Cancer-secreted hsa-miR-940 induces an osteoblastic phenotype in the bone metastatic microenvironment via targeting ARHGAP1 and FAM134A

Kyoko Hashimoto, Hiroki Ochi, Satoko Sunamura, Nobuyoshi Kosaka, Yo Mabuchi, Toru Fukuda, Kenta Yao, Hiroaki Kanda, Keisuke Aes, Atsushi Okawa, Chihiro Akazawa, Takahiro Ochiya, Mitsuuru Futakuchi, Shu Takeda, and Shingo Sato

*Department of Physiology and Cell Biology, Tokyo Medical and Dental University (TMDU), Graduate School, 113-8510 Tokyo, Japan; Division of Molecu- lar and Cellular Medicine, National Cancer Center Research Institute, 104-0045 Tokyo, Japan; Department of Biochemistry and Biophysics, Tokyo Medical and Dental University (TMDU), Graduate School, 113-8510 Tokyo, Japan; Department of Food Science, Tokyo Sei- ni College, 124-8530 Tokyo, Japan; Department of Pathology, The Cancer Institute of the Japanese Foundation for Cancer Research, 135-8550 Tokyo, Japan; Department of Orthopaedic Oncology, Cancer Institute Arika Hospital, 135-8550 Tokyo, Japan; Department of Orthopaedic Surgery, Tokyo Medical and Dental University (TMDU), Graduate School, 113-8510 Tokyo, Japan; Department of Pathology, Nagasaki University Graduate School of Biomedical Sciences, 852-8523 Nagasaki, Japan; and Division of Endocrinology, Toranomon Hospital Endocrine Center, 105-8470 Tokyo, Japan

Edited by Owen N. Witte, Howard Hughes Medical Institute and University of California, Los Angeles, CA, and approved January 12, 2018 (received for review October 3, 2017)

Bone metastatic lesions are classified as osteoblastic or osteolytic lesions. Prostate and breast cancer patients frequently exhibit osteoblastic-type and osteolytic-type bone metastasis, respectively. In metastatic lesions, tumor cells interact with many different cell types, including osteoblasts, osteoclasts, and mesenchymal stem cells, resulting in an osteoblastic or osteolytic phenotype. However, the mechanisms responsible for the modification of bone remodeling have not been fully elucidated. MicroRNAs (miRNAs) are transferred between cells via exosomes and serve as intercellular communication tools, and numerous studies have demonstrated that cancer-secreted miRNAs are capable of modifying the tumor microenvironment. Thus, cancer-secreted miRNAs can induce an osteoblastic or osteolytic phenotype in the bone metastatic microenvironment. In this study, we performed a comprehensive expression analysis of exosomal miRNAs secreted by several human cancer cell lines and identified eight types of human miRNAs that were highly expressed in exosomes from osteoblastic phenotype-inducing prostate cancer cell lines. One of these miRNAs, hsa-miR-940, significantly promoted the osteoblastic phenotype-inducing prostate cancer cell lines. Our previous reports have demonstrated that mmu-miR-206 and mmu-miR-145 play important roles in osteoblast differentiation in vivo (11, 12). To date, numerous studies have shown that multiple miRNAs regulate the differentiation of osteoblasts and osteoclasts. In addition, a recent study showed that miRNAs transfer between cells via exosomes to serve as intercellular communication tools (13, 14).

**Significance**

Prostate cancer is one of the most common cancers in men worldwide, and osteoblastic bone metastasis is frequently observed in prostate cancer patients. However, the mechanisms responsible for the predominantly osteoblastic phenotype have not been fully elucidated. Cancer-secreted microRNAs (miRNAs) were recently shown to be significant in the modification of the tumor microenvironment. Here, hsa-miR-940, which was highly secreted by prostate cancer cells, promoted osteogenic differentiation of human mesenchymal stem cells in vitro, and induced extensive osteoblastic lesions in the bone metastatic microenvironment in vivo. Our study provides a demonstration that osteoblastic bone metastasis can be induced by miRNAs secreted by cancer cells in the bone microenvironment.

Author contributions: A.O., C.A., T.O., M.F., S.T., and S. Sato designed research; K.H. and S. Sunamura performed research; K.H., H.O., S. Sunamura, N.K., Y.M., T.F., K.Y., H.K., K.A., and S. Sato analyzed data; and K.H. and S. Sato wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

1To whom correspondence may be addressed. Email: takeda.phy2@tmd.ac.jp or satoshin.phy2@tmd.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1717363115/-/DCSupplemental.

Published online February 12, 2018.
into the surrounding stromal cells, resulting in the modification of bone remodeling.

Results

**Identifying Exosomal miRNAs Markedly Secreted by Osteoblastic Phenotype-Inducing Cancer Cell Lines.** Based on previous studies, we divided human cancer cell lines into two groups: osteoblastic phenotype- and osteolytic phenotype-inducing cell lines. The osteoblastic phenotype-inducing cancer cell lines included human prostate cancer cell lines (e.g., C4, C4-2, and C4-2B) and represent the osteoblastic phenotype when the cells are implanted into the tibia of immunodeficient mice (Fig. 1A) (15). In contrast, human breast cancer cell lines (e.g., MDA-MB-231-Luc) and human myeloma cell lines (e.g., KMS11 and U266) are known to induce the osteolytic phenotype in bone metastatic lesions (Fig. 1A) (6, 16).

We first examined the expression profile of miRNAs in exosomes secreted by the above osteoblastic or osteolytic phenotype-inducing cell lines. The cancer-secreted exosomes were isolated by ultracentrifugation of the culture supernatant (Fig. S1A), and total RNAs, including miRNAs, were extracted from the isolated exosomes and subjected to miRNA microarray. In advance of the analysis, we confirmed that the exosomal RNAs had an abundance of small RNAs compared with cellular RNAs (Fig. S1B). The comprehensive microarray analysis showed that eight human miRNAs, hsa-miR-99a-5p, 125b-2-3p, 141-3p, 200a-3p, 200b-3p, 200c-5p, 940, and 1260a, were highly expressed in the exosomes secreted by osteoblastic phenotype-inducing prostate cancer cell lines compared with the exosomes from osteolytic phenotype-inducing cancer cell lines (Fig. 1B).

**hsa-miR-940 Promotes the Osteogenic Differentiation of Human Mesenchymal Stem Cells.** To visualize the transfer of miRNA-containing exosomes from cancer cells to mesenchymal cells, we constructed a retroviral vector containing CD63, which is a well-known exosome marker (13), fused with improved yellow fluorescent protein (Venus). We then established a CD63-Venus stably expressing cancer cell line, C4-2B-CD63-Venus, using a retroviral infection system. Venus-labeled exosomes were isolated from the supernatant of the cells by ultracentrifugation and added to dtTomato-expressing immortalized human mesenchymal stem cell lines (hMSCs) (Fig. 2A, Left). After a 24-h culture, we detected Venus-labeled exosomes incorporated into hMSCs (Fig. 2A, Center). We also confirmed that the expression of ALPL, which is significantly up-regulated in hMSCs cultured with the exosomes for 48 h (Fig. 2A, Right). These findings suggest that miRNA-containing exosomes derived from osteoblastic phenotype-inducing cell lines have the potential to promote the osteogenic differentiation of hMSCs.

To examine the effect of the cancer-secreted miRNAs that were identified in Fig. 1B on osteogenic differentiation of hMSCs, each miRNA mimic was subsequently transfected into hMSCs, and the cells were cultured in osteogenic induction medium. After a 14-d osteogenic induction, an alkaline phosphatase (ALP) assay revealed that the overexpression of miR-940 or miR-1260a significantly promoted the osteogenic differentiation of hMSCs as shown by the increase in the ALP activity (Fig. 2B). After a 28-d induction, von Kossa staining was performed to investigate the effect of the miRNAs on the matrix mineralization of hMSCs (Fig. 2C). We also established stable miR-940-overexpressing hMSCs using a lentiviral infection system, and the osteogenic potential of the cells was examined. After a 7-d osteogenic induction, miR-940-overexpressing hMSCs showed significantly increased ALP activity, as well as a higher expression of osteogenic markers than the empty vector-infected hMSCs (Fig. 2D and Fig. S2A). The mineralized areas of the cultured cells were also significantly increased in miR-940-overexpressing hMSCs after a 28-d osteogenic induction (Fig. S2B). In contrast, miR-940 overexpression in human osteoclast precursor cells, which were isolated from CD14+ peripheral blood mononuclear cells, did not affect osteoclastogenesis. The number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts was not altered by miR-940 overexpression (Fig. S2C). The gene expression of an osteoclast marker, CTSK, was also not changed (Fig. S2D).

**ARHGAP1 and FAM134A Are Targets of hsa-miR-940 to Promote Osteogenic Differentiation.** To identify the target genes of hsa-miR-940 to regulate osteogenic differentiation, we performed a silico analysis using four target prediction databases: TargetScan (17), miRDB (18), miRanda (19), and mirWalk (20). According to the analysis, 19 candidate genes were identified as targets of miR-940 (Fig. 3A). To determine whether miR-940 regulates osteogenic differentiation through targeting these genes, hMSCs were transfected with siRNA of each candidate gene and cultured in osteogenic induction medium. After a 14-d osteogenic induction, the down-regulation of ARHGAP1 and FAM134A significantly increased the ALP activity of hMSCs (Fig. 3B). In contrast, hMSCs with stable overexpression of ARHGAP1 or FAM134A also showed a significant decrease in the ALP activity of the cells (Fig. 3C). Moreover, we also confirmed that miR-940 overexpression in hMSCs decreased ARHGAP1 and FAM134A protein levels, as well as their mRNA expression levels (Fig. 3D and E), indicating that ARHGAP1 and FAM134A were targets of miR-940. According to the in silico analysis, ARHGAP1 and
FAM134A have the binding sites for miR-940 in each 3′UTR region (Fig. S3A). To confirm that miR-940 binds to the lesions, we performed a luciferase assay using a reporter plasmid in which the above putative miR-940 binding sites were cloned into the 3′UTR of the luciferase gene. In accordance with the in silico prediction, transient overexpression of miR-940 significantly decreased the luciferase activity of both binding sites of ARHGAP1 and FAM134A (Fig. S3B, Left), whereas each mutation in these binding sites abrogated the response to miR-940 (Fig. S3B, Right). miR-940 also significantly decreased the luciferase activity of a construct harboring the entire length of the ARHGAP1 or FAM134A 3′UTR (Fig. S3C, Left), and mutations in the binding sites of the 3′UTR diminished the response to miR-940 (Fig. S3C, Right). These findings suggest that miR-940 promotes osteogenic differentiation of hMSCs by targeting ARHGAP1 and FAM134A.

Cancer-Secreted hsa-miR-940 Is Transferred via Exosomes to Mesenchymal Stem Cells and Promotes Osteogenesis. To investigate whether miR-940 overexpression in the osteolytic phenotype-inducing cancer cells would induce the characteristics of osteoblastic phenotype-inducing cancer cells, we established hsa-miR-940-overexpressing MDA-MB-231 cells using a lentiviral infection system (Fig. 4A, Upper). Empty vector-infected MDA-MB-231 cells were also established as a control. As MDA-MB-231 cells were transduced with the genomic fragment of pri-miR-940, the overexpressing cells showed higher expression of mature miR-940 as well as miR-940 compared with the control cells (Fig. 4A, Left). Exosomes from the miR-940-overexpressing MDA-MB-231 cells (Exo-miR-940) or exosomes from the empty vector-infected cells (Exo-control) were added to the culture medium of hMSCs. Exo-miR-940–incorporated hMSCs showed a higher expression of mature miR-940 than Exo-control-incorporated cells. In contrast, the expression level of pri-miR-940 was not altered (Fig. 4A, Right), suggesting that the increased expression of miR-940 in the Exo-miR-940–incorporated hMSCs was due to the transfer of mature miR-940 via exosomes. Indeed, to investigate whether miR-940 is enriched in the exosomes from miR-940–overexpressing cells, MDA-MB-231–CD63–Venus cells were transfected with miR-940 mimic, and Venus+ exosomes were sorted from the culture supernatant by flow cytometry using magnetic beads (Fig. S4A). qPCR analysis showed that miR-940 was significantly expressed in the exosomes from miR-940–overexpressing cancer cells (Fig. S4B). We also examined the expression of ALPL and targets of miR-940 in the Exo-miR-940–incorporated hMSCs. qPCR analysis showed that the expression of ALPL was significantly up-regulated in hMSCs cultured with Exo-miR-940 (Fig. 4B). Western blot analysis revealed that the protein levels of ARHGAP1 and FAM134A were down-regulated in the cells (Fig. 4C), suggesting that the exosomes from miR-940–overexpressing cells were incorporated into hMSCs, promoting their osteogenic differentiation.

Fig. 3. ARHGAP1 and FAM134A are targets of hsa-miR-940 to promote osteogenic differentiation. (A) A diagram illustrating in silico analysis. (B and C) The effects of ARHGAP1 or FAM134A knockdown (B) or overexpression (C) on the osteogenic differentiation of hMSCs. (D and E) The down-regulation of miRNA levels (D) and protein levels (E) of target genes by the transient overexpression of miR-940. All of the data are the means ± SEMs (n = 3). n.s., not significant, *P < 0.05, **P < 0.01 by one-way ANOVA with Tukey’s HSD test (B and C) and Student’s t test (D).
Prostate cancer is one of the most common cancers in men worldwide, and osteoblastic-type bone metastasis is observed in up to 70% of patients with prostate cancer. Bone metastasis can cause severe pain, pathological fractures, and spinal cord compression. An improved understanding of the mechanisms underling bone metastasis can facilitate the development of new therapeutic options and improve patient survival.

The bone microenvironment comprises osteoblasts, osteoclasts, and many other cell types, including MSCs. Therefore, it is believed that the cross-talk between metastasized cancer cells and the surrounding bone cells is critical for the formation of the osteoblastic or osteolytic phenotype. Previous studies have demonstrated that metastasized prostate cancer cells secrete several osteotropic factors, such as BMPs, VEGF, PDGF, and ET-1, and promote osteoblastic-type bone metastasis (22, 23). These findings raise the possibility that bone remodeling in the bone metastatic lesions can be regulated by miRNAs secreted by cancer cells. However, the role of cancer-secreted miRNAs in the bone metastatic phenotype is not yet understood.

In our study, miRNA microarray analysis identified eight human miRNAs that were highly expressed in the exosomes isolated from the prostate cancer cells. In vitro analysis showed that hsa-miR-940 significantly promoted the osteogenic differentiation of hMSCs (Fig. 2B and C). To date, several studies have shown that miR-940 plays diverse roles in cell proliferation, migration, metastasis, and apoptosis in various types of cancers (24, 25). However, the role of miR-940 in bone metabolism has not previously been reported. Therefore, our study reveals the osteogenic effect of cancer-secreted miR-940. To investigate whether miR-940 is generally expressed in prostate cancer cell lines that induce osteoblastic bone metastasis, we also examined the expression of miR-940 in other osteoblastic phenotype-inducing prostate cancer cell lines in vitro...

Hashimoto et al.  

PNAS | February 27, 2018 | vol. 115 | no. 9 | 2207
miR-940 expression level comparable to C4 lineage cell lines (Fig. 4A and Fig. S7), and implanted the cells on the calvaria of mice. Interestingly, miR-940 overexpression could be microenvironment-dependent and that additional factors in the bone microenvironment may mediate the formation of the osteoblastic lesions.

Finally, in preliminary analyses, we also showed that miR-940 enhanced the resistance of cancer cells against chemotherapy agents. Clinically, advanced prostate or breast cancers become hormone-independent and rapidly develop resistance to chemotherapy, leading to aggressive bone metastasis. Recent numerous studies have shown the significance of miRNAs in cancer therapeutic response or drug resistance. Several miRNAs were also identified as predictive biomarkers of drug resistance (31, 32). Our high-throughput inhibitor screening revealed that miR-940 increased resistance to several chemotherapy agents, such as 5-FU, methotrexate, and vinblastine (Fig. S9).

In conclusion, we demonstrated that prostate cancer-secreted hsa-miR-940 promoted the osteogenic differentiation of hMSCs in vitro and induced extensive osteoblastic lesions in the bone metastatic microenvironment in vivo. This study provides a comprehensive understanding of the role of miR-940 in the bone microenvironment and its potential therapeutic implications for cancer treatment.
demonstration that a cancer-secreted miRNA induced osteoblastic-type bone metastasis, serving as an osteotropic factor in the bone microenvironment.

Materials and Methods

Cell Culture and Osteogenic Differentiation. The human prostatic carcinoma cell lines C4, C4-2, and C4-2B, the human breast adenocarcinoma cell line MDA-MB-231-Luc, and the immortalized human mesenchymal stem cell line UCB408E6E7ERTT-33 were cultured as previously reported (15, 16, 33). The osteogenic differentiation potential of hMSCs was examined using the ALP assay and von Kossa staining, as previously described (12). Further details are provided in the SI Materials and Methods.

Exosome Isolation and miRNA Microarray Analysis. Exosomes were isolated from the culture medium by ultracentrifugation as previously reported (34). Microarray analysis was performed using an Agilent Human miRNA Microarray Kit (V3). The data were subsequently normalized and analyzed using GeneSpring GX software (Agilent Technologies). Additional details are provided in the SI Materials and Methods.

qPCR and Western Blot Analysis. Relative miRNA expression levels were determined by qPCR using microscript SYBR Green PCR Kit (Qiagen). Western blot analysis was performed as previously described (12). Additional details are provided in the SI Materials and Methods.

Cancer Cell Implantation and Microcomputed Tomography Analysis. MDA-MB-231 cells were implanted on the calvaria of BALB/c-Null-nu mice, as previously reported (21). Microcomputed tomography (Micro-CT) analysis was performed using R_micT2 (Nigaku) and TRIFCS-BDN (Ratoc System Engineering). Additional details are provided in the SI Materials and Methods. All animal experiments were performed with approval from the Animal Study Committee of Tokyo Medical and Dental University and conformed to the relevant guidelines and legislations.

Human Bone Metastasis Specimens. The Cancer Institute of the Japanese Foundation for Cancer Research stocks frozen samples of bone metastatic lesions that were previously harvested from prostate cancer patients. Informed consent for their use in medical research was obtained under an Institutional Review Board-approved protocol. The ethics committee of the Cancer Institute approved the use of such samples in this study. The expression of miR-940 in human samples was examined by qPCR.

Statistical Analyses. All of the data are presented as the means ± SEMs. The values were considered significant at P < 0.05. The results are representative of more than three individual experiments. Additional details are provided in the SI Materials and Methods.

ACKNOWLEDGMENTS. The methods for human osteocalcium isolation and differentiation were instructed by Dr. Toru Yago (Tokyo Women’s Medical University). The anti-alkaline phosphatase primary antibody was generously provided by Dr. Kimimitsu Oda (Niigata University). This work was supported by Grants-in-Aid for Scientific Research (KAKENHI, 24791567, 26893068, and 16H06276). This work was also supported by the Core Research for Evolutional Science and Technology (JP17gm0610008) and the Japan Agency for Medical Research and Development (JP17gm0210008).

1. Kingsley LA, Fournier PG, Chirgwin JM, Guise TA (2007) Molecular biology of bone metastasis. Mol Cancer Ther 6:2609–2617.
2. Papachristou DJ, Basdra EK, Papavassiliou AG (2012) Bone metastases: Molecular mechanisms and novel therapeutic interventions. Med Res Rev 32:611–636.
3. Dai J, et al. (2004) Vascular endothelial growth factor contributes to the prostate cancer-induced osteoblast differentiation mediated by bone morphogenic protein-4. Cancer Res 64:984–999.
4. Dooloff NG, et al. (2005) Bone-metastatic potential of human prostate cancer cells correlates with Akt/ERK activation by alpha platelet-derived growth factor receptor. Oncogene 24:6848–6854.
5. Ji YJ, et al. (2003) A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. Proc Natl Acad Sci USA 100:10954–10959.
6. Farrugia AN, et al. (2003) Receptor activator of nuclear factor-kappa ligand expression by human myeloma cells mediates osteoclast formation in vitro and correlates with bone destruction in vivo. Cancer Res 63:5438–5445.
7. Bouchabara A, et al. (2004) Platelet-derived lysophosphatic acid supports the progression of osteolytic bone metastases in breast cancer. J Clin Invest 114:1714–1725.
8. Bartel DP (2004) MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116:281–297.
9. Hobert O (2008) Gene regulation by transcription factors and microRNAs. Science 319:1785–1788.
10. Kloosterman WP, Plasterk RH (2006) The diverse functions of microRNAs in animal development and disease. Dev Cell 11:441–450.
11. Inose H, et al. (2009) A microRNA regulatory mechanism of osteoblast differentiation. Proc Natl Acad Sci USA 106:20794–20799.
12. Fukuda T, et al. (2015) MicroRNA-145 regulates osteoblastic differentiation by targeting the transcription factor CbfB. FEBS Lett 589:3302–3308.
13. Suetugu A, et al. (2013) Imaging exosome transfer from breast cancer cells to stroma at metastatic sites in orthotopic nude-mouse models. Adv Drug Deliv Rev 65:383–390.
14. Kosaka N, Yoshikoa Y, Fujita Y, Ochiai T (2016) Versatile roles of extracellular vesicles in cancer. J Clin Invest 126:1163–1172.
15. Thalmann GN, et al. (1994) Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. Cancer Res 54:2577–2581.
16. Yoneya T, Williams PJ, Hiraga T, Niewolna M, Nishimura R (2001) A bone-seeking clonal cell line exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone in vivo and in vitro. J Bone Miner Res 16:1486–1495.
17. Agarwal V, Bell GW, Nam JW, Bartel DP (2015) Predicting effective microRNA target sites in mammalian mRNAs. elife 4:e05005.
18. Wong N, Wang X (2015) miRDB: An online resource for microRNA target prediction and functional annotations. Nucleic Acids Res 43:D146–D152.
19. Betel D, Koppal A, Agius P, Sander C, Leslie C (2010) Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. Genome Biol 11:R90.
20. Deveh H, Grett N (2015) miRWalk2.0: A comprehensive atlas of microRNA-target interactions. Nat Methods 12:697.
21. Futakuchi M, et al. (2009) Transforming growth factor-beta signaling at the tumor-bone interface promotes mammary tumor growth and osteoclast activation. Cancer Cell 100:71–81.