The miR-199a/Brm/EGR1 axis is a determinant of anchorage-independent growth in epithelial tumor cell lines

Kazuyoshi Kobayashi1, Kouhei Sakurai1, Hiroaki Hiramatsu1, Ken-ichi Inada3, Kazuya Shiogama3, Shinya Nakamura1, Fumiko Suemasa1, Kyosuke Kobayashi1, Seiya Imoto2, Takeshi Haraguchi1, Hiroaki Ito1, Aya Ishizaka1, Yutaka Tsutsumi3 & Hideo Iba1

1Division of Host-Parasite Interaction, Department of Microbiology and Immunology, University of Tokyo, Tokyo, Japan, 2Laboratory of DNA Information Analysis, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan, 3First Department of Pathology, Faculty of Medicine, Fujita Health University, Aichi, Japan.

In epithelial cells, miRNA-199a-5p/-3p and Brm, a catalytic subunit of the SWI/SNF complex were previously shown to form a double-negative feedback loop through EGR1, by which human cancer cell lines tend to fall into either of the steady states, types 1 [miR-199a(-)/Brm(+)/EGR1(+)] and 2 [miR-199a(+)/Brm(-)/EGR1(+)]. We show here, that type 2 cells, unlike type 1, failed to form colonies in soft agar, and that CD44, MET, CAV1 and CAV2 (miR-199a targets), all of which function as plasma membrane sensors and can co-localize in caveolae, are expressed specifically in type 1 cells. Single knockdown of any of them suppressed anchorage-independent growth of type 1 cells, indicating that the miR-199a/Brm/EGR1 axis is a determinant of anchorage-independent growth. Importantly, two coherent feedforward loops are integrated into this axis, supporting the robustness of type 1-specific gene expression and exemplifying how the miRNA-target gene relationship can be stably sustained in a variety of epithelial tumors.

Chromatin remodeling factors play vital roles in epigenetical regulation via genome-wide gene transcription1. On the other hand, microRNAs (miRNAs) are post-transcriptional regulatory molecules that are involved in diverse biological processes, including development, differentiation, and homeostasis2. Growing evidence indicates that the robustness of gene expression is often supported by coordinated transcriptional and miRNA-mediated regulatory networks3,4. In addition, improper use of these networks may lead to human diseases such as cancer. However, the interplay between chromatin remodeling factors and miRNA, as well as its biological outcome, is not fully understood in the context of gene regulatory networks common to a wide variety of cell lines.

The human SWI/SNF-A complex (also known as the BAF complex), a member of a family chromatin remodeling factors5 composed of about 10 proteins, regulates gene transcription, either positively or negatively. The SWI/SNF complex contains a single molecule of either Brm or BRG1 as ATP-dependent catalytic subunits. Brm and BRG1 regulate target promoters that do not fully overlap and show clear differences in their biological activities6–9. This SWI/SNF complex interacts with various proteins, including transcriptional regulators, through many specific and varied associations with its several subunits. For example, the d4-family proteins DPF2 (REQ) and DPF3a/3b function as efficient adaptor proteins for RELB/p5210 and RELA/p5011 dimers to induce SWI/SNF-dependent NFκB target genes.

In terms of human cancers, we and other groups have reported that Brm is frequently undetectable in various cancer cell lines12, and in primary tumors of the lung13, stomach14, and prostate15. We found in nuclear run-on transcription assays that a functional Brm gene was present and actively transcribed in all of the Brm-deficient cancer cell lines tested12–14, indicating that Brm expression is largely suppressed by post-transcriptional gene silencing. Brm was later shown to be efficiently targeted by both mir-199a-5p and mir-199a-3p15. In addition, Brm acts as a potent negative regulator of endogenous EGR1 gene expression. EGR1 activates the miR-199a (2) gene locus, which is mainly responsible for the biogenesis of mature miR-199a-5p and -3p in these cancer cell lines. Overall, these findings suggest that, in the cell lines examined, Brm and mir-199a form a robust double-negative feedback loop that includes EGR116. By examining a panel of human cell lines that were derived from a wide variety of cancer tissues, we found that they tend to fall into either of the steady states, mir-199a(-)/Brm(+)/
EGR1(-) cells and miR-199a(+)/Brm(-)/EGR1(+) cells\(^\text{17}\), denoted hereafter as type 1 and type 2, respectively. These regulatory networks may explain why variable (either higher or lower) expression of miR-199a-5p/-3p\(^\text{18}\) or EGR1\(^\text{18}\) has been reported among many carcinomas when compared with the normal epithelial tissues from which they originated.

In the early stage of our current study, we noticed clear differences in the biological properties between type 1 and type 2 cells: all of the type 1 cell lines tested (8 lines), but no type 2 cell lines (4 lines), could grow in soft agar, providing us with an unprecedented opportunity to unravel the robust regulatory networks involved in anchorage-independent growth common to these cancer cell lines. Of course, the gene expression patterns of each cancer cell line would be expected to be largely cell-line-specific and dependent on a wide variety of factors, including the originating tissue type, mutated genes, and pathological properties, such as the tumor stage. However, in our current study, we speculated that epithelial tumors would share regulatory networks that control their basic biological activities. In addition, we hypothesized that several genes would be specifically expressed in type 1 cancer cells, but not in type 2, and, further, that some of them would be crucial for their anchorage independency. Here, we have identified several genes specifically expressed in type 1 cells and show that single knockdown of some of these genes is sufficient to suppress the colony-forming activity of type 1 cells in soft agar. We further examined the underlying molecular mechanisms of the all-or-none regulation of these type 1-specific genes in the two cell types, leading to the identification of two coherent feedforward loops associated with the miR199a/Brm/EGR1 axis. We finally present evidence that these type-specific gene expression patterns can be recapitulated in tumor some lesions of non-small-cell lung carcinomas (NSCLCs).

**Results**

**Type 1, but not type 2, cells grow efficiently in soft agar.** For type 1 and type 2 cells, we chose 12 cell lines (8 for type 1 and 4 for type 2) originating from various human epithelial tumors (Fig. 1a). To ensure that we only examined cell lines originating from epithelial tumors, PA-1 (originating from tridermic teratocarcinoma), MDA-MB435 (recently discovered to originate from melanoma), and HEK-293FT (originating from human embryonic kidney) were removed from the panel of cancer cell lines previously used for categorization\(^\text{17}\). In addition, cell lines from one pancreatic (Panc-1) and three colon cancers (DLD-1, HT29, and HCT116) were added to the current analysis.

The results of a series of quantitative reverse transcription-polymerase chain reaction (RT-PCR) experiments (Fig. 1a) confirmed.

---

**Figure 1** | Basic properties of 12 human cell lines originating from various epithelial tumors. (a) Relative expression levels of Brm, BRG1, EGR1, EGR2, EGR3, and EGR4 mRNA and mature miR-199a-5p, -3p, and miR-214 were determined by quantitative RT-PCR. The data represent the means ± S.D. (n = 3). (b) Numbers of colonies formed in soft agar by each cell line. Numbers of colonies (more than 150 μm in diameter) in soft agar were counted 21–28 days after 1,000 cells were seeded in 60-mm plates. The data represent the means ± S.D. (n = 3) In a–b, P values were determined using Mann-Whitney test. (c) Four type 1 cell lines transduced with retroviral vectors expressing shBrm (#4 or #5) or shCre#4 (negative control) were seeded as in b. Colony numbers of shBrm-expressing cells were compared with those of shCre#4-expressing cells and the ratio was shown as a percentage. The data represent the means ± S.D. (n = 4). Asterisks indicate P value, compared with those transduced with shCre#4. *P < 0.05, **P < 0.01, ***P < 0.001.
our previous observations that epithelial tumor cell lines can be classified into two types according to the expression levels of Brm, EGR1, and miR-199a; type 1 cells specifically express Brm mRNA, whereas expressions of EGR1 mRNA and miR-199a-5p and -3p, as well as miR-214, which is also generated from the miR-199a (2) gene locus, are restricted to type 2 cells. Notably, like EGR1, EGR2, EGR3, and EGR4, which are the other members of EGR family gene and which recognize the same DNA sequence, were shown to be type 2-specific (Fig. 1a) by the parallel analysis. This might indicate EGR2, EGR3, and EGR4 are involved in the miR-199a/Brm axis in a similar manner to EGR1.

After several preliminary comparative analyses between type 1 and type 2 cells, we noticed clear differences in terms of anchorage-independent growth. Type 1 cells formed 25–300 colonies (more than 150 μm in diameter) in soft agar when 1,000 cells were seeded per 60-mm plate and kept for 21–28 days (Fig. 1b). None of the four type 2 cells formed clear colonies in the same conditions. Notably, all of the type 2 cancer cell lines tested—SW1321, HuTu8022, H52223, and C33A24—have shown clear tumor-forming activity in mouse xenograft models.

To test whether the anchorage-independent growth of type 1 cells requires Brm, we performed Brm knockdown experiments in several type 1 cells—A549, H1229, HeLaS3, and Panc-1—using a set of retroviral vectors containing shBrm (#4 and #5). We confirmed that all shBrm vectors significantly suppressed the levels of Brm mRNA and its product (see below) and that cells transduced with these vectors reduced colony-forming activity in soft agar when compared with that of negative control cells transduced with the shCre#4 vector (Fig. 1c). These results reveal the pivotal role of Brm in anchorage-independent growth in type 1 cells. In several cases, strong suppression in colony formation in soft agar was observed by Brm knockdown, whereas the same culture grow normally when kept in monolayer culture (A549 cells expressing shBrm#5 and HeLaS3 and Panc-1 cells expressing either shBrm#4 or #5, Supplementary Fig. 1). In the case of H1299, however, we cannot exclude the possibility that reduction in the growth rate of monolayer culture partly contributed reduction in anchorage-independent growth. These findings provided us with an excellent opportunity to uncover the critical genes required for anchorage-independent growth of type 1 cells and suggested that these candidate genes would be expressed in a type 1-specific manner.

Several genes are preferentially expressed in type 1 cells. Whereas we know that suppression of the expression of a target protein by a certain miRNA is usually moderate and is not unconditionally retained in steady states, we selected the candidates of type 1-specific genes from various targets of miR-199a-5p (10 genes tested), miR-199a-3p (11 genes tested), and miR-214 (6 genes tested). These target genes were identified in previous reports or were predicted by target prediction algorithms as well as by our own analysis (Supplementary Table 1). Of 32 candidate genes tested by quantitative RT-PCR (Fig. 2a and Supplementary Figs. 2 and 3), CAV1, KRT80, MET, and CD44 were expressed in both type 1 and type 2 cells, whereas CAV2 and CAV4 were expressed in none of the type 2 cells (Fig. 2a). We also performed FACS analysis for CD44 and MET using

---

**Figure 2 | Detection of five type 1-specific genes.** (a) Relative expression levels of five type 1-specific genes—CAV1, KRT80, MET, CD44 (all transcripts and variant types v8–v10), and CAV2—in 12 cancer cell lines were determined by quantitative RT-PCR. The data were normalized by taking the highest levels as 1.0. The data represent the means ± S.D. (n = 3). P values were determined using Mann Whitney test. (b) Protein expression levels of Brm, BRG1, and four type 1-specific genes products as well as five type-independent gene products were analyzed by western blotting using 10 gels. The full-length blots were presented in the supplementary Figure 6. β-actin was used as the loading control for each gel. Analysis of KRT80 protein was not possible because of the lack of a specific antibody. Relative expression levels of each protein were quantified in Supplementary Figure 4.
H1299 cells (type 1), and found that the entire population showed high levels of both CD44 and MET (data not shown). We thus designated them as type 1-specific genes. Interestingly, two other epithelial-type keratin genes\textsuperscript{29,30}, KRT7\textsuperscript{31} (an miR-199a-3p target) and KRT19 (an miR-199a-5p target), were not expressed in any of the type 2 cells but were expressed in some of the type 1 cells (Supplementary Fig. 2 and 3).

The protein expression profiles of most of these type 1-specific genes, as well as four genes whose mRNA was expressed in both types, were examined by western blotting (Fig. 2b) and relative amounts of each protein were quantified (Supplementary Fig. 4). The protein expression profiles were generally very similar to those of the corresponding mRNA (Fig. 2a). Mann-Whitney test further confirmed that these miR-199a target gene products (CD44, MET, CAV1 and CAV2) are specifically expressed in type 1 cells (Supplementary Fig. 4). KRT80 protein was not analyzed because of the lack of a specific antibody. PTEN protein, a well-known miR-214 target, was clearly expressed in all of the cell lines except C33A cells, because C33A have homozygous nonsense mutation of this gene. It is noteworthy that among these type 1-specific proteins, CD44, MET, CAV1 and CAV2 function as plasma membrane sensors and signaling platforms and can be colocalized in caveolae in several physiological conditions\textsuperscript{32}.

A single knockdown of CD44, MET, CAV1, or CAV2 is sufficient to suppress anchorage-independent growth of type 1 cell lines. To evaluate whether the type 1-specific genes identified above contribute to the anchorage-independent growth of this cell type, we developed pairs of shRNA constructs for CD44, MET, CAV1, and CAV2 capable of efficiently suppressing their target gene products (Supplementary Fig. 5). Specific knockdown of KRT80 by short hairpin was not possible because there are too many conserved regions among the large keratin gene family paralogues. After A549, H1299, HeLaS3, and Panc-1 cells were transduced with the shRNA-expressing retroviral vectors, their colony-forming activity in soft agar was evaluated (Fig. 3). Single knockdown of CD44, MET, CAV1, or CAV2 efficiently suppressed colony formation compared with negative control cells expressing shRNA for Cre\#4. As an exception, the colony number of A549 cells expressing shCAV2\#2 was slightly increased, probably due to off-target effects. Knockdown of CD44, MET, CAV1, and CAV2 did not significantly affect cell growth. These results indicate that all of the four type 1-specific genes tested significantly contribute to the anchorage-independent growth of these type 1 cell lines.

All-or-none expression patterns of some type 1-specific genes in the cell line panel are supported by two coherent feedforward loops that associate with the axis. Given that miRNA usually suppresses its target protein in a modest manner, it might be somewhat unexpected that some miR-199a targets were regulated in an all-or-none manner between type 1 and type 2 cells (Fig. 2a,b). We speculated that this all-or-none phenomenon could be reflecting regulation by the molecular switch through miR-199a/Brm/EGR1 axis, where Brm and miR-199a expressions manifest a mutually exclusive pattern. Therefore, we first tested whether these type-1 specific genes, CD44, MET, CAV1, CAV2, and KRT80 genes are under the positive control of the Brm-type SWI/SNF complex, for which type 1 cells are competent.

To test whether type 2 cells can induce type 1-specific genes when Brm is exogenously introduced, we transfected SW13 cells (type 2) with Brm expression plasmid or empty plasmid (EV1). In these experiments, some parallel cultures were cotransfected with the expression plasmids for representative NFκB dimers—RELα/p50 (canonical pathway) and RELB/p52 (noncanonical pathway)—or...
empty plasmid (EV2) to determine whether the activation is enhanced by NFκB dimers. As shown in Fig. 4a, CD44, MET, CAV1, and KRT80 mRNA were induced by Brm, as judged by quantitative RT-PCR, although the Brm induction effect varied among the genes. CD44 and CAV1 mRNA levels were increased by cotransfection with NFκB dimers and Brm, whereas expression of MET gene was not NFκB dependent at all. CD44 expression was more strongly dependent upon the noncanonical dimer RelB/p52 than RelA/p50, consistent with a recent report33. CAV1 expression was further increased by cotransfection with RelA/p50 and Brm but was also significantly induced by RelA/p50 alone. Therefore, CAV1 would only require Brm for its full expression. On the other hands, the

Figure 4  Brm is required for the expression of some type 1-specific genes. (a) Expression of Brm and type 1-specific (CD44, MET, CAV1, CAV2, and KRT80) and non-type-specific (GSK3β, SIRT1 and PTEN) genes in SW13 cells transfected with a Brm expression vector or an empty vector (EV1;pCAG-IG) with or without NFκB dimers (RelA/p50, RelB/p52) or another empty vector (EV2;pRK5). The highest expression level was taken as 1.0. The data represent the means ± S.D. (n = 3). (b) Relative expression levels of CD44 (all transcripts), MET, CAV1, CAV2, and KRT80 as well as Brm mRNA in three cell lines of type 1 cells transduced with shBrm-expressing retroviral vector. The expression levels of cells transduced with shCre#4-expressing vector was taken as 1.0. The data represent the means ± S.D. (n = 3). Asterisks indicate P value, compared with those transduced with shCre#. NS, not significant. *P<0.05, **P<0.01, ***P<0.001 (c) Protein analysis of the parallel A549 cultures prepared as shown in b. β-actin was used as the internal control. The full-length blots were presented in the supplementary Figure 7.
expression levels of GSK3β and Sirt1 (miR-199a-5p targets), and PTEN (a miR-214 target), which did not show type 1-specific expression patterns, were not affected by high levels of either Brm or NFκB in SW13 cells (Fig. 4a). Next, A549, H1299, and HeLaS3 cells (type 1) were transduced with retroviral vector encoding Brm shRNA, and the steady-state expression of CD44, MET, CAV1, CAV2, and KRT80 was evaluated by quantitative RT-PCR (Fig. 4b). The levels of CD44, MET, and KRT80 mRNA were suppressed to various extents by Brm knockdown. Western blot analysis of parallel A549 cultures also indicated that CD44 and MET, but not CAV1 and CAV2, required Brm for expression (Fig. 4c). This strong Brm-dependency of CD44 expression is consistent with the previous report that assorted tissues from Brm null/BRG1-positive mice lack CD44 expression. Overall, these findings indicated that genes that are suppressed by miR-199a and simultaneously require the Brm-type SWI/SNF complex for efficient expression show distinct expression patterns: expression in type 1 cells but no expression in type 2 cells. But CAV1 and CAV2 expression failed to show clear Brm dependency in A549 and H1299 cells.

We next tested whether type 1-specific genes are under the negative control of EGR1. When HeLaS3 and A549 cells were stably transduced with EGR1-expressing retrovirus, endogenous miR-199a-3p levels were elevated as expected from the axis (Fig. 5a). In HeLaS3, levels of MET, CAV1 and CAV2 mRNA (Fig. 5a) and their gene products (Fig. 5b and Supplementary Table 2) were reduced by exogenous EGR1 expression. In the case of A549 cells, slight reduction of CAV1 and CAV2 mRNA and reduction of MET, CAV1 and CAV2 proteins were observed (Fig. 5ab and Supplementary Table2). These results are consistent with previous reports indicating that MET([a]) and CAV1([b]) genes are negatively regulated by EGR1: the MET and CAV1 promoters have one and three EGR/SPI-1 binding sites, respectively. We also found EGR1 binding sites on the CAV1, CAV2 and MET promoter regions (from -1600 to +500bp of TSS) by using ChIP-seq data obtained by ENCODE. These results suggest that CAV1/2 and possibly MET are specifically expressed in type 1 cells by evading transcriptional suppression by EGR1 proteins and also post-transcriptional suppression by miR-199a-5p/3p.

Overall, these results suggest that there are at least two feedforward loops. One is composed of miR-199a-5p/3p, Brm and CD44, MET and KRT80 (Fig. 6a left) and another is composed of EGR1, miR-199a-5p/3p and CAV1, and CAV2 (and possibly MET) (Fig. 6a right). Type 1-specific genes would be regulated in an all-or-none manner by either of these two feedforward loops that associate with the robust miR-199a/Brm/EGR1 axis that dictates cancer cell lines to either of the steady states, [miR-199(−)/Brm(+)/EGR1(−)] and [miR-199a(+)/Brm(−)/EGR1(+)] (Fig. 6b).

The miR-199a/Brm/EGR1 axis persists in an extended panel of cell lines originating from epithelial tumors. Because our panel of cancer cell lines used for the development of the cell line typing was limited to 14 cell lines, we intended to increase the number of cell lines by directly performing quantitative RT-PCR of Brm mRNA using totally 4 PCR primer pairs, EGR1 mRNA (using totally 4 PCR primer pairs) and miR-199a-3p by adding 12 new cell lines prepared in a by western blotting. β-actin was used as the loading control. The full-length blots were presented in the supplementary Figure 8 and relative expression levels of each protein including two additional sets of blots were quantified in Supplementary Table 2.

Sanger database (Genomics of Drug Sensitivity in Cancer http://www.cancerrxgene.org), and their expression profiles obtained from the database was compared with those of the qRT-PCR data shown in Fig. 7a (Supplementary Fig. 9). We found that the expression profiles of Brm and EGR1 are not correlates well between them. Since Brm mRNA levels of even Brm-deficient cell lines such as SW13, H522, C33A, A427, and H23—previously reported by our[6,11] and other groups[7,16] by RT-PCR or Northern blotting analysis—were significantly high according to Sanger database, there would be limitations.

Figure 5 | Effects of exogenous EGR1 expression in type 1 cells. (a) Relative expression levels of miR-199a-3p and type 1-specific mRNAs were determined by quantitative RT-PCR in A549 and HeLaS3 cells which were transduced with retroviral vectors expressing EGR1. The expression levels of cells transduced with empty vector was taken as 1.0. The data represent the means ± S.D. (n = 3). Asterisks indicate P value, compared with those transduced with empty vector. *P<0.05, **P<0.01, ***P<0.001 (b) Analysis of type 1-specific gene products and EGR1 in the parallel cultures prepared in a by western blotting. β-actin was used as the loading control. The full-length blots were presented in the supplementary Figure 8 and relative expression levels of each protein including two additional sets of blots were quantified in Supplementary Table 2.
in microarray data to estimate mRNA levels of such transcriptional regulatory genes as Brm and EGR1 accurately.

Since we found expression profiles of CD44, MET, CAV1 and CAV2 mRNA by our qRT-PCR and those obtained from Sanger Database are correlated well, we showed both of them in Fig. 7b. The expression levels of CD44 and MET were high in type 1 cell lines, whereas they were mostly undetectable in the type 2 cell lines even in these extended panels, and specific CD44 and MET expression in type 1 cells were statistically supported in both data of qRT-PCR and the Database. CAV1 and CAV2 expression was not detected in most type 2 cells with a clear exception of A427. Because of this, type 1 specific expression of CAV1 and CAV2 was not supported statistically. Relatively low EGR1 expression in A427 among type1 cells (Fig. 7a, Supplementary Table 3) might partly explain this exception.

Expression patterns observed in type 1 or type 2 cell lines are recapitulated in some cancer lesions of NSCLCs. We finally examined whether the distinct expression patterns observed between the two cell types are reflected in human primary tumors. Since the cell lines originating from NSCLCs in the cell line panel used here can be categorized as both type 1 and type 2 (Supplementary Table 3), we pathologically analyzed surgically resected, formalin-fixed, paraffin-embedded tissues from human cancer lesions of NSCLCs. Among NSCLCs, we especially focused upon squamous cell carcinoma (SCC), because in this type of cancer, we can easily understand activity of proliferation or the status/direction of differentiation two-dimensionally in the histological section. After preparing sequential thin sections of total 21 SCC cases, they were immunohistochemically stained with antibodies against Brm, CD44, MET, and CAV1 and also probed for miR-199a-5p by in situ hybridization and interrelationships among their expression patterns in the coincident area of the each section were analyzed by comparing lower and higher differentiation status.

In the area of lower differentiation status where cancer cells are crowded by the active proliferation and have increased nuclear/cytoplasmic ratio without keratinization, we clearly observed a Brm⁺, CD44⁺, MET⁺, and CAV1⁺ phenotype in almost all cases. In some of these areas, expression of miR-199a was undetectable as shown in Fig. 8 (surrounded by solid line), which recapitulates the expression patterns of type 1 cells. However, in the other areas expressing these 4 proteins, we detected also miR-199a expression, indicating expression heterogeneity in cancer lesions.

As for the areas of highly differentiation status, we observed them in many so-called cancer pearls in 4 cases of SCC, where cancer cells are sparse with large cytoplasm. Even in cancer pearls, significant population at the periphery retains clearly recognized nuclei indicating that cells are still alive, but in the central regions, cells are gradually losing their nuclei on their process of keratinization. We detected a Brm⁺, CD44⁺, MET⁺, and CAV1⁺, and miR-199a⁺ phenotype in all of the cancer pearls where the cell retained nuclei, which recapitulated that of type 2 cells (Fig. 8 within the broken line). In the area between solid line and the cancer pearl in Fig. 8, where tumor cells assumed intermediate differentiation status, these 4 proteins and miR-199a were weakly expressed with various extents. At least in these regions, tumor cells might be undergoing changes from the type 1 cells into the type 2 cells through the process of cellular differentiation.

Discussion

Using 12 cell lines that were strictly derived from human epithelial tumors, we can confirm the findings of our previous report that these cells can be classified into type 1 [mir-199a(−)/Brm(+)/EGR1(−)] (8 lines) or type 2 [mir-199a(+)/Brm(−)/EGR1(+)] (4 lines) cells. In our current study, we were able to efficiently identify the type 1-specific genes by setting the reported miR-199a and miR-214 target genes as the candidates. Some of the identified type 1-specific genes (CD44, MET, and KRT80) required Brm, whereas others (CAV1, CAV2 and probably MET) required the absence of EGR1 for their efficient expression, indicating that two coherent feedforward loops are formed (Fig. 6a). These two feedforward loops are integrated into the robust double-negative feedback loop forming a regulatory network that functions as an efficient switch that determines the expression levels of these type 1-specific genes in an all-or-none manner (Fig. 6b). Thus, the current situation would be a good example of a network formed by multiple miRNA-mediated feedback and feedforward loops37,38, which are commonly present in a wide variety of cell lines. Importantly, we observed regions whose expression patterns recapitulated those of type 1 or type 2 cells by pathological analysis of SCC lesions of NSCLC tissues. It should be pointed out, however, that there are several lesions whose expression patterns do not belong to either of them. We speculate that in the process of cell line establishment from primary tumors, they would tend to fall into either of steady states, type 1 or type 2 cells.
Among the type 1-specific genes shown here, CD44, MET, CAV1, and CAV2 alone significantly contributed to anchorage-independent growth of type 1 cells when tested in knockdown experiments using four type 1 cell lines (Fig. 3). Several previous reports indicated that CD44, MET, and CAV1 are potentially important for colony formation in some epithelial tumor cell lines. We observed that these four genes are all simultaneously suppressed to a marginal level in type 2 cells by the regulatory network shown here, ensuring the anchorage dependency of type 2 cells. Whereas CD44, MET, CAV1, and CAV2 have their own multiple downstream signaling

**Figure 7 | Gene expression analysis on an extended epithelial tumor cell line panel.** (a) Expression profiles of Brm and EGR1 mRNA and mature miR-199a-3p RNA of 26 cell lines, as determined by quantitative RT-PCR. Three additional primer pairs were designed and for Brm and EGR1 mRNA quantification other than used in Fig. 1 (Brm-1, EGR1-1). The relative expression levels are shown by taking the highest as 1.0. Two red vertical break lines indicate the boundary among each type cell lines. Detailed criteria for type 1-3 cells were indicated in Supplementary Table 3. (b) Expression profiles of CD44, MET, CAV1 and CAV2 mRNA of 17 cell lines, determined by quantitative RT-PCR (blue bars) or obtained from Sanger database (red bars).
pathways, their interplay would also contribute to anchorage-indepen-
dent growth or metastasis. Importantly, CD44 and MET were
reported to co-localize with CAV1 in caveolae, and CAV2 is also
localized in caveolae when it forms hetero-oligomers with CAV1. In
normal epithelial cells, caveolae function as plasma membrane
sensors, responding to changes in extracellular matrix via integrin
signaling and also as interacting domains with cytoskeletons. Un-
regulated expression of these four proteins in type 1 cells, their
intimate and coordinated interactions would generate strong down-
stream signaling preferable to grow in soft agar. Therefore, in normal
epithelial cells, we expect miR-199a-5p and -3p would fine-tune
caveolin function such as homeostasis for plasma membrane integ-
urity, signaling platforms, cytoskeleton remodeling and cell migra-
tion. In addition to overlapping subcellular localizations of these
gene products, it should also be pointed out that the genetic loci of
MET, CAV1, and CAV2 all reside in the fragile chromosomal region,
FRA7G at 7q31.2. These regions seem to be neither amplified nor
deleted based on the copy number data compiled in CCLE (https://
ccele.ucla.edu/); copy number data of eight cell lines (data of SW13,
C33A and HeLaS3 and KB are not available) and the copy numbers of
this region are distributed from 1.72 to 2.61.

Currently, we cannot fully explain why type 2 cells can form
tumors when tested in xenograft models using immunodeficient
mice but fail to form colonies in soft agar in vitro. Our preliminary
analyses indicated that type 2 cells express TNF-α, IL-1α, or CCL5,
whereas they do not express Brm-dependent genes, IL-6 and IL-8,
whose products support cell-autonomous growth in soft agar. When
type 2 cells are introduced into mice, their production of
TNF-α, IL-1α, or CCL5 is expected to induce the production of
cytokines such as IL-6 and IL-8 in the associated fibroblast-like cells,
which might in turn function as paracrine factors in tumor
formation.

Stable Brm knockdown in type 1 cell lines by short hairpin retro-
viral vectors reduced the expression levels of several type 1-specific
genes when examined within 2 weeks after the transduction, but
cloning of these shBrm-expressing cells in culture is usually dif-
ficult. These genes are induced by the transient expression of Brm in
type 2 cells (Fig. 4a), but type 2 cells stably expressing exo-
genous Brm are also difficult to establish, as observed by our group*2
and others. These observations indicate that transition from either
type 2 to type 1 or from type 1 to type 2 is inevitably partial and
transient, suggesting that both type 1 and type 2 cells are strongly tied
to their own state after the loops are established and therefore stable
switching to the opposite type would be difficult. This robustness of
each type may reflect some aspects of “oncogene addiction” or
“oncomiR addiction”.

The results in our present study reveal that in normal cells, the
interplay between chromatin remodeling factors and miRNAs would
fine-tune plasma membrane sensors by several motifs including the
miR-199a/Brm/EGR1 axis and two feedforward motifs detected
here. These motifs, once misapplied during the process of carcino-
genesis, would finally fix the cancer cells to extreme steady states,
which cannot be easily reversed. We believe our current findings will
give us clues to elucidate how the homeostatic balance is abrogated at
cancer initiation to establish type 1 or type 2 tumors and how to guide
the development of distinct therapeutic strategies in each case.

Methods
Cell culture. The following human cell lines were used in this study: SW13
(adrenocortical carcinoma) [SW13(vim-) was used as a subtype of SW13 that is
deficient in Brm and BRG1]16; HuTu80 (duodenum carcinoma); the previous
nomenclature, AZ521, was corrected according to the instructions of the American
Type Culture Collection); NCI-H522, A549, and NCI-H1299 (non-small-cell lung
carcinoma); C33A and HeLaS3 (cervical carcinoma); KB (recently shown to be a
derivative of HeLaS3); Panc-1 (pancreatic carcinoma); and DLD-1, HT29, and
HCT116 (colon carcinoma). All cultures were maintained in Dulbecco’s modified
Eagle’s medium containing 10% fetal calf serum. A549, NCI-H522, NCI-H1299,
C33A, A549, KB, Panc-1, DLD-1, HT29, and HCT116 cell lines were purchased from
the American Type Culture Collection. AZ521 (HuTu80) and HeLaS3 cell lines were
obtained from the Cell Resource Center for Biomedical Research, Institute of
Development, Aging and Cancer, Tohoku University, Japan.

Expression vectors. Expression vectors for Brm (pCAG-Brm-IG) and NF-kB-
expressing vectors (pRKS-RelA, -RelB, -p50, and p52) used in this study have been
described previously. To generate EGR1 expressing retrovirus vector, a EcoRI-NcoI
DNA fragment of pCMV-SPORT6-EGR117 was inserted to the corresponding
cloning site of pmXs-ires-puro or -Bla.

Plasmid preparation for retroviral vectors expressing shRNA. Pairs of
oligonucleotides encoding gene-specific short hairpin RNA (shRNA) were
synthesized (Supplementary Table 4) and inserted between the BbsI/EcoRI sites of
pmU6. The pmU6 derivatives shCre4* [used as negative control (NC)] and
shBrm4#1 were previously described. These pmU6-based plasmids were digested
with BamHI and EcoRI and inserted between these sites in pSSP for the retroviral
directors.

DNA transfection and preparation of retroviruses. For the transfection of plasmid
directors into cell lines, Lipofectamine 2000 (Invitrogen Corp.) was used in accordance
with the manufacturer’s instructions. The preparation and transduction of vesicular
stomatitis virus-G (VSV-G) pseudotyped retroviral vectors were performed as
described previously.8

Quantitative RT-PCR. Total RNA was extracted using a mirVana microRNA
Isolation Kit (Ambion). To detect coding gene cDNAs, cDNA was synthesized with a
PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa Bio)
in accordance with the manufacturer’s instructions. Quantitative (real-time) RT-PCR
was performed using a SYBR® Select Master Mix (Applied Biosystems). GAPDH
was used as an internal control. The primer pairs used are listed in Supplementary Table 4.
For the detection of miRNA, miRNA-specific looped RT-primers and TaqMan
probes were used as described by the manufacturer’s protocol (Applied Biosystems).
RNU6 RNA was used as an internal control. PCRs were performed in triplicate using a
7300 Real-Time PCR system (Applied Biosystems).

Western blotting. Total protein extracts were prepared by boiling the cells in SDS
sample buffer for 5 min at 95°C. The proteins were then separated by 10% SDS-PAGE

---

Figure 8 | SCC categorized as NSCLC analyzed by in situ hybridization for miR-199a-5p or immunohistochemistry for Brm, CD44, MET, CAV1. The bar indicates 200 μm. In the HE staining slide, less differentiated cells are surrounded by white solid lines and highly differentiated cells which still retain cellular nuclei at the periphery of a cancer pearl are shown by a white broken line, respectively.
and transferred onto Immobilon-P PVDF membranes (Millipore). Immunoblotting was performed by incubating the membrane overnight at 4°C with primary antibodies against the following proteins: Brm (ab15597; Abcam), BRG1 (sc-10768; Santa Cruz), EGR1 (#4153; Cell Signaling), CD44 (#3570; Cell Signaling), MET (#8198; Cell Signaling), CAV1(#3267; Cell Signaling), CAV2 (#8522; Cell Signaling), SIRT1 (sc-7446; Santa Cruz), GSK3β (#2126; Cell Signaling), RELA (ab7971; Abcam), PTEN (#9552; Cell Signaling), IKKβ (#493; Cell Signaling), and β-actin (sc-4777; Santa Cruz). For the secondary antibody, membranes were incubated overnight at room temperature with the corresponding antibody used for western blotting and washed in phosphate-buffered saline (TBS) containing Tween 20.

In situ histochemical staining. Deparaffinization, endogenous peroxidase inactivation, and antigen retrieval of formalin-fixed, paraffin-embedded clinical tissues and immunostaining of Brm were performed as described previously [17,20]. For CD44 (#3570; Cell Signaling), MET (ab51067; Abcam), and CAV1 (sc-894; Santa Cruz) immunostaining, the sections were incubated overnight at room temperature with the corresponding antibody used for western blotting and washed in phosphate-buffered saline. N-HistoFix® Simple Stain® MAX PO (MULTI) (414145; Nichirei Biosciences Inc.) were then applied to the slides for 30 min at room temperature, followed by three washes in TBS. The reaction products were visualized using 50 mg/dl 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.003% H2O2. The immunostained sections were evaluated independently by two pathologists in conjunction with hematoxylin and eosin-stained sections from the same lesions.

In situ hybridization. In situ hybridization analysis to detect miR-19a-5p in formalin-fixed, paraffin-embedded sections was performed using LNA-modified oligonucleotide probes as described previously [17,20]. Use of the clinical tissue sections in this study was approved by the Fujita Health University ethical review board for human investigation.

Statistical analysis. Results are presented as means ± S.D. Statistical significance for quantitative RT-PCR assays was determined using a two-tailed Student’s t-test. Statistical significance for the differences of a parameter between type 1 and type 2 cell lines was determined using Mann-Whitney test. In both cases, P-values<0.05 were considered statistically significant.

1. Wilson, B. G. & Roberts, C. W. SWI/SNF nuclease remodelers and cancer. Nat Rev Cancer 11, 481–92 (2011).
2. Ebert, M. S. & Sharp, P. A. Roles for microRNAs in conferring robustness to biological processes. Cell 149, 515–34 (2012).
3. Ilipoulos, D., Hirsch, H. A. & Stuhl, K. An epigenetic switch involving NF-kB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cancer Res 70, 1155–68 (2010).
4. Gurtan, A. M. & Sharp, P. A. The role of miRNAs in regulating gene expression. J Mol Biol 425, 3582–600 (2013).
5. Yam, M. Chromatin remodeling: from transcription to cancer. Cancer Genet 207, 352–375 (2014).
6. Mizutani, T. et al. Maintenance of integrated proviral gene expression requires Brm, a catalytic subunit of SWI/SNF complex. J Biol Chem 277, 15859–64 (2002).
7. Reisman, D. N. et al. Comcomitant down-regulation of BRM and BRG1 in human tumor cell lines: differential effects on RB-mediated growth arrest vs CD44 expression. Cancer Res 202, 1196–207 (2011).
8. Kadam, S. & Emerson, B. M. Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes. Mol Cell 11, 377–89 (2003).
9. Mizutani, T. et al. Loss of the Brm-type SWI/SNF chromatin remodeling complex is a strong barrier to the Tat-independent transcriptional elongation of human immunodeficiency virus type 1 transcripts. J Virol 83, 11569–80 (2009).
10. Tando, T. et al. Requiem protein links RbE/p52 and the Brm-type SWI/SNF complex in a noncanonical NF-kB/p52 pathway. J Biol Chem 285, 21951–60 (2010).
11. Ishizaka, A. et al. Double plant homeodomain (PHD) finger proteins DPF3a and -b are required as transcriptional co-activators in SWI/SNF complex-dependent transcriptional activation of NF-kB. J Biol Chem 287, 11924–33 (2012).
12. Yamamichi, N. et al. The Brm gene is suppressed at the post-transcriptional level in various human cell lines is inducible by transient HDAC inhibitor treatment, which shows antitumorigenic potential. Oncogene 24, 5471–81 (2005).
13. Reisman, D. N., Sciarrotta, J., Wang, W., Funkhouser, K. W. & Weissman, B. E. Loss of BRG1/BRM in human lung cancer cell lines and primary lung cancers: correlation with poor prognosis. Cancer Res 63, 560–6 (2003).
14. Yamamichi, N. et al. Frequent loss of Brm expression in gastric cancer correlates with histologic features and differentiation state. Cancer Res 67, 10727–35 (2007).
15. Shen, H. et al. The SWI/SNF ATPase Brm is a gatekeeper of proliferative control in prostate cancer. Cancer Res 68, 10134–62 (2008).
45. Bolanos-Garcia, V. M. MET meet adaptors: functional and structural implications in downstream signalling mediated by the Met receptor. *Mol Cell Biochem* **276**, 149–57 (2005).

46. Lloyd, P. G. Caveolin-1, antiapoptosis signaling, and anchorage-independent cell growth. Focus on "Caveolin-1 regulates McI-1 stability and anokisis in lung carcinoma cells". *Am J Physiol Cell Physiol* **302**, C1282–3 (2012).

47. Kwon, H., Lee, J., Jeong, K., Jang, D. & Pak, Y. A novel actin cytoskeleton-dependent nonaveolar microdomain composed of homo-oligomeric caveolin-2 for activation of insulin signaling. *Biochim Biophys Acta* **1833**, 2176–89 (2013).

48. Fujisaki, T. et al. CD44 stimulation induces integrin-mediated adhesion of colon cancer cell lines to endothelial cells by up-regulation of integrins and c-Met and activation of integrins. *Cancer Res* **59**, 4427–34 (1999).

49. Elliott, V. A., Rychahou, P., Zaytseva, Y. Y. & Evers, B. M. Activation of c-Met and upregulation of CD44 expression are associated with the metastatic phenotype in the colorectal cancer liver metastasis model. *PLoS One* **9**, e97432 (2014).

50. Singleton, P. A. et al. CD44 regulates hepatocyte growth factor-mediated vascular integrity. Role of c-Met, Tiam1/Rac1, dynamin 2, and cortactin. *J Biol Chem* **282**, 20643–57 (2007).

51. Mora, R. et al. Caveolin-2 localizes to the golgi complex but redistributes to plasma membrane, caveolae, and rafts when co-expressed with caveolin-1. *J Biol Chem* **274**, 25708–17 (1999).

52. Tatarella, C., Linnenbach, A., Mimori, K. & Croce, C. M. Characterization of the human TESTIN gene localized in the FRA7G region at 7q31.2. *Genomics* **68**, 1–12 (2000).

53. Hartman, Z. C. et al. Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the proinflammatory cytokines IL-6 and IL-8. *Cancer Res* **73**, 3470–80 (2013).

54. Ito, T. et al. Brm transactivates the telomerase reverse transcriptase (TERT) gene and modulates the splicing patterns of its transcripts in concert with p54(nrb). *Biochem J* **411**, 201–9 (2008).

55. Hoffman, G. R. et al. Functional epigenetics approach identifies BRM/SMARCA2 as a critical synthetic lethal target in BRG1-deficient cancers. *Proc Natl Acad Sci U S A* **111**, 3128–33 (2014).

56. Weinstein, I. B. & Joe, A. Oncogene addiction. *Cancer Res* **68**, 3077–80 (2008).

57. Cheng, C. J. & Slack, F. J. The duality of oncmiR addiction in the maintenance and treatment of cancer. *Cancer J* **18**, 232–7 (2012).

58. Watanabe, H. et al. SWI/SNF complex is essential for NRSF-mediated suppression of neuronal genes in human nonsmall cell lung carcinoma cell lines. *Oncogene* **25**, 470–9 (2006).

59. Haraguchi, T. et al. SiRNAs do not induce RNA-dependent transcriptional silencing of retrovirus in human cells. *FEBS Lett* **581**, 4949–54 (2007).

60. Yamamichi, N. et al. Locked nucleic acid in situ hybridization analysis of miR-21 expression during colorectal cancer development. *Clin Cancer Res* **15**, 4009–16 (2009).

**Acknowledgments**

We thank S. Kawaura and A. Kato for their assistance in preparing the manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (22000318) and on Innovative Areas (24115007) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

**Author contributions**

Kazuyoshi K., K. Sakurai, and H. Iba, designed all the experiments and wrote the manuscript. Kazuyoshi K. performed and analyzed a large part of the experiments. H.H., S.N., F.S. and Kyosuke K. contributed protein analysis and plasmid construction. K.I., K. Shiogama and Y.T. prepared clinical samples and performed pathological analysis. S.I. conducted database analysis. T.H., H. Ito and A.I. gave important advices for vector construction, protein analysis and cellular preparation. All authors reviewed the manuscript.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Kobayashi, K. et al. The miR-199a/Brm/EGR1 axis is a determinant of anchorage-independent growth in epithelial tumor cell lines. *Sci. Rep.*, 5; 8428; DOI:10.1038/srep08428 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/