Biochemical and Biophysical Research Communications 447 (2014) 531–536

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Systemic identification of estrogen-regulated genes in breast cancer cells through cap analysis of gene expression mapping

Ryonosuke Yamaga [a,b,c], Kazuhiro Ikeda [a], Joost Boele [d,g], Kuniko Horie-Inoue [a], Ken-ichi Takayama [b,c], Tomohiko Urano [b,c], Kaoru Kaida [e,g], Piero Carninci [e,g], Jun Kawai [f,g], Yoshihide Hayashizaki [f,g], Yasuyoshi Ouchi [b], Michiel de Hoon [e,g], Satoshi Inoue [a,b,c,*]

[a] Division of Gene Regulation and Signal Transduction, Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan
[b] Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[c] Department of Anti-Aging Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[d] Department of Systems Bioinformatics, Amsterdam Institute for Molecules, Medicines and Systems, VU University Amsterdam, Amsterdam, the Netherlands
[e] RIKEN Center for Life Science Technologies, Division of Genomic Technologies, Yokohama, Kanagawa, Japan
[f] RIKEN Preventive Medicine and Diagnosis Innovation Program, Wako, Saitama, Japan
[g] RIKEN Omics Science Center, Yokohama, Kanagawa, Japan[1]

ARTICLE INFO

Article history:
Received 23 March 2014
Available online 16 April 2014

Keywords:
Estrogen
Breast cancer
Cap analysis of gene expression
Transcriptome

ABSTRACT

To explore the estrogen-regulated genes genome-widely in breast cancer, cap analysis of gene expression (CAGE) sequencing was performed in MCF-7 cells under estrogen treatment. Estrogen-regulated expressional changes were found in 1537 CAGE tag clusters (TCs) ($\geqslant$1.5 or $\leqslant$0.66-folds). Among them, 15 TCs were situated in the vicinity of ($\leqslant$10 kb) reported estrogen receptor-binding sites. Knockdown experiments of the 15 TC-associated genes demonstrated that the genes such as RAMP3, ISOC1 and GPRC5C potentially regulate the growth or migration of MCF-7 cells. These results suggest that CAGE sequencing will reveal novel estrogen target genes in breast cancer.

1. Introduction

Breast cancer is the most common cancer among women worldwide [1]. Previous reports have evidenced that estrogen, a steroid hormone, is a key factor for breast cancer progression and the development of normal breast tissues [2]. Estrogen exerts biological effects via its interaction with estrogen-dependent nuclear estrogen receptors (ERs). The most physiologically active form of estrogen, 17β-estradiol (E_2), binds to and activates the ERs, which in turn bind to the estrogen responsive elements (EREs) on the genome and regulate the transcription of target genes that mediate estrogen-induced effects [3]. However, the landscape of estrogen-mediated intracellular signaling has not been fully elucidated, because estrogen regulates many genes, and its signaling pathway is so complicated that it is not easy to detect the really important genes responsible for the progression of breast cancer.

For elucidating the complicated estrogen signaling in breast cancer cells, previous studies were performed targeting specific genes, and various genes important for breast cancer were detected in each case [4,5]. However, it was impossible to survey the whole genome by this approach. For whole-genome study, microarray analysis of human breast cancer cells has been performed and various estrogen-responsive genes have been identified by this method [6,7]. Although microarray analysis has led to the development of genome-wide expression studies, it has limited sensitivity owing to the number of pre-designed probes and it is susceptible to errors in hybridization and reading the signal intensity. Recently, cap analysis of gene expression (CAGE) was developed to map the transcription start sites (TSSs) and their promoters. CAGE employs cap-trapping as the first step to capture the 5′-ends of the cDNAs, which are then transformed into short sequences (tags) of 20 nt, corresponding to the mRNA TSSs [8,9]. In combination with new high-throughput or next generation sequencing technology, CAGE enables a comprehensive genome-wide mapping of TSSs [10].

In this study, we treated human breast cancer MCF-7 cells with estrogen and performed CAGE to investigate novel TSSs important for breast cancer cells. Upon analyzing the expression levels of TSSs after estrogen treatment, we detected novel estrogen-responsive

* Corresponding author at: Department of Geriatric Medicine, Graduate School of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Fax: +81 3 5800 6530.
E-mail address: INOUE-GER@h.u-tokyo.ac.jp (S. Inoue).

[1] RIKEN Omics Science Center ceased to exist as of April 1st, 2013, due to RIKEN reorganization.

http://dx.doi.org/10.1016/j.bbrc.2014.04.033
0006-291X/

genes, which were validated by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). Furthermore, we examined the effects of these genes on growth, migration, and ER-mediated transcription in MCF-7 cells using the small interfering RNA (siRNA) technique. Several novel estrogen-responsive genes were found to be involved in the proliferation or migration of breast cancer cells. Thus, our approach provides new insights into the molecular mechanism of breast cancer development.

2. Materials and methods

2.1. Cell culture and RNA extraction

ERα-positive human breast cancer MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan), supplemented with 10% fetal bovine serum (FBS; Nichirei Bioscience, Tokyo, Japan), in a humidified incubator at 37 °C and under 5% CO_2. Prior to 17β-estradiol (E_2) treatment, cells were starved under hormone-free conditions for 72 h. The hormone-free medium consisted of phenol red-free DMEM (Sigma Aldrich, St. Louis, MO) and 5% dextran-charcoal stripped FBS. After hormone starvation, MCF-7 cells were treated with 100 nM E_2 for 0, 2, 4, 8, 12, and 24 h, and total RNAs were isolated from the cells at each time point using ISOGEN (Nippon Gene, Toyama, Japan) in accordance with the manufacturer's instructions.

2.2. Cap analysis of gene expression (CAGE)

CAGE libraries were generated as previously described [8]. Briefly, the 5′-end of capped RNAs were transcribed to first-strand cDNAs, attached to CAGE "bar code" tags, and digested into 20-nt tags by *Mme*I (New England Biolabs, Ipswich, MA). The CAGE tags were concatenated, ligated to sequencer-specific DNA adaptors, and analyzed with the Illumina GAIIx system (Illumina, CA). The positions of the CAGE tags on the human genome (NCBI, version 36) were determined by using the Vmatch alignment tool (developed by Professor Stefan Kurtz, University of Hamburg, Germany). CAGE tags are grouped into tag clusters (TCs), in which member tags map to the same strand of a chromosome and overlap by at least 1 bp.

2.3. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

The isolated RNA samples were reverse transcribed using SuperScript II reverse transcriptase (Life Technologies, Carlsbad, CA), as recommended by the supplier. qRT-PCR was carried out on a StepOnePlus system (Life Technologies) using FAST SYBR Green Master Mix (Life Technologies) and 150 nM each of gene-specific forward and reverse primers (RAMP3: 5′-GGGAAGGCTTTCGCAGACA-3′ and 5′-CGGACAGGTTGCACCACTT-3′; ISOC1: 5′-CAACAAACTGCCCT GGAGCTA-3′ and 5′-AGGTGGCATCAGCAACAATG-3′; and GPRC5C 5′-GGCAGCGCCTATGTTTCTCT-3′ and 5′-TGAGCGCCTGGGAAGTCT-3′). The cycling conditions were 95 °C for 2 min, followed by 40 cycles at 95 °C for 2 s and 60 °C for 30 s. Evaluation of the relative differences of the PCR product amounts was carried out with the comparative cycle threshold (Ct) method, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control [11]. The experiments were carried out in triplicate.

2.4. Cell growth assay

Cell growth was estimated by the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay using the CellTiter 96 AQueous One Solution Cell Proliferation kit (Promega, Madison, WI). MCF-7 cells were seeded in 96-well plates (1.5×10^3 cell/well) with 200 μL of DMEM containing 10% FBS. After incubation for 24 h, 5 nM siRNA targeting each gene (Life Technologies) or control siRNA (siControl) [12] was added to the medium, using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Then, 10 μL of MTS solution was added to each well at the indicated times (0, 24, 48, 72, and 96 h). The plates were incubated for an additional 2 h at 37 °C, after which the absorbance at 490 nm was recorded using a Multiskan FC microplate photometer (Thermo Fisher Scientific, Waltham, MA). The effects of each siRNA on cell growth were estimated by comparison with the siControl. The experiments were carried out in triplicate.

2.5. Cell migration assay

The cell migration assay was performed using the Cell Culture Insert system with an 8.0-μm-pore-size PET filter (Becton Dickinson, Franklin Lakes, NJ). Prior to the assay, MCF-7 cells were treated with 5 nM siRNA containing Lipofectamine 2000 for 24 h. The lower surface of the filter was immersed for 30 min in 10 μg/mL fibronectin (Sigma Aldrich) diluted in PBS. Next, 700 μL of DMEM containing 10% FBS was added to the lower chamber. Then, 5×10^4 cells were suspended in 300 μL of DMEM containing 10% FBS and added to the upper chamber. After incubation for 48 h at 37 °C, the cells on the upper surface of the filter were completely removed by wiping with cotton swabs. The cells on the lower surface of the filter were fixed in methanol for 30 min, washed with PBS, and then stained with Giemsa's stain solution (Sigma Aldrich) for 30 s. After washing 3 times with PBS, the filters were mounted on a glass slide. The cells on the lower surface were counted from photographs taken of at least 3 fields at a magnification of 200× under the microscope. The effects of each siRNA on migration were estimated by comparison with the siControl. The experiments were carried out in triplicate.

2.6. Luciferase assay

The luciferase assay was performed as described previously, with some modifications [13]. Briefly, 200 ng of ptk-ERE-Luc plasmid and 20 ng of pRL-CMV control plasmid (Promega) were used to measure the transcription activity of ERs. Transient transfections were carried out in MCF-7 cells, using Lipofectamine 2000 with 5 nM siRNAs. After incubation for 24 h with hormone-free medium containing 100 nM E_2 or vehicle, the luciferase activity was assayed as previously described [11]. The experiments were carried out in triplicate.

3. Results

3.1. Identification of estrogen-responsive CAGE TCs in MCF-7 cells

To identify the estrogen-regulated TSSs of genes in breast cancer cells, we performed CAGE of RNAs derived from MCF-7 cells with or without E_2 treatment. Throughout the observation time from 0 to 24 h after hormone stimulation, a total of 34,861 CAGE TC were located in the human genome (NCBI, build 36). Among these, 1537 TCs (4.4%) were defined as being estrogen-responsive, based on the number of tags per million (tpm) determined by sequencing ($\geqslant$1.5 or $\leqslant$0.66-folds change at any time point versus basal control) (Fig. 1A). Of the estrogen-responsive CAGE TCs, 870 (56.6%) were up-regulated, 401 (26.1%) were down-regulated, and 266 (17.3%) were both up- and down-regulated during the time course of study (Fig. 1B).

Based on the CAGE data of 1537 TCs, we selected a subgroup of 15 Refseq genes with estrogen-responsive TCs in the vicinity of their TSSs and nearby (⩽10 kb) estrogen receptor-binding sites that were reported by Carroll et al. (Fig. 1C) [14].

3.2. Functional analysis of estrogen-regulated genes in MCF-7 cells

To investigate the contribution of the 15 selected genes to estrogen signaling in MCF-7 cells, we performed a knockdown study of the genes using specific siRNAs (Table 1). In the experiments, siRNAs targeting ERα (ESR1) and p53 (TP53) were used as references. All of the specific siRNAs for the 15 genes down-regulated the target mRNA levels by >40%, compared with the control siRNA (siControl). In the MTS assay to assess the effects of the siRNAs on the growth of MCF-7 cells, siRNAs targeting isochorismatase domain-containing protein 1 (ISOC1) and voltage-dependent anion channel 2 (VDAC2) significantly reduced cell growth, whereas the siRNA targeting the G-protein-coupled receptor, family C, group 5, member C (GPRC5C) significantly promoted growth. We next performed the transwell migration assay in MCF-7 cells. siRNAs for receptor activity-modifying protein 3 (RAMP3), RAB5B member RAS oncogene family (RAB5B), and VDAC2 substantially repressed cell migration. We also performed the luciferase reporter assay using the ERE-Luc vector in MCF-7 cells, although none of the siRNAs against the 15 genes substantially affected the ERα-mediated transcription.

As described above, these results implied that ISOC1 and VDAC2 stimulate, whereas GPRC5C inhibits, the growth of MCF-7 cells. In addition, RAMP3, VDAC2, and RAB5B appeared to up-regulate the migration of MCF-7 cells. Among these, RAMP3 was reported to

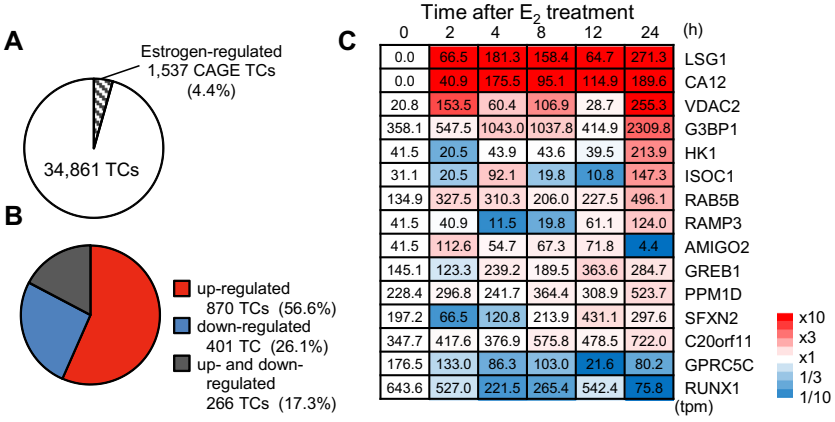

Fig. 1. CAGE transcriptome in breast cancer MCF-7 cells and identification of estrogen-responsive genes based on the data of CAGE and estrogen receptor occupancy. Percentage of estrogen-responsive CAGE tag clusters (TCs) mapped to the human genome (build 36), and expression alteration manners of estrogen-responsive CAGE TCs. (A) Of the total 34,861 CAGE TCs, 1537 were estrogen-dependently altered by >1.5-fold in at least 1 time point after estradiol (E_2) treatment versus basal control. (B) Of the 1537 estrogen-regulated genes, 870 TCs (56.6%) were up-regulated, 401 TCs (26.1%) were down-regulated, and 266 TCs (17.3%) were up- and down-regulated by E_2 treatment during the observation time up to 24 h. (C) Fifteen selected estrogen-regulated genes, including CAGE TCs in their transcription start sites (TSSs), which are situated at <10 kb from the estrogen receptor binding sites (ERBSs) previously reported by Carroll et al. [14]. The number in the chart represents tags per million (tpm) counts at each time point, and the color represents the relative expression level compared with the basal level, as analyzed by CAGE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
siRNA-mediated knockdown study of estrogen-regulated genes, which were identified using CAGE-seq.

siRNA	Target gene	Knockdown efficiency of siRNA[a]	Absorbance at 490 nm [b]	Number of migrated cells[c]	Fold change of luciferase activity [d]
siESR1	ESR1	0.42 ± 0.01**	0.85 ± 0.07*	13.9 ± 2.0**	0.88 ± 0.07**
siTP53	TP53	0.51 ± 0.06*	1.26 ± 0.01**	34.9 ± 12.8	2.11 ± 0.10
siGREB1	GREB1	0.42 ± 0.04*	1.02 ± 0.15	28.2 ± 9.9	1.69 ± 0.27
siLSG1	LSG1	0.46 ± 0.03*	0.76 ± 0.23	29.7 ± 5.5	1.97 ± 0.51
siISOC1	ISOC1	0.58 ± 0.05*	0.79 ± 0.03**	25.2 ± 10.2	2.11 ± 0.04
siG3P1	G3P1	0.54 ± 0.01**	0.98 ± 0.08	28.7 ± 3.0	2.03 ± 0.10
siRAMP3	RAMP3	0.47 ± 0.07*	1.12 ± 0.05	20.1 ± 4.9*	1.88 ± 0.07
siHK1	HK1	0.46 ± 0.02**	1.00 ± 0.14	27.9 ± 8.0	1.83 ± 0.08
siVDAC2	VDAC2	0.50 ± 0.07*	0.72 ± 0.14*	19.8 ± 3.6*	1.90 ± 0.14
siSFXN2	SFXN2	0.54 ± 0.05*	1.05 ± 0.13	18.6 ± 15.1	1.99 ± 0.39
siAMIGO2	AMIGO2	0.43 ± 0.10*	1.04 ± 0.06	30.6 ± 3.9	1.75 ± 0.15
siRAB5B	RAB5B	0.47 ± 0.01**	1.00 ± 0.28	16.1 ± 1.1**	1.75 ± 0.17
siCA12	CA12	0.58 ± 0.01**	0.77 ± 0.16	26.6 ± 1.5	1.94 ± 0.03
siPPM1D	PPM1D	0.60 ± 0.06*	0.96 ± 0.18	30.4 ± 5.2	1.89 ± 0.33
siGPRC5C	GPRC5C	0.46 ± 0.07*	1.25 ± 0.03**	29.9 ± 5.3	1.97 ± 0.25
siC20orf11	C20orf11	0.45 ± 0.07*	0.92 ± 0.19	32.4 ± 6.6	2.12 ± 0.37
siRUNX1	RUNC1	0.42 ± 0.01**	0.91 ± 0.08	31.0 ± 3.3	2.45 ± 0.33
siControl	None		1.00 ± 0.08	31.0 ± 3.9	2.12 ± 0.26

[a] Effect of a specific siRNA versus control siRNA (siControl) for the relative target gene mRNA level was determined by qRT-PCR.
[b] Growth ability of MCF-7 cells was analyzed by MTS assay after 4 days of siRNA treatment.
[c] Migration ability of MCF-7 cells was analyzed by the transwell assay.
[d] Effect of siRNA on estrogen activity was analyzed by the luciferase assay.
* $P < 0.05$.
** $P < 0.01$ by Student's t-test.

be an estrogen-responsive gene that may also be involved in the migration of breast cancer cells [15]. In the present study, the human RAMP3 located on chromosome 7 (Fig. 2A) was shown by both CAGE (Fig. 1C) and qRT-PCR (Fig. 2B) to be time-dependently up- and down-regulated by estrogen. Silencing of RAMP3 expression by siRNA had no effect on growth, but the migration of MCF-7 cells was impaired (Fig. 2C and D). Four other genes were newly identified as estrogen-responsive genes. Human ISOC1, located on chromosome 5 (Fig. 3A), was found to be up-regulated in response to estrogen by using CAGE (Fig. 1C) and qRT-PCR (Fig. 3B). Knockdown of GPRC5C did not affect migration, but it impaired the growth of MCF-7 cells (Fig. 3C and D). On the other hand, human GPRC5C, located on chromosome 17 (Fig. 4A), was found to be gradually down-regulated in response to estrogen by using CAGE (Fig. 1C) and qRT-PCR (Fig. 4B). Knockdown of GPRC5C did not affect migration, but it did promote the growth of MCF-7 cells (Fig. 4C and D).

4. Discussion

Estrogen is known to play a critical role in the pathogenesis of breast cancer, although the landscape of its signaling has not been fully elucidated. In the present study, we investigated the genome-wide estrogen-regulated transcription network in estrogen-sensitive MCF-7 cells using CAGE in combination with high-throughput deep sequencing. This analysis effectively identified known and novel estrogen-responsive genes. Furthermore, through a functional assay using siRNAs, we newly identified 4 estrogen-responsive genes critical for the growth and migration of MCF-7 cells.

Among the identified estrogen-responsive genes by CAGE, RAMP3 has been previously reported as an estrogen target gene directly regulated by ERs [15]. RAMP3 is strongly expressed in human breast cancer cells, but not in normal epithelial cells [16]. In human breast cancer cells, knockdown of RAMP3 by small hairpin RNA (shRNA) impairs the invasive ability of cancer cells *in vitro*

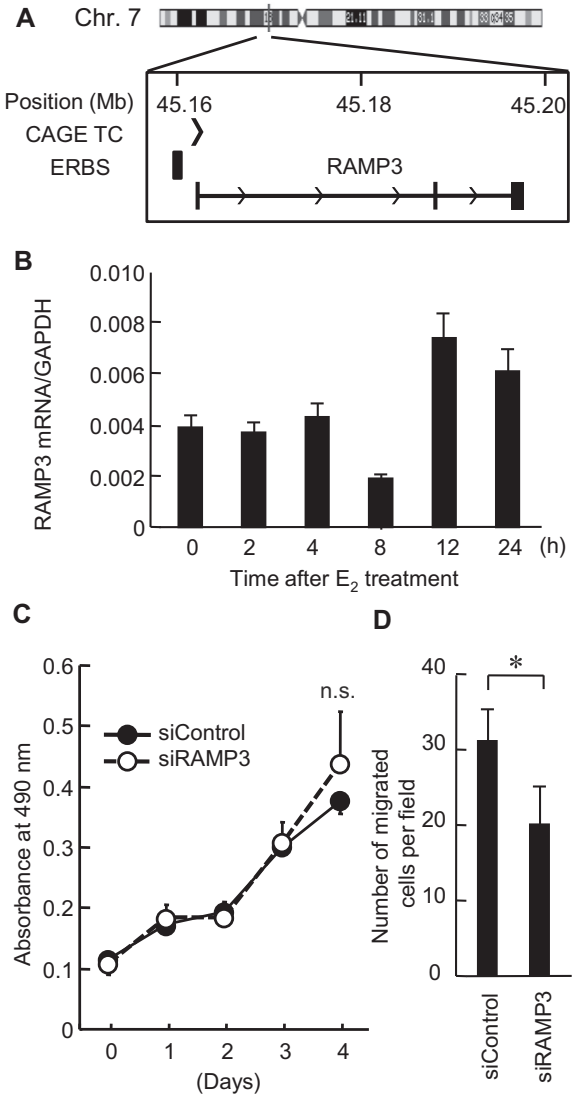

Fig. 2. RAMP3 as an estrogen-regulated gene that promotes the migration of MCF-7 cells. (A) Genome mapping in the vicinity of RAMP3, with a CAGE TC and a functional estrogen receptor binding site (ERBS) close to its annotated transcription start site (TSS). (B) Estrogen-dependent alteration of RAMP3 mRNA expression in MCF-7 cells, as validated by qRT-PCR. (C) siRNA-mediated knockdown of RAMP3 did not affect the cell growth. (D) Knockdown of RAMP3 significantly reduced the migration ability ($^{*}P < 0.05$ by Student's *t*-test).

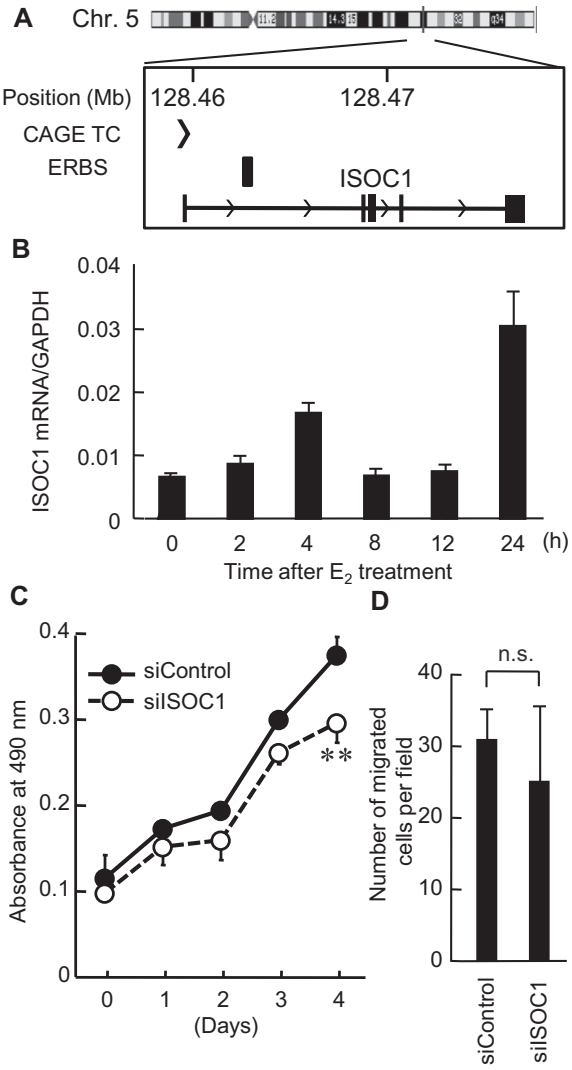

Fig. 3. ISOC1 as an estrogen-regulated gene that promotes the proliferation of MCF-7 cells. (A) Genome mapping in the vicinity of ISOC1, with a CAGE TC close to its annotated TSS and a functional ERBS in its intronic region. (B) Estrogen-dependent alteration of ISOC1 mRNA expression in MCF-7 cells, as analyzed by qRT-PCR. (C) siRNA-mediated knockdown of ISOC1 significantly reduced the cell growth ($^{**}P < 0.01$ by Student's *t*-test). (D) Knockdown of ISOC1 did not affect the migration ability.

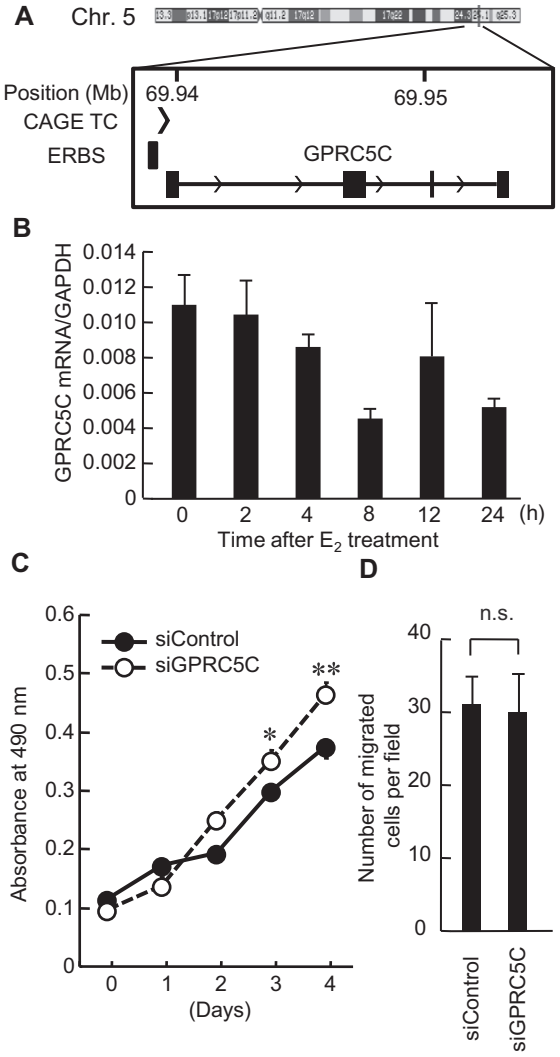

Fig. 4. GPRC5C as an estrogen-repressed gene that inhibits the growth of MCF-7 cells. (A) Genome mapping in the vicinity of GPRC5C, with a CAGE TC and a functional ERBS close to its annotated TSS. (B) Estrogen-dependent down-regulation of GPRC5C mRNA expression in MCF-7 cells, as analyzed by qRT-PCR. (C) The siRNA-mediated knockdown of GPRC5C significantly reduced the cell growth ($^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test). (D) Knockdown of GPRC5C did not affect the migration ability.

and attenuates tumor development in the mouse model [16]. As RAMP3 has been shown as one of the estrogen-responsive genes in our screening system, it is assumed that the rest of the genes identified in the present study will play some roles in the pathogenesis of breast cancer.

We identified VDAC2, ISOC1, GPRC5C, and RAB5B as novel estrogen-responsive genes in MCF-7 cells, because the siRNA-mediated knockdown of these genes impaired either growth or migration of the cells. VDAC2 is one of several voltage-dependent anion channels and is thought to be involved in the mitochondrial apoptotic pathway. VDAC2 binds BAX and inhibits BAK-mediated apoptosis [17,18]. In addition, VDAC2 is more abundantly expressed in clinical breast cancer samples derived from patients with poor outcome, compared with those from patients with good outcome [19]. In the present study, VDAC2 was implicated in the growth and migration of MCF-7 cells. As VDAC2 has been previously reported as an oncogenic factor in human breast cancer [19], our results show that VDAC2 may function as an oncogenic estrogen-responsive gene in breast cancer cells.

ISOC1 is a molecule containing isochorismatase-like domain. Isochorismatase belongs to the family of hydrolases and catalyzes the conversion of isochorismate to 2,3-dihydroxy-2,3-dihydrobenzoate and pyruvate. Besides its putative isochorismatase activity, precise function of ISOC1 in human has not been well elucidated. The association of ISOC1 with ERα signaling has been previously suggested as ISOC1 was more abundantly expressed in ERα-positive breast cancer tissues than in ERα-negative ones in quantitative proteome analysis [20]. In this study, we demonstrated that ISOC1 expression was up-regulated by estrogen, and that siRNA targeting ISOC1 inhibited the growth of MCF-7 cells. Thus, we consider that ISOC1 will be also involved in the growth of breast cancer cells.

GPRC5C is one of the G-protein-coupled receptors (GPCRs) that possess 7 membrane-spanning domains. Although many GPCRs have been identified as signal transducers [21,22], the specific function of this protein is unknown. In our study, GPRC5C expression was shown to be negatively regulated by estrogen, and may down-regulate the growth of breast cancer cells. This result implies that estrogen may exert its oncogenic action by suppressing the expression of tumor-suppressive genes in breast cancer cells.

We found that RAB5B, a member of the small GTPase RAS oncogene family, was transcriptionally regulated by estrogen, and was related to the migration of breast cancer cells. Of the RAS oncogene family, RAB11a, RAB25, and RAB27b have been shown to have roles in the development or progression of breast cancer [23–25]. RAB5B was previously reported to be involved in the trafficking and degradation of epidermal growth factor receptor (EGFR) [26], which is a well-known signal transducer of breast cancer and important for cancer development [27,28]. We considered that RAB5B may mediate estrogen function through the regulation of EGFR in breast cancer cells.

We compared the estrogen-regulated profiles in MCF-7 cells identified by our CAGE ($\geqslant$1.5 or $\leqslant$0.66-folds) and by a previous our RNA-seq study (>2 or <0.5-folds) [29]. In the case of the CAGE, 1067 out of 1537 estrogen-regulated TCs were included in this comparison due to their localizations in exon regions. In terms of upregulated genes, 666 and 765 were identified by our CAGE and the RNA-seq analyses, respectively. Eighty-nine upregulated genes were overlapped between the studies including growth regulation by estrogen in breast cancer 1 (GREB1) and carbonic anhydrase XII (CA12). GREB1 is one of the prototypic estrogen-responsive genes and overexpressed in ER-positive breast cancers [30]. CA12 is also known to be transcriptionally regulated by ERα and the expression is associated with ERα in human breast cancers [31,32]. CA12-positive breast tumors were associated with a lower relapse rate while CA12 is considered to promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH [33,34]. In terms of downregulated genes, twelve overlapped genes were observed in comparison between 217 and 90 downregulated genes by the CAGE and the RNA-seq studies, respectively. Although there are some overlapped genes in CAGE and RNA-seq, it is rather interesting that the rest of estrogen-regulated genes are independently identified either by the CAGE or the RNA-seq. The difference of gene profiles may be due to the methodology of each technique. Considering the present results, CAGE will have implications as an additional source of transcriptome to identify novel estrogen-responsive genes that have not been determined by other transcriptome sequencing or expression analyses.

In summary, CAGE and subsequent knockdown screening using siRNA were useful for identifying genes involved in estrogen signaling throughout the genome of breast cancer cells. This approach can provide a clearer picture of the transcriptional regulation of breast cancer as well as identify the physiologically significant genes, thereby facilitating the diagnosis and treatment of this disease.

Acknowledgments

This work was supported by Grants of the Genome Network Project (S.I., Y.H.), Cell Innovation Program (S.I.), Grants-in-Aid, and Support Project of Strategic Research Center in Private Universities (S.I.) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan; by Grants from the Japan Society for the Promotion of Science, Japan; by Grants-in-Aid (S.I.) from the Ministry of Health, Labour and Welfare, Japan; by the Advanced Research for Medical Products Mining Program (S.I., K.I.) of the National Institute of Biomedical Innovation, Japan; by a Research Grant for RIKEN Omics Science Center from MEXT to Y.H. The authors wish to acknowledge RIKEN GeNAS for the sequencing of the CAGE libraries using the Genome Analyzer IIx (Illumina), as well as for subsequent data processing.

References

[1] J. Ferlay, H.R. Shin, F. Bray, D. Forman, et al., Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008, Int. J. Cancer 127 (2010) 2893–2917.
[2] H.S. Feigelson, B.E. Henderson, Estrogens and breast cancer, Carcinogenesis 17 (1996) 2279–2284.
[3] C.M. Klinge, Estrogen receptor interaction with estrogen response elements, Nucleic Acids Res. 29 (2001) 2905–2919.
[4] A.J. Stewart, M.D. Johnson, F.E. May, B.R. Westley, Role of insulin-like growth factors and the type I insulin-like growth factor receptor in the estrogen-stimulated proliferation of human breast cancer cells, J. Biol. Chem. 265 (1990) 21172–21178.
[5] M. Razandi, A. Pedram, E.M. Rosen, E.R. Levin, BRCA1 inhibits membrane estrogen and growth factor receptor signaling to cell proliferation in breast cancer, Mol. Cell. Biol. 24 (2004) 5900–5913.
[6] R. Mo, Y.T. Zhu, Z. Zhang, et al., GAS6 is an estrogen-inducible gene in mammary epithelial cells, Biochem. Biophys. Res. Commun. 353 (2006) 189–194.
[7] A. Inoue, Y. Omoto, Y. Yamaguchi, et al., Transcription factor EGR3 is involved in the estrogen-signaling pathway in breast cancer cells, J. Mol. Endocrinol. 32 (2004) 649–661.
[8] R. Kodzius, M. Kojima, H. Nishiyori, et al., CAGE: capanalysis of gene expression, Nat. Methods 3 (2006) 211–222.
[9] T. Shiraki, S. Kondo, S. Katayama, et al., Cap analysis gene expression for high-throughput analysis of transcriptional starting point and identification of promoter usage, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 15776–157781.
[10] P. Carninci, A. Sandelin, B. Lenhard, et al., Genome-wide analysis of mammalian promoter architecture and evolution, Nat. Genet. 38 (2006) 626–635.
[11] K. Takayama, K. Kaneshiro, S. Tsutsumi, et al., Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis, Oncogene 26 (2007) 4453–4463.
[12] K. Ueyama, K. Ikeda, W. Sato, et al., Knockdown of Efp by DNA-modified small interfering RNA inhibits breast cancer cell proliferation and in vivo tumor growth, Cancer Gene Ther. 17 (2010) 624–632.
[13] T. Watanabe, S. Inoue, S. Ogawa, et al., Agonistic effect of tamoxifen is dependent on cell type, ERE-promoter context, and estrogen receptor subtype: functional difference between estrogen receptors alpha and beta, Biochem. Biophys. Res. Commun. 236 (1997) 140–145.
[14] J.S. Carroll, C.A. Meyer, J. Song, et al., Genome-wide analysis of estrogen receptor binding sites, Nat. Genet. 38 (2006) 1289–1297.
[15] H. Watanabe, E. Takahashi, M. Kobayashi, et al., The estrogen-responsive adrenomedullin and receptor-modifying protein 3 gene identified by DNA microarray analysis are directly regulated by estrogen receptor, J. Mol. Endocrinol. 36 (2006) 81–89.
[16] V. Brekhman, J. Lugassie, S. Zaffryar-Eilot, et al., Receptor activity modifying protein-3 mediates the protumorigenic activity of lysyl oxidase-like protein-2, FASEB J. 25 (2011) 55–65.
[17] E.H. Cheng, T.V. Sheiko, J.K. Fisher, et al., VDAC2 inhibits BAK activation and mitochondrial apoptosis, Science 25 (2003) 513–517.
[18] M. Lazarou, D. Stojanovski, A.E. Frazier, et al., Inhibition of Bak activation by VDAC2 is dependent on the Baktransmembrane anchor, J. Biol. Chem. 285 (2010) 36876–36883.
[19] Y. Oishi, K. Nagasaki, S. Miyata, et al., Functional pathway characterized by gene expression analysis of supraclavicular lymph node metastasis-positive breast cancer, J. Hum. Genet. 52 (2007) 271–279.
[20] K. Rezaul, J.K. Thumar, D.H. Lundgren, et al., Differential protein expression profiles in estrogen receptor-positive and -negative breast cancer tissues using label-free quantitative proteomics, Genes Cancer 1 (2010) 251–271.
[21] J. Meijer, J. Ogink, B. Kreike, et al., The chemokine receptor CXCR6 and its ligand CXCL16 are expressed in carcinomas and inhibit proliferation, Cancer Res. 68 (2007) 4701–4708.
[22] M. Zajac, J. Law, D.D. Cvetkovic, et al., GPR54 (KISS1R) transactivates EGFR to promote breast cancer cell invasiveness, PLoS ONE 6 (2011) e21599.
[23] D. Palmieri, A. Bouadis, R. Ronchetti, et al., Rab11a differentially modulates epidermal growth factor-induced proliferation and motility in immortal breast cells, Breast Cancer Res. Treat. 100 (2006) 127–137.
[24] J.M. Cheng, L. Volk, D.K. Janaki, et al., Tumor suppressor function of Rab25 in triple-negative breast cancer, Int. J. Cancer 126 (2010) 2799–2812.
[25] A. Hendrix, D. Maynard, P. Pauwels, et al., Effect of the secretory small GTPase Rab27B on breast cancer growth, invasion, and metastasis, J. Natl. Cancer Inst. 102 (2010) 866–880.
[26] P.I. Chen, C. Kong, X. Su, et al., Rab5 isoforms differentially regulate the trafficking and degradation of epidermal growth factor receptors, J. Biol. Chem. 284 (2009) 30328–30338.
[27] J.R. Sainsbury, J.R. Farndon, G.K. Needham, et al., Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer, Lancet 20 (1987) 1398–1402.
[28] A. Agrawal, E. Gutteridge, J.M. Gee, et al., Overview of tyrosine kinase inhibitors in clinical breast cancer, Endocr. Relat. Cancer 12 (2005) S135–S144.
[29] R. Yamaga, K. Ikeda, K. Horie-Inoue, et al., RNA sequencing of MCF-7 breast cancer cells identifies novel estrogen-responsive genes with functional estrogen receptor-binding sites in the vicinity of their transcription start sites, Horm. Cancer 4 (2013) 222–232.
[30] M.G. Ghosh, D.A. Thompson, R.J. Weigel, PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer, Cancer Res. 60 (2000) 6367–6375.
[31] S. Tozlu, I. Girault, S. Vacher, et al., Identification of novel genes that co-cluster with estrogen receptor alpha in breast tumor biopsy specimens, using a large-scale real-time reverse transcription-PCR approach, Endocr. Relat. Cancer 13 (2006) 1109–1120.
[32] D.H. Barnett, S. Sheng, T.H. Charn, et al., Estrogen receptor regulation of carbonic anhydrase XII through a distal enhancer in breast cancer, Cancer Res. 68 (2008) 3505–3515.
[33] P.H. Watson, S.K. Chia, C.C. Wykoff, et al., Carbonic anhydrase XII is a marker of good prognosis in invasive breast carcinoma, Br. J. Cancer 88 (2003) 1065–1070.
[34] J. Chiche, K. Ilc, J. Laferrière, et al., Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH, Cancer Res. 69 (2009) 358–368.