibroblast genes, upregulated cardiac gene expression, and induced more contracting iCMs with GMT transduction, recapitulating the effects of miR-133 overexpression. In contrast, overexpression of Snai1 in GMT/miR-133-transduced cells maintained fibroblast signatures and inhibited generation of beating iCMs. MiR-133-mediated Snai1 repression was also critical for cardiac reprogramming in adult mouse and human cardiac fibroblasts. Thus, silencing fibroblast signatures, mediated by miR-133/Snai1, is a key molecular roadblock during cardiac reprogramming.

Keywords cardiomyocyte; microRNA; reprogramming; Snai1; transcription factor

Subject Categories Development & Differentiation; Stem Cells

DOI 10.15252/embj.201387605 | Received 16 December 2013 | Revised 18 April 2014 | Accepted 5 May 2014 | Published online 11 June 2014

The EMBO Journal (2014) 33: 1565–1581

Introduction

Direct reprogramming of mature cells from one lineage to another without passing through a stem cell state has emerged as a new strategy for generating cell types of interest and may hold great potential for regenerative medicine. Thus far, neurons, cardiomyocytes, hepatocytes, blood precursor cells, and neural progenitors were successfully induced from fibroblasts by overexpression of lineage-specific transcription factor (Ieda *et al*, 2010; Szabo *et al*, 2010; Vierbuchen *et al*, 2010; Sekiya & Suzuki, 2011; Han *et al*, 2012; Wada *et al*, 2013). Suppression of the starting-cell signature is a recognized characteristic of cell fate conversion, although the molecular mechanisms underlying this process and its importance during direct reprogramming remain poorly understood (Marro *et al*, 2011; Muraoka & Ieda, 2014).

It was reported that induced cardiomyocyte-like cells (iCMs) can be directly generated from mouse fibroblasts by the combination of transcription factors, Gata4, Mef2c, and Tbx5 (GMT), GMT plus Hand2 (GHMT), or Mef2c, Myocd, and Tbx5 *in vitro* (Ieda *et al*, 2010; Protze *et al*, 2012; Song *et al*, 2012). Recently, we and others reported that iCMs can be directly generated from human fibroblasts by overexpression of GMT plus Mesp1 and Myocd (GMTMM) or other combinations of reprogramming factors (Fu *et al*, 2013; Nam *et al*, 2013; Wada *et al*, 2013). However, induction of functional cardiomyocytes *in vitro* was inefficient and slow, possibly hindering our investigations of the molecular events during cardiac reprogramming (Chen *et al*, 2012; Srivastava & Ieda, 2012; Addis & Epstein, 2013). We and others also showed that endogenous mouse cardiac fibroblasts (CFs) can be converted into iCMs *in vivo* by gene transfer of GMT or GHMT (Inagawa *et al*, 2012; Qian *et al*, 2012;

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