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厚生労働科学研究費補助金

第３次対がん総合戦略研究事業

脳転移性エクソソームによる前転移ニッシェの解明

平成２４年度　総括・分担研究報告書

研究代表者　落谷　孝広

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目　次

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

脳転移性エクソソームによる前転移ニッシェの解明および研究の総括
研究代表者　落谷孝広　国立がん研究センター研究所分子細胞治療研究分野・分野長

研究要旨
本研究の目的は、乳がん細胞の血液脳関門(BBB)通過の分子メカニズムや、その前転移ニッシェの分子機構の解明、脳転移機構におけるトロピズムを乳がん細胞の分泌する小胞顆粒であるエクソソームを中心に明らかにすることで、癌の脳転移を予防する新しい方策を開発することである。平成２４年度は、おもに脳転移における癌細胞が BBB を通過する仕組みを、細胞間の新たなコミュニケーションツールであるエクソソームの本体解明を中心に解析する目的で、脳に高転移性を示すヒト乳がん細胞株を樹立し（落谷、小野）、この乳がん細胞株が分泌するエクソソームが BBB にどのような影響を与えるかを解析した。その結果，脳に高転移する乳がん細胞から分泌されたエクソソームが，BBB の機能を破壊し，もともと BBB を通過しないはずの低転移乳がん細胞を BBB 通過させうることがわかった。さらに、このエクソソームの BBB 破壊は，脳血管内皮細胞のタイトジャンクションを司る分子の制御にかかわっていた。

A．研究背景、目的
（背景）

癌の脳転移は近年増加傾向にある。とりわけ、乳がんでは、ある特定のサブタイプに脳転移を多く認め、その生物学的特性と転移臓器におけるトロピズムの存在が示唆される。近年の分子標的治療薬の進歩により生存期間が延長した癌患者に脳転移は今後頻発すると予測され、我が国において脳転移の治療と管理法の開発は緊急かつ重要な課題である。癌転移のメカニズムには、癌細胞が脳血管関門(BBB)を通過し、脳内で腫瘍を形成できるよう、あらかじめ血管内皮細胞や間質成分などがニッシェ（前転移ニッシェ）を形成することが癌細胞の定着や増殖の最初のプロセスに重要である。本研究の目的は、BBB通過の分子メカニズムや、その前転移ニッシェの分子機構の解明、脳転移機構におけるトロピズムを乳がん細胞の分泌する小胞顆粒であるエクソソームを中心に明らかにすることで、癌の脳転移を予防する新しい方策を開発することである。

B．研究方法

平成２４年度は、おもに脳転移における癌細胞が BBB を通過する仕組みを、細胞間の新たなコミュニケーションツールであるエクソソームの本体解明を中心に解析する目的で、脳に高転移性を示すヒト乳がん細胞株を樹立し（落谷、小野）、この乳がん細胞株が分泌するエクソソームが BBB にどのような影響を与えるかを、BBB の電気抵抗値の測定による破壊の有無の判定および BBB の本質である脳血管内非細胞の形成するタイトジャンクションへの影響を免疫組織学的に解析する方法を選択した。

（倫理面への配慮）

遺伝子組み換え生物等の使用等の規制による生物多様性の確保に関する法律（「バイオセーフティに関するカルタヘナ議定書」に基づくカルタヘナ法）」の定める細則と、文部科学省・厚生労働省・経済産業省の定める細則、ならびに施設内の組み換え DNA 実験指針の基準に従って、定められた基準に適合することを確認し、指針に従って DNA 組み換え実験委員会等の倫理審査委員会の審査を経る手続きを適切に行う。動物実験は、国立がん研究センターの定める動物実験指針に従うとともに、動物倫理委員会の承認を得たうえで、動物の苦痛の低減に務め、動物愛護の精神に基づく実験を行う。ヒトの臨床サンプル解析に関しては、センターの倫理審査委員会の承認を得て実施する。

C．研究結果

まず、ヒト乳がん細胞株 MDA-MB-231LN 細胞（低い脳転移率）を scid マウスに左心室投与で移植し，1/5 の低頻度で脳転移したマウスから転移腫瘍を摘出し，培養，移植のサイクルを繰り返した結果，3/5 の高頻度で脳転移する細胞株を２株樹立した。これらの脳高転移乳がん細胞株の分泌するエクソソームを超高速遠心法にて分離精製し、その生物学的性状を BBB に与える影響を観察する事で検討した。BBB は、サルの脳血管内被細胞，pericyte（血管周囲細胞）、及びアストロサイトから構成される BBB を in vitro で模倣したシステムを用いた。結果の解釈は，BBB の形成により生まれる電気的抵抗値を変動させるかどうかで判断した結果，低転移の細胞由来のエクソソームに比較して，高転移の細胞由来のエクソソームを添加する事で，電気抵抗値が顕著に低下するこ

とが明らかとなった。さらに small compound の透過性を検証したところ、やはり高転移の細胞由来のエクソソームの処理によって，BBB の物質透過性が更新する事実が判明した。以上の成果は，高転移の細胞由来のエクソソームには BBB を破綻させる能力が有る事が示唆された。さらに、血管内皮細胞のタイトジャンクションを形成する occludin, claudin 等の分子の免疫染色を実施した結果，高転移の細胞由来のエクソソーム処理によって，これらの分子の細胞表面の局在が失われることが判明した。

D．考察

初年度の計画に照らし合わせると，こうしたエクソソームによって引き起こされる BBB 破壊のメカニズムを明らかにするところまでは至らなかったが，すでに低転移および高転移に由来する２種類のエクソソームの解析を，プロテオームおよび microRNA の２つの手法で分析を終え，その分析を急いでいる。初年度の結果はこれまでに知られていなかった、がん細胞の分泌するエクソソームによる BBB の破綻の存在を示唆する結果であり，今後も分子メカニズムの解明や，臨床けんたいにおける BBB 破壊能力を持つエクソソームの発見を注意新に研究を続ける必要が有る。

E．結論

転移性乳癌細胞の分泌するエクソソームには、血液脳関門を破壊し，乳がん細胞を脳に転移するし機構が存在する事が示唆された。

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G. 知的財産権の出願・登録状況（予定を含む。）

1.特許取得
特になし。
2.実用新案登録
特になし。
3.その他
特になし。

厚生労働科学研究費補助金（第3次対がん総合戦略研究事業）
分担研究報告書

脳転移を規定するnon-coding RNAのエピゲノムプロファイリング

研究分担者　畑田出穂　群馬大学生体調節研究所

研究要旨

転移能、薬剤耐性を獲得した癌細胞でのエピゲノムの変化、特にDNAのメチル化の変化は癌細胞の性質を決定づける重要な因子である。脳転移の関連したmiRNAの転写調節領域でのDNAのメチル化の変化はエクソソーム内でのmiRNAの量的変化への関連が予想される。本研究ではmiRNAの転写調節領域でのDNAのメチル化を次世代シーケンサーを用いて調べた。調べた対象は乳癌細胞のMCF7と高転移性をもつ乳癌細胞のMCF7/ADRである。その結果、様々な既知の転移能に関連したmiRNAの転写調節領域のDNAのメチル化がMCF7とMCF7/ADRで異なっていることがわかった。このことから今回みつかってきたDNAメチル化が異なるmiRNAの中に未知の転移能と関連をもったものが含まれていることが示唆される。

A. 研究背景、目的
（背景）

癌細胞におけるエピゲノムの変化、特にDNAメチル化の変化は癌化、癌の転移能、薬剤耐性など悪性化に関連した様々な性質において重要な働きをしていることが知られている。小分子RNAのひとつであるmiRNAの発現変化は癌化やその悪性化において重要な働きをしていることがわかってきている。これらmiRNAをコードする遺伝子においてもDNAのメチル化の変化は通常の遺伝子と同様、癌細胞で変化が見られることが知られているが、その網羅的な解析はこれまであまりおこなわれていない。

一方、細胞が分泌するエンドソーム由来の小胞顆粒であるエクソソームの中に、miRNAが安定して存在することが発見され、細胞間のメッセンジャーとして機能することが示唆されている。特にがん患者の血清中のエクソソームには健常人と異なる種類と量のmiRNAが含まれていることが報告されており、バイオマーカーとしても注目されている。このような変化は癌細胞中におけるmiRNAの発現量の変化とも関係しており、癌細胞におけるエピゲノムの研究が重要であることがわかる。またがん細胞は、比較的たくさんのエクソソームを分泌する性質をもつ傾向にあり、転移とも密接にかかわっていることがわかってきている。

B. 研究方法

本研究では乳癌細胞のMCF7と高転移性をもつ乳癌細胞のMCF7/ADRを用いて網羅的なDNAメチル化解析をおこなった。方法としては今回我々が開発したMBD1-DIP Seq法を用いた。この方法ではメチル化されたDNAに結合するたんぱく質であるMBD1のメチル化結合ドメインをクローニングしてHisTagに融合したたんぱく質がメチル化DNAに結合することを利用してメチル化DNAを濃縮する。そして回収したメチル化されたDNAからライブラリーを作成し次世代シーケンサー(Illumina)で解析した。ゲノムにマップされたリードの数がメチル化量を表す。今回の目的ではmiRNAをコードする遺伝子の転写開始点近傍のDNAメチル化の解析をおこなった。

（倫理面への配慮）

今回の解析では該当しない。

C. 研究結果

解析の結果、様々なmiRNAにおいてDNAのメチル化がみられた。またDNAのメチル化は発現量と逆相関していることもわかった。さらに個々のmiRNAについてbisulfite sequencing法で次世代シーケンスの結果が正しいことも立証された。

さて乳癌細胞のMCF7と高転移性をもつ乳癌細胞のMCF7/ADRのDNAメチル化の違いをみていくと多くのmiRNAにおい

て差が見られることがわかった。またそれらは発現の違いとも逆相関していた。これらの中にはこれまで転移能と関係しているものも含まれていた。例えばmiR-10bは今回、MCF7/ADRで脱メチル化され発現が上昇していることがわかったが、このmiRNAは乳癌の転移能と関係していることが報告されている。また転移の見られる乳癌患者の血清中でmiR-10bの量が増えていることも知られている。miR-222も上皮間葉移行(EMT)や転移に関与することが知られているmiRNAであるが、MCF7/ADRで脱メチル化され発現が上昇していることがわかった。miR-222も乳癌患者の血清中で量が増えていることが報告されている。

D. 考察

今回の結果から次世代シーケンサーを用いたメチル化解析法であるMBD1-DIP Seq法でmiRNA遺伝子のメチル化が良好に解析できることがわかった。さらにこの方法により高転移能を持つ乳癌細胞でメチル化と発現が変化している遺伝子を多数みつけることができた。これらの中には転移に関与して乳癌患者の血清中での量が変化しているものも存在し、新たな転移能に関係したエクソソームmiRNAの発見につながることが期待される。

E. 結論

転移性乳癌細胞の網羅的DNAメチル化解析をMBD1-DIP Seq法を用いておこない、転移に関連するmiRNAの候補をみつけだした。

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(予定を含む。)

1.特許取得
特になし。
2.実用新案登録
特になし。
3.その他
特になし。

別紙４

研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

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Competitive Interactions of Cancer Cells and Normal Cells via Secretory MicroRNAs*[S]

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Nobuyoshi Kosaka[‡1], **Haruhisa Iguchi**[‡§1], **Yusuke Yoshioka**[‡2], **Keitaro Hagiwara**[‡¶], **Fumitaka Takeshita**[‡], **and Takahiro Ochiya**[‡3]

From the [‡]*Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan,* [§]*Pharmacology Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd., 1-98, Kasugadenaka 3-chome, Konohana-ku, Osaka 554-0022, Japan, and the* [¶]*Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Kanagawa 226-8501, Japan*

Background: Homeostatic cell competitive system between cancerous cells and non-cancerous cells is considered as the reason for tumor initiation.
Results: Exosomal tumor-suppressive microRNAs secreted by non-cancerous cells inhibit the proliferation of cancerous cells.
Conclusion: Exosomal tumor-suppressive microRNAs act as an inhibitory signal for cancer cells in a cell-competitive process.
Significance: This provides a novel insight into a tumor initiation mechanism.

Normal epithelial cells regulate the secretion of autocrine and paracrine factors that prevent aberrant growth of neighboring cells, leading to healthy development and normal metabolism. One reason for tumor initiation is considered to be a failure of this homeostatic cell competitive system. Here we identify tumor-suppressive microRNAs (miRNAs) secreted by normal cells as anti-proliferative signal entities. Culture supernatant of normal epithelial prostate PNT-2 cells attenuated proliferation of PC-3M-luc cells, prostate cancer cells. Global analysis of miRNA expression signature revealed that a variety of tumor-suppressive miRNAs are released from PNT-2 cells. Of these miRNAs, secretory miR-143 could induce growth inhibition exclusively in cancer cells *in vitro* and *in vivo*. These results suggest that secretory tumor-suppressive miRNAs can act as a death signal in a cell competitive process. This study provides a novel insight into a tumor initiation mechanism.

Competitive interactions among cells are the basis of many homeostatic processes in biology. In *Drosophila*, normal epithelial cells compete with transformed ones for individual survival, which is a process called cell competition (1, 2). If a given group of cells was exposed to some stress, it would be separated into subpopulations of cells with different levels of damage. In noncompetitive conditions, cells with severe damage die in a short time, whereas moderately damaged cells survive to the next generation, indicative of the transduction of a negative phenotype. On the other hand, in competitive conditions even slightly damaged cells are eliminated from the cell group because healthy cells, the "winners," convey death signals to damaged cells, the "losers," and the losers reciprocally confer growth signals to the winners. This feed-forward regulation enables the cell population to eradicate abnormal cells and maintain the same number of normal cells in a limited niche.

Oncogenesis is characterized by genetic and metabolic changes reprogramming living cells to undergo uncontrolled proliferation (3). This suggests that the abnormal cells that are originally destined for elimination can survive and expand against the cell competitive regulation, leading to the formation of a tumor mass. Consistently with this concept, Bondar and Medzhitov (4) showed that the cell competition process involves p53, a tumor-suppressive gene, between the hematopoietic stem cells and progenitor cells, suggesting that gene modifications of p53 could disturb the homeostatic mechanism and give rise to tumor initiation. It is conceivable that p53 target genes could be associated with intercellular communication between winners and losers; however, this literature has not answered the question of whether this regulatory system is mediated by contact-dependent or contact-independent manner. More than 10 years ago a pioneer study suggested that non-cancerous cells co-cultured with cancer cells inhibit the growth of cancer cells *in vitro* (5). This result indicated that humoral factors could be involved in cell competition as intercellular communicators (6).

As recently as a few years ago it was believed that RNAs could not behave as extracellular signal molecules because of their vulnerability to the attack of ribonucleases largely existing in body fluid. Evidence is presently increasing to show that miRNAs[4] contained in exosomes are released from mammalian

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[1] Both authors contributed equally to this work.
[2] A Research Fellow of the Japan Society for the Promotion of Science.
[3] To whom correspondence should be addressed: Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 1-1, Tsukiji, 5-chome, Chuo-ku, Tokyo 104-0045, Japan. Tel.: 81-3-3542-2511 (ext. 4800); Fax: 81-3-3541-2685; E-mail: tochiya@ncc.go.jp.

[4] The abbreviations used are: miRNA, microRNA; CM, conditioned medium; luc, luciferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; QRT-PCR, quantitative real time PCR.

cells and act as a signal transducer (7). It is important that many different tumor-suppressive miRNAs, such as miR-16 and miR-143, are down-regulated in cancer cells, resulting in tumorigenesis, tumor progression, and metastasis (8–11). Taken together, these findings suggest that secretory miRNAs may have favorable aspects for anti-proliferative signals mediating cell competition.

In this report we show that miR-143 expression in normal prostate cells, PNT-2 cells, is higher than that in prostate cancer cells, PC-3M-luc cells, and that miR-143 released from non-cancerous cells transfers growth-inhibitory signals to cancerous cells *in vitro* and *in vivo*. These results suggest that secretory tumor-suppressive miRNAs might be a death signal from winners to losers in the context of cell competition. Secretory miRNAs can be conducive to the maintenance of normal growth and development.

EXPERIMENTAL PROCEDURES

Reagents—Mouse monoclonal anti-KRAS (F234) (sc-30) was purchased from Santa Cruz. Rabbit polyclonal anti-ERK5 (#3372) was purchased from Cell Signaling. Mouse monoclonal anti-actin, clone C4 (MAB1501), was obtained from Millipore. Mouse monoclonal ant-human-CD63 antibody (556019) was purchased from BD Pharmingen. Peroxidase-labeled anti-mouse and anti-rabbit antibodies were included in the Amersham Biosciences ECL PLUS Western blotting Reagents Pack (RPN2124) (GE Healthcare). Synthetic *Caenorhabditis elegans* miRNA cel-miR-39 was synthesized by Qiagen (Valencia, CA). Synthetic hsa-miR-143 (pre-miR-143), the negative control 1 (NC1), has-miR-143 inhibitor molecule (anti-miR-143), and the negative control inhibitor molecule (anti-NC) were purchased from Ambion (Austin, TX). GW4869 was purchased from Calbiochem. Geneticin was purchased from Invitrogen.

Cell Culture—PNT-2 cells, immortalized normal adult prostatic epithelial cell line, were purchased from the DS Pharma Biomedical Co., Ltd. (Osaka, Japan). HEK293 cells, a human embryonic kidney cell line (CRL-1573), were obtained from American Type Culture Collection (Manassas, VA). HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS) and an antibiotic-antimycotic (Invitrogen) at 37 °C in 5% CO_2. PNT-2 and the prostate cancer cell line, PC-3M-luc cells, continuously expressing firefly luciferase (Xenogen, Alameda, CA), were cultured in RPMI containing 10% heat-inactivated FBS and an antibiotic-antimycotic at 37 °C in 5% CO_2.

Preparation of Conditioned Medium and Exosomes—Before the collection of culture medium, cells were washed 3 times with Advanced RPMI containing an antibiotic-antimycotic and 2 mM L-glutamine (medium A), and the medium was switched to fresh medium A. After incubation for 3 days, medium A was collected and centrifuged at 2000 × *g* for 10 min at room temperature. To thoroughly remove cellular debris, the supernatant was centrifuged again at 12,000 × *g* for 30 min at room temperature or filtered through a 0.22-μm filter (Millipore). The conditioned medium (CM) was then used for miRNA extraction and functional assays as well as exosome isolation.

For exosome preparation the CM was ultracentrifuged at 110,000 × *g* for 70 min at 4 °C. The pellets were washed with 11 ml of PBS, and after ultracentrifugation they were resuspended in PBS. The exosome fraction was measured for its protein content using the Micro BCA Protein Assay kit (Thermo Scientific, Wilmington, DE).

Isolation of MicroRNAs—Isolation of extracellular and cellular miRNAs was performed using the miRNeasy Mini Kit (Qiagen). Two hundred microliters of conditioned medium or cell lysate was diluted with 1 ml of Qiazol Solution. After 5 min of incubation, 10 μl of 0.1 nM cel-miR-39 was added to each aliquot followed by vortexing for 30 s. Subsequent extraction and filter cartridge work were carried out according to the manufacturer's protocol.

Quantitative Real Time PCR (QRT-PCR)—The method for QRT-PCR has been previously described (7). PCR was carried out in 96-well plates using the 7300 Real Time PCR System (Applied Biosystems). All reactions were done in triplicate. All TaqMan MicroRNA Assays were purchased from Applied Biosystems. Cel-miR-39 and RNU6 were used as an invariant control for the CM and cells, respectively.

Immunoblot Analysis—SDS-PAGE gels, SuperSep Ace 5–20% (194–15021) (Wako), were calibrated with Precision Plus Protein Standards (161–0375) (Bio-Rad), and anti-KRAS (1:100), anti-ERK5 (1:1000), anti-CD63 (1:200), and anti-actin (1:1000) were used as primary antibodies. The dilution ratio of each antibody is indicated in parentheses. Two secondary antibodies (peroxidase-labeled anti-mouse and anti-rabbit antibodies) were used at a dilution of 1:10,000. Bound antibodies were visualized by chemiluminescence using the ECL PLUS Western blotting detection System (RPN2132) (GE Healthcare), and luminescent images were analyzed by a LuminoImager (LAS-3000; Fuji Film, Inc.). Only gels for CD63 (BD Biosciences) detection were run under non-reducing conditions.

Plasmids—The primary-miR-143 expression vector was purchased from TaKaRa BIO. For luciferase-based reporter gene assays, pLucNeo was constructed by inserting a firefly luciferase gene derived from the pGL3-control (Promega) into the pEYFP-1 vector (Clontech) at BglII and AflII sites. The sensor vector for miR-143 was constructed by introducing tandem binding sites with perfect complementarity to miR-143 separated by a four-nucleotide spacer into the NotI site of psiCHECK2 (Promega). The sequences of the binding site are as follows: 5′-AAACCTAGAGCGGCCGCGAGCTACAGTGCT***TCATCTC***AAAGAATTCTTGAGCTACAGTGCT***TCATCTC***AGCGGCCGCTGGCCGCAA-3′ (sense) and 5′-TTGCGGCCAGCGGCCGCTGAGATGAAGCACTGTAGCTCAAGAATTCTTTGAGATGAAGCACTGTAGCTCGCGGCCGCTCTAGGTTT-3′ (antisense). The "seed" sequence of miR-143 is indicated by bold italics. In a mutated miR-143 sensor vector, the seed sequence, TCATCTC, was displaced with GACGAGA. All the plasmids were verified by DNA sequencing.

Transient Transfection Assays—Transfections of 10 nM miR-143 mimic and 3 nM anti-miR-143 were accomplished with the DharmaFECT Transfection Reagent (Thermo Scientific) according to the manufacturer's protocol. The total amounts of miRNAs for each transfection were equally adjusted by the addition of NC1 and anti-NC, respectively.

Establishment of Stable Cell Lines—Stable HEK293 cell lines that express miR-143 were generated by selection with 300 μg/ml Geneticin. HEK293 cells were transfected with 0.5 μg of the pri-miR-143 expression vector at 90% confluency in 24-well dishes using a Lipofectamine LTX reagent in accordance with the manufacturer's instructions. Twelve hours after the transfection, the cells were re-plated in a 10-cm dish followed by a 3-week selection with the antibiotic. Ten surviving single colonies were picked up from each transfectant and then cultured for another 2 weeks. The cells expressing the largest amount of miR-143 among transfectants were used as miR-143 stably expressing cells.

Luciferase Reporter Assay—HEK293 cells were cultured at a density of 1×10^4 cells/well in 96-well tissue culture plates overnight, and miRNA transfections or the addition of CM was performed. The cells were harvested, and renilla luciferase activity was measured and normalized by firefly luciferase activity (10). All assays were performed in triplicate and repeated at least three times, and the most representative results are shown.

Cell Growth Assay—PC-3M-luc cells were seeded at a density of 2×10^3 cells/well in a 96-well plate. The following day the cells were transfected with mature miRNAs or incubated with a CM. Twenty-four hours later the culture medium of the transfected cells was switched to medium A, whereas the conditioned medium was not changed. After a 3-day culture, cells were harvested for the measurement of firefly luciferase activity. To know the cellular proliferation by the tetrazolium-based colorimetric MTT assay, 20 μl CM of TetraColor ONE (SEIKAGAKU Corp., Tokyo, Japan) was added to each well after 72 h of culture. After 2–4 h of incubation at 37 °C, the optical density was measured at a wavelength of 450 nm using a microplate reader.

PKH67-labeled Exosome Transfer—Purified exosomes derived from PNT-2 CM were labeled with a PKH67 green fluorescent labeling kit (Sigma). Exosomes were incubated with 2 μM PKH67 for 5 min, washed 4 times using a 100-kDa filter (Microcon YM-100, Millipore) to remove excess dye, and incubated with PC-3M-luc cells at 37 °C.

Co-culture Experiment—In co-culture experiments, 2×10^5 cells/well of PNT-2 cells were plated in 6-well plates. To stain the PNT-2 cells with BODIPY-TR-ceramide (Invitrogen), 5 μM BODIPY-TR-ceramide in a non-serum culture medium was added and incubated with the cells at 37 °C. After 30 min the cells were rinsed several times with a non-serum culture medium and incubated in a fresh medium at 37 °C for an additional 30 min. After the staining of PNT-2 cells by BODIPY-TR-ceramide, labeling of PC-3M-luc cells with PKH67 was performed in accordance with the manufacturer's instructions. After that, labeled PC-3M-luc cells were added and co-cultured with PNT-2 cells for 12 h at 37 °C.

Microarray Analysis—To detect the miRNAs in exosomes and cells derived from PNT-2 and PC-3M-luc cells, 100 ng of total RNA was labeled and hybridized using a human microRNA microarray kit (Agilent Technologies) according to the manufacturer's protocol (Protocol for Use with Agilent MicroRNA Microarrays Version 1.5). Hybridization signals were detected using a DNA microarray scanner (Agilent Technologies), and the scanned images were analyzed using Agilent Feature Extraction software.

Evaluation of Tumor-suppressive miRNA Delivery to Subcutaneously Implanted Prostate Cancer Cell Line in Mice—Animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. Seven-week-old male Balb/c athymic nude mice (CLEA Japan, Shizuoka, Japan) were anesthetized by exposure to 3% isoflurane for injections and *in vivo* imaging. Four days ahead of the first CM injection, the anesthetized animals were subcutaneously injected with 5×10^5 PC-3M-luc cells suspended in 100 μl of sterile Dulbecco's phosphate-buffered saline into each dorsal region. Five hundred μl of CM derived from miR-143-overexpressing HEK293 cells and control cells were daily injected into each tumor from day 0 to 6. For *in vivo* imaging, the mice were administered D-luciferin (150 mg/kg, Promega) by intraperitoneal injection. Ten minutes later, photons from animal whole bodies were counted using the IVIS imaging system (Xenogen) according to the manufacturer's instructions. Data were analyzed using LIVINGIMAGE 2.50 software (Xenogen).

RESULTS

Suppression of Prostate Cancer Cell Proliferation by Conditioned Medium Isolated from Non-cancerous Prostatic Cell—Cell competition is a homeostatic mechanism for the accommodation of an appropriate number of cells in a limited niche or stroma (1). Based on this idea it is possible that the cell competition between normal and abnormal cells frequently occurs in a precancerous state. Of note is that non-cancerous cells suppress cancer cell development by contact-independent interaction (12). For instance, endothelial cells provide the major extracellular heparan sulfate proteoglycan as anti-proliferative signals (12); however, the molecular mechanism by which the other types of cells in a tumor environment associate with cancer cells is not fully understood.

To analyze the mechanism, we treated a hormone-insensitive prostatic carcinoma cell line, PC-3M-luc cells, with a CM from the non-cancerous prostate cell line PNT-2 cells. After a 3-day incubation, the PNT-2 CM inhibited the growth of the PC-3M-luc cells up to ~10% compared with the cell growth treated by fresh culture medium (Fig. 1*A*; compare *lanes 1* and *3*). In contrast, the growth of PC-3M-luc cells incubated in the CM of PC-3M-luc cells themselves showed no inhibitory effect (Fig. 1*A*; compare *lanes 1* and *2*). To determine that the performed treatments did not affect the luciferase activity, we also used the colorimetric MTT assay to measure the cell growth of PC-3M-luc cells. As shown in supplemental Fig. 1*A*, not only luciferase assay but also MTT assay show the inhibition of PC-3M-luc cell proliferation by the addition of PNT-2 cells derived CM, indicating that our treatment did not affect the luciferase activity. These results indicate that the non-cancerous cells may secrete some molecules that can suppress cancer cell proliferation.

In a recent report we showed that miRNAs contained in exosomes are secreted and that their secretion is tightly regulated by neutral sphingomyelinase 2, which is known to hydrolyze sphingomyelins to generate ceramides and trigger the budding

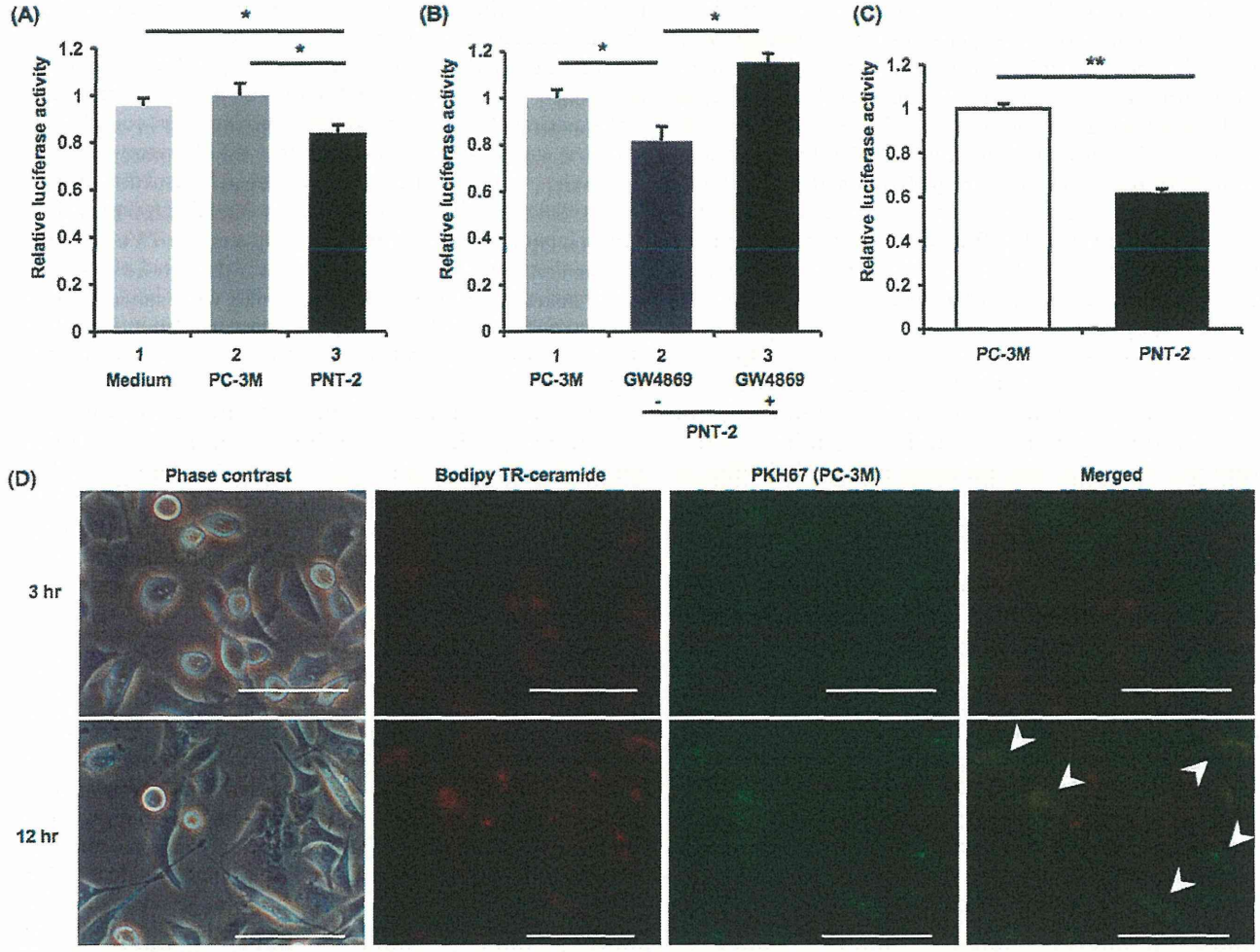

FIGURE 1. **Suppression of cancerous cell proliferation by exosome isolated from non-cancerous cells.** *A*, cell growth inhibition by a conditioned medium derived from PNT-2 cells is shown. PC-3M-luc cells were incubated for 3 days in a conditioned medium isolated from PC-3M-luc cells, PNT-2 cells, or a culture medium followed by a cell growth assay as described under "Experimental Procedures." The values on the *y* axis are depicted relative to the normalized luciferase activity of culture medium-treated cells, which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). *, $p < 0.05$ as compared with culture medium-treated PC-3M-luc cells; Student's *t* test. *B*, treatment with GW4869 to donor cells restored the reduced cell growth by the PNT-2-derived CM is shown. Donor PNT-2 cells were incubated in the presence or absence of 10 μM GW4869 for 2 days. The conditioned medium from PC-3M-luc cells was used as a control. The values on the *y* axis are depicted relative to the normalized luciferase activity of PC-3M-luc-conditioned medium-treated cells, which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). *, $p < 0.05$; Student's *t* test. *C*, cell growth inhibition by exosomes derived from PNT-2 cells is shown. PC-3M-luc cells were incubated in exosomes isolated from PNT-2 cells or PC-3M-luc cells followed by a cell growth assay, as described under "Experimental Procedures." The values on the *y* axis are depicted relative to the normalized luciferase activity of cells treated with exosomes derived from PC-3M-luc cells is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). **, $p < 0.005$, as compared with exosomes isolated from PC-3M-luc cells; Student's *t* test. *D*, shown are fluorescent photos of BODIPY-ceramide-labeled PNT-2 and PC-3M-luc cells marked by PKH67. PNT-2 cells and PC-3M-luc cells were labeled with red fluorescent BODIPY-ceramide and green fluorescent PKH67, respectively, as described under "Experimental Procedures." After treatment of PNT-2 by BODIPY-ceramide, PKH67-labeled PC-3M-luc cells were added. After co-culturing for 3 or 12 h, images were obtained. Fluorescent photos were detected with the Eclipse TE 2000 Inverted Research Microscope, and images were produced using NIS-Elements BR software. *Arrowheads* show *yellow colored* cancer cells. The *size bar* indicates 100 μm.

of exosomes. We collected two separate aliquots of CM from PNT-2 cells incubated with or without GW4869, a specific inhibitor for neutral sphingomyelinase 2. The isolated exosomes were verified by the detection of CD63 protein, a well established exosome marker, with immunoblotting (supplemental Fig. 1*B*), and the activity of GW4869 was confirmed by the decreased amount of exosomal protein (supplemental Fig. 1*C*). The CM prepared in the presence of the GW4869 compound cancelled most tumor-suppressive activity of the non-treated PNT-2 CM (Fig. 1*B*; compare *lanes 1–3*). Furthermore, proliferation of PC-3M-luc cells was inhibited by the addition of the exosome fraction isolated from the PNT-2 CM by ultracentrifugation (Fig. 1*C*). These observations suggest that exosomal miRNAs derived from non-cancerous cells were transferred to cancerous cells, resulting in the inhibition of their proliferation.

To visualize the transfer of ceramide-containing exosome from PNT-2 to PC-3M-luc *in vitro*, a co-culture experiment was performed. Before the co-culture, 2×10^5 PNT-2 cells were incubated for 30 min with red fluorescent BODIPY-ceramide dye, which can label the exosomes inside the cells (13, 14). After washing five times with PBS, equal numbers of PC-3M-luc cells labeled by green fluorescent PKH67, a cellular membrane indicator, were added into the culture dishes. Three hours later we did not observe any PC-3M-luc cells with a yellow color (*Merged* photo in *upper panel* of Fig. 1*D*), indicating that car-

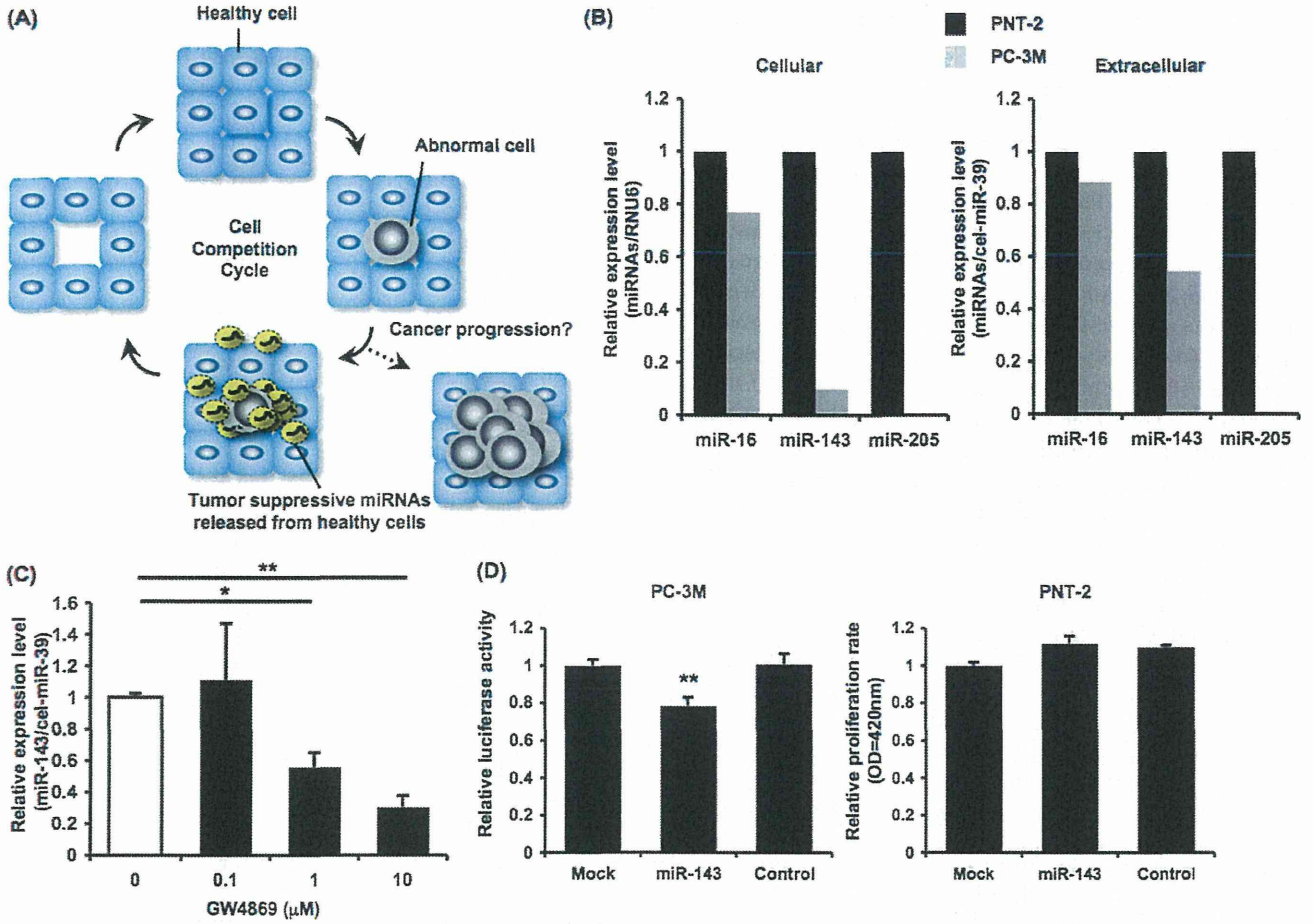

FIGURE 2. **Down-regulation of cellular and extracellular tumor-suppressive miRNAs in PC-3M-luc cells.** *A*, shown is a schematic representation of hypothetical tumor initiation process. Neighboring healthy cells (*blue*) secrete tumor-suppressive miRNAs (*light yellow*) to inhibit the proliferation of abnormal cells (gray), and this cell population returns to the initial healthy condition (a homeostatic cycle). Once the cell competitive cycle is compromised, this niche become susceptible to tumor initiation (indicated by a *dashed arrow*). *B*, comparison of cellular and extracellular miRNAs expression in PNT-2 and PC-3M-luc cells is shown. miRNA expression levels were determined by a Taq-Man QRT-PCR. The values on the *y* axis are depicted relative to the normalized expression level of PNT-2 cells, which is defined as 1. *C*, secretion of miR-143 was suppressed by the treatment with GW4869. PNT-2 cells were seeded and cultured in a 24-well plate for 48 h in the indicated concentrations of GW4869. After the incubation, the medium was subjected to QRT-PCR for miR-143. The values on the *y* axis are depicted relative to the amount of miR-143 at 0 μM GW4869, which is defined as 1. *D*, shown is cell growth inhibition by miR-143 in PC-3M-luc cells but not in PNT-2 cells. PNT-2 and PC-3M-luc cells were transfected with 10 nM miR-143 molecules (miR-143) or 10 nM negative control molecules (control) or without RNA molecules (*Mock*). The values on the *y* axis are depicted relative to the normalized luciferase activity of untreated cells (*Mock*), which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). *, $p < 0.05$; **, $p < 0.005$, as compared with untreated PC-3M-luc cells; Student's *t* test.

ried-over red dyes were thoroughly removed as 3 h is enough time for the dye to be incorporated directly into the cells. By contrast, after 12 h of co-culture, yellow fluorescence was observed in green-labeled PC-3M-luc cells (indicated by *arrowheads* in Merged photo in the *lower panel* of Fig. 1*D*), suggesting that ceramide-containing exosomes from PNT-2 cells were transferred to the PC-3M-luc cells. This result is corroborated by the uptake experiment using the PKH67-labeled exosomes purified from PNT-2 culture medium (supplemental Fig. 1*D*). Green fluorescence was detected in PC-3M-luc cells after 16 h of incubation, providing a direct evidence for exosome uptake by cancerous cells.

Tumor-suppressive miRNAs Down-regulated in Cancerous Cells Were Secreted from Non-cancerous Cells—We propose a hypothetical model of tumor initiation involving cell competition and anti-proliferative secretory miRNAs (Fig. 2*A*). In a cell competition cycle, as illustrated in the *bottom part* of Fig. 2*A*, growth inhibitory miRNAs are actively released from non-cancerous cells to kill abnormal cells with a partial oncogenic ability, thereby restoring them to a healthy state. Indeed, inhibitory capacity of these miRNAs appears to be limited in the setting of single treatment with the PNT-2 CM (Fig. 1*A*); however, they can potentially prevent emergence of tumor cells in a physiological condition. Because abundantly existing healthy cells continuously provide nascent overproliferative cells with tumor-suppressive miRNAs for a long period, a local concentration of secretory miRNAs can become high enough to restrain a tumor initiation. A *dashed arrow* in Fig. 2*A* indicates the way whereby the disruption of the homeostatic system leads to tumor expansion. If precancerous cells acquire resistance to anti-proliferative secretory miRNAs or normal cells cannot supply an adequate amount of miRNAs, then this defensive system will fail to maintain the healthy condition.

Secretory miR-143 as an Anti-cancer Signal

To test this hypothesis we checked the secretion amount of representative tumor-suppressive miRNAs by comparing PNT-2 and PC-3M-luc cells with Taq-Man QRT-PCR analysis. As shown in Fig. 2*B*, miR-16, miR-205, and miR-143, which are already reported to be dysregulated in prostate cancer (10, 15, 16), were down-regulated in PC-3M-luc cells at a cellular and extracellular level. The GW4869 inhibitor suppressed the secretion of miR-143 from PNT-2 cells in a dose-dependent manner (Fig. 2*C*), whereas its cellular level was not altered (supplemental Fig. 2*A*). Additionally, the application of small interfering RNAs specific for human neutral sphingomyelinase 2 gene knocked down its mRNAs, resulting in profound decrease in miR-143 secretion (supplemental Fig. 2, *B* and *C*). On the contrary, the expression of miR-143 in the cells was not changed after the transfection of neutral sphingomyelinase 2 siRNA (supplemental Fig. 2*D*). Taken with the result of Fig. 1*B*, these results suggest that the secreted tumor-suppressive miRNAs are implicated in the process of growth inhibition by PNT-2 CM.

For a global understanding of the expression change of non-cancerous and cancerous cells, we performed an miRNA microarray analysis against cellular and exosomal RNAs purified from PNT-2 and PC-3M-luc cells. In the sub-dataset of secretory exosomal miRNAs from PNT-2 cells, we found 40 miRNAs whose cellular amounts were lowered by one-half in PC-3M-luc cells (Table 1). The selected miRNAs expectedly include several types of tumor-suppressive miRNAs, such as miR-15a, miR-200 family, miR-148a, miR-193b, miR-126, and miR-205 (10, 15, 17–20). This observation supports the idea that secretory tumor-suppressive miRNAs are transferred from non-cancerous cells to cancerous cells, in accordance with the concentration gradient of the miRNA.

We have so far demonstrated that normal cells have a higher secretion of tumor-suppressive miRNAs than cancerous cells; however, it remains unclear whether or not these secreted miRNAs affect the proliferation of cells of their origin. To answer this question, we introduced synthesized miR-143 to both PNT-2 and PC-3M-luc cells and assessed their proliferation rates. After 3 days of transfections, the miR-143 analog induced growth inhibition of PC-3M-luc cells compared with mock and control small RNA transfection (Fig. 2*D*, *left panel*). In contrast, the exogenously transduced miR-143 did not show its anti-proliferative effect in PNT-2 cells (Fig. 2*D*, *right panel*), indicating that excessive miR-143 did not confer an additional growth inhibitory effect on normal cells in which expression of miR-143 is maintained to a physiological level. This finding suggests that animal cells may have their own threshold amount for miRNA activity. The different sensitivity found in different cell types can help secretory miRNAs fulfill their purpose to combat exclusively precancerous cells. It is possible that secretory miRNAs, at least, derived from non-cancerous cells such as PNT-2 cells could supplement growth-suppressive signals that are decreased in cancerous cells. Thus, secreted miR-143 might be involved in the cell competitive regulatory system.

TABLE 1
A list of PNT-2-derived secretory miRNAs that were down-regulated less than 0.5-fold in PC-3M cells compared with PNT-2 cells

miRNAs	Fold change[a]
hsa-miR-141	0.0
hsa-miR-200c	0.0
hsa-miR-886–3p	0.0
hsa-miR-30a*	0.0
hsa-miR-155	0.0
hsa-miR-205	0.0
hsa-miR-224	0.0
hsa-miR-148a	0.0
hsa-miR-130a	0.0
hsa-miR-30a	0.1
hsa-miR-663	0.1
hsa-miR-181a-2*	0.1
hsa-miR-484	0.1
hsa-miR-10a	0.1
hsa-miR-192	0.1
hsa-miR-193b	0.1
hsa-miR-200a	0.1
hsa-miR-429	0.1
hsa-miR-769–5p	0.1
hsa-miR-200b	0.2
hsa-miR-195	0.2
hsa-miR-203	0.2
hsa-miR-7	0.2
hsa-miR-200a*	0.2
hsa-miR-200b*	0.2
hsa-miR-30c	0.2
hsa-miR-126	0.3
hsa-miR-149	0.3
hsa-miR-30d	0.3
hsa-miR-181a	0.3
hsa-miR-30e*	0.3
hsa-miR-365	0.4
hsa-miR-135b	0.4
hsa-miR-454*	0.4
hsa-miR-129*	0.4
hsa-miR-30b	0.4
hsa-miR-181b	0.4
hsa-miR-210	0.4
hsa-miR-455–3p	0.5
hsa-miR-15a	0.5

[a] Fold change of the expression of miRNAs in PC-3M cells compared with PNT-2 cells is indicated.

Secretory miR-143 Inhibited Prostate Cancer Cell Proliferation in Vitro—To examine whether miR-143 released from normal cells exert an anti-proliferative activity, we generated HEK293 cells overexpressing miR-143 by nearly 200-fold compared with control (supplemental Fig. 3*A*). After a 3-day incubation with the CM derived from the miR-143-overproducing HEK293 cells and control HEK293 cells, PC-3M-luc cells showed an ~50% decrease in proliferation (Fig. 3*A*, *lanes 1* and *3*). Importantly, the decrease was recovered by the transfection of anti-miR-143 in PC-3M-luc cells (Fig. 3*A*, *lane 3* and *4*). These data indicate that the growth inhibition is attributable to secretory miR-143 contained in the supernatant of miR-143-overexpressing HEK293 cells. In agreement with the exosome-dependent machinery of miRNA secretion, we observed a similar result by using exosome fractions purified from miR-143-transduced HEK293 cells (Fig. 3*B*).

To further study miRNA transfer on a molecular level, we performed a target gene expression analysis and an miRNA-responsive reporter assay. The immunoblotting analysis shows that the addition of the CM isolated from miR-143-overexpressing HEK293 cells significantly knocked down expression of KRAS, a target gene for miR-143 (21), in PC-3M-luc cells (Fig. 3*C*). In addition, we implemented luciferase analyses using

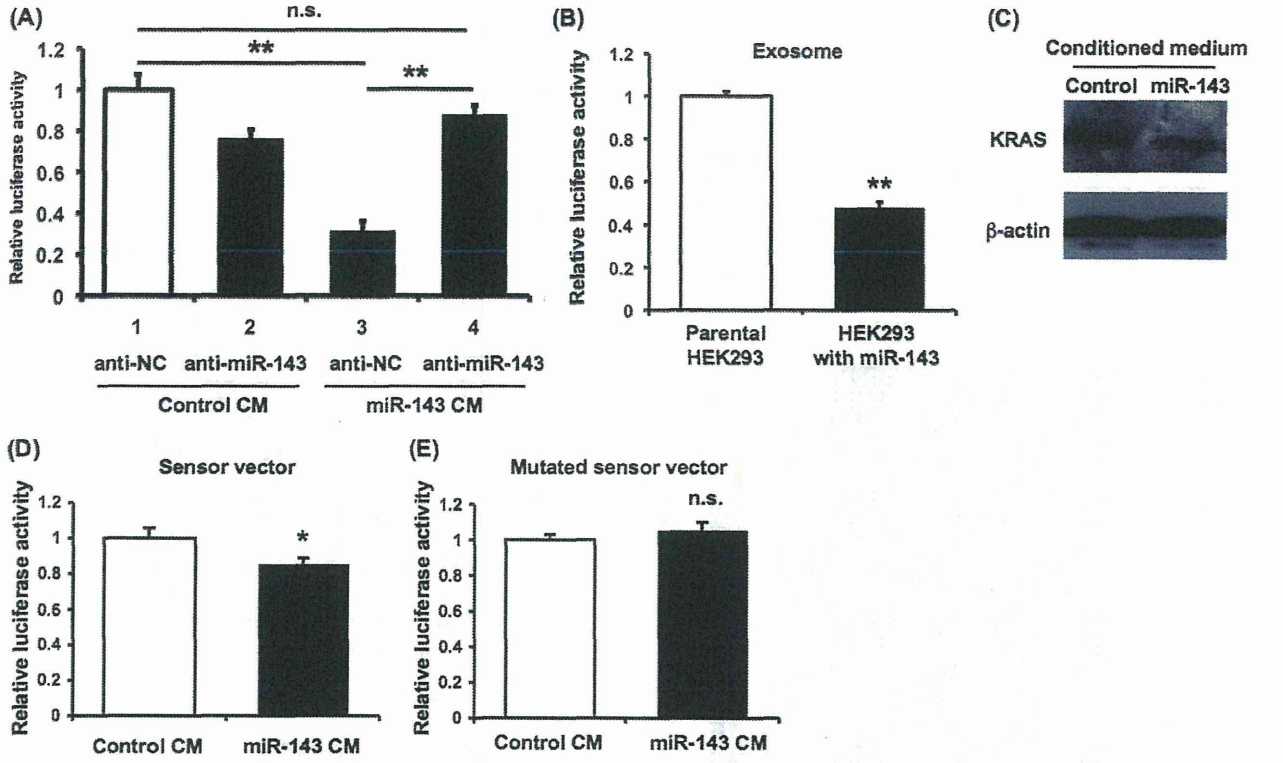

FIGURE 3. **Transfer of secretory miR-143 to PC-3M-luc cells *in vitro*.** *A*, the transfection of anti-miR-143 to PC-3M-luc cells restored the reduced cell growth by the CM derived from miR-143 overproducing cells. After the transfection with 3 nM miR-143 inhibitor molecule (anti-miR-143) (*lanes 2* and *4*) or its control molecule (*anti-NC*) (*lanes 1* and *3*), PC-3M-luc cells were incubated for 3 days in a control conditioned medium (*lanes 1* and *2*) and CM containing extracellular miR-143 (*lane 3* and *4*) followed by a cell growth assay as described under "Experimental Procedures." The values on the *y* axis are depicted relative to the normalized luciferase activity of cells treated in a culture medium, which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). (*, $p < 0.05$; Student's *t* test; *n.s.*, not significant). *B*, cell growth inhibition by exosomes derived from miR-143-transduced HEK293 cells is shown. PC-3M-luc cells were incubated in the exosomes followed by cell growth assay as described under "Experimental Procedures." The values on the *y* axis are depicted relative to the normalized luciferase activity of cells treated with exosomes derived from original HEK293 cells, defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). (**, $p < 0.005$; Student's *t* test). *C*, secretory miR-143-mediated KRAS suppression in PC-3M-luc cells is shown. Ten micrograms of protein of whole cell lysates prepared from PC-3M-luc cells treated with or without secretory miR-143 were applied to electrophoresis. Immunoblotting was performed with KRAS and actin antibodies and visualized by LAS-3000 system. *D*, extracellular miR-143 derived from HEK293 cells suppressed the luciferase activity of the sensor vector. HEK293 cells transfected with an miR-143 sensor vector were used as recipient cells. The recipient cells were incubated in a CM containing extracellular miRNAs. After a 2-day incubation, a luciferase reporter assay was performed as described under "Experimental Procedures." The values on the *y* axis are depicted relative to the normalized luciferase activity of original HEK293-conditioned medium-treated cells, which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). *, $p < 0.05$; Student's *t* test). *E*, extracellular miR-143 did not reduce the luciferase activity of the mutated sensor vector. HEK293 cells transfected with the mutated miR-143 sensor vector were used as recipient cells. The recipient cells were incubated in a conditioned medium containing extracellular miRNAs. The luciferase assay was carried out as described above. The values on the *y* axis are depicted relative to the normalized renilla luciferase activity of control cells, which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 3$). *n.s.* represents not significant.

a sensor vector harboring renilla luciferase fused in tandem with miR-143 seed sequence in the 3′-UTR. As shown in Fig. 3*D*, the normalized renilla luciferase activities were reduced by the treatment of miR-143-enriched CM derived from HEK293 cells stably expressing miR-143. In contrast, we did not detect any changes of luminescence by using a mutated vector instead of the intact sensor vector (Fig. 3*E*). Furthermore, we quantified cellular amounts of miR-143 in PC-3M-luc cells incubated with CM derived from HEK293 cells or miR-143 overproducing HEK293 cells by QRT-PCR. As shown in supplemental Fig. 3*B*, miR-143 was clearly increased at a cellular level by the treatment of the miR-143 enriched CM. These results indicate that secretory miR-143 exhibits its on-target growth-inhibitory effect in neighboring precancerous cells, thereby suppressing their disordered growth.

Secretory miR-143 Functions as Tumor Suppressor in Vivo—To our knowledge it has never been demonstrated that extracellular tumor-suppressive miRNAs can be transferred into living cells and induce phenotypic change *in vivo*. To address this possibility, we injected CM derived from miR-143 overproducing HEK293 cells or parental HEK293 cells into nude mice implanted with PC-3M-luc cells. Four days after the subcutaneous implantation, we carried out *in vivo* imaging and CM injections according to the timetable shown in Fig. 4*A*. Tumor expansions have been restrained for 8 days with intratumor administrations of miR-143 enriched CM, and consequently the tumor masses shrank by ~0.5-fold on day 8 (Fig. 4*B*). The representative luminescent images of inoculated PC-3M-luc cells on day 8 were shown in Fig. 4*C*. Consistent with the finding that miR-143 did not impair growth activity of non-cancer cells *in vitro* (Fig. 2*D*), no toxicity was observed in these mice (data not shown). In addition, the expressions of miR-143 target genes, such as KRAS and ERK5 (16, 21), were decreased after miR-143-transduced CM injections, indicative of intercellular miRNA transfer *in vivo* (Fig. 4*D*). Thus, our prostate cancer xenograft model suggests that the tumor-suppressive miRNAs

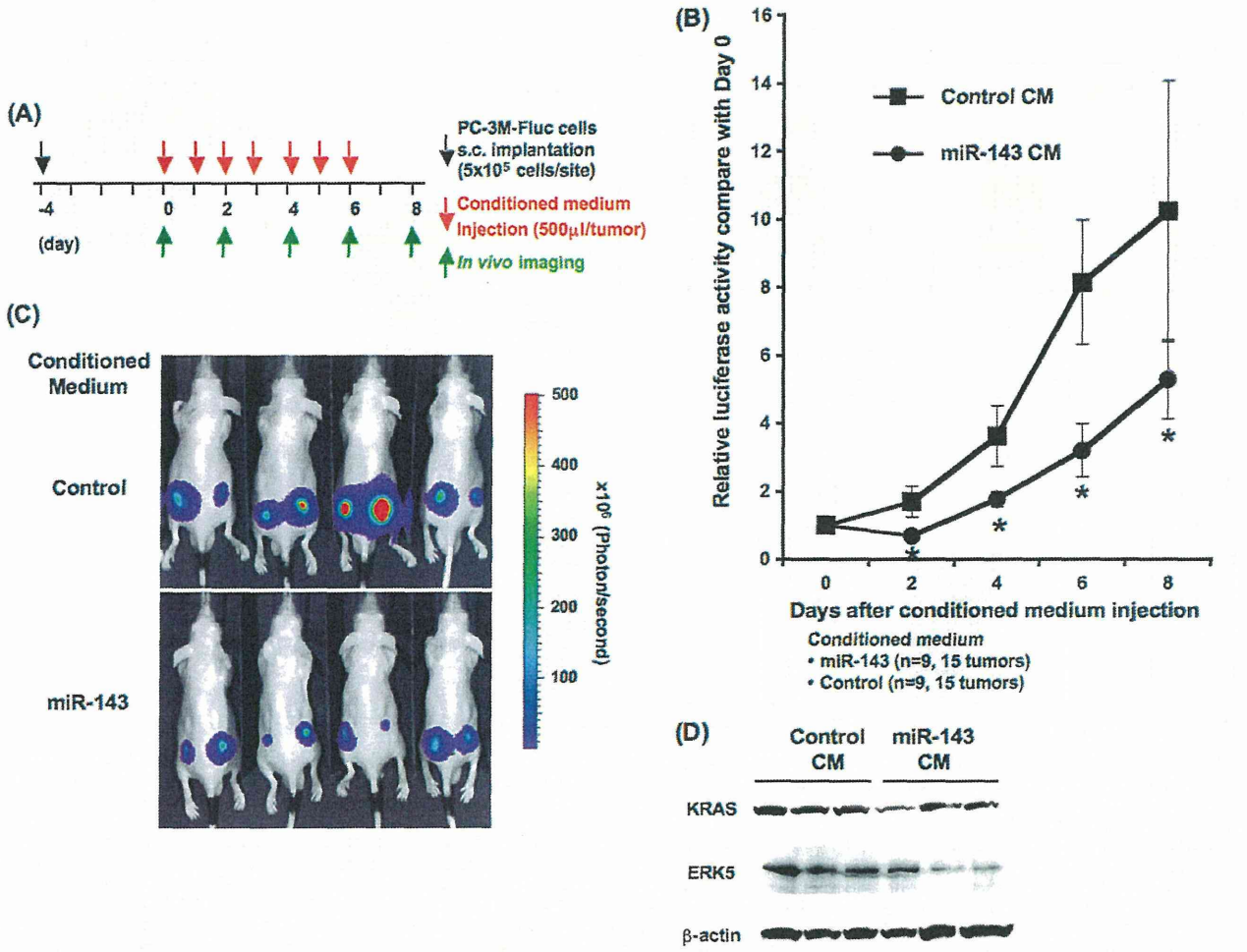

FIGURE 4. **Transfer of secretory miR-143 to PC-3M-luc cells *in vivo*.** *A*, shown is the timetable for conditioned medium injections and *in vivo* imaging. *B*, shown are tumor growth ratios of the inoculated PC-3M-luc cells during the secretory miR-143 treatment. *Closed circles* and *closed squares* indicate the tumor mass administrated with CM from miR-143-overproducing HEK293 cells or parental HEK293 cells, respectively. The values on the *y* axis are depicted relative to the luciferase activity of each tumor on day 0, which is defined as 1. Each *bar* is presented as the mean S.E. ($n = 9$). *, $p < 0.05$; Student's *t* test. *C*, representative images are shown of tumor cells in the skin of mice. Bioluminescence of firefly luciferase from miR-143-enriched CM treated mice and control mice were detected on day 8 with IVIS imaging system. *D*, shown is secretory miR-143-mediated KRAS and ERK5 suppression in inoculated tumor cells. On day 8 the inoculated tumor masses were isolated and applied to immunoblotting analysis for the quantification of KRAS and ERK5 on a protein level.

secreted from normal cells could be efficiently delivered into their neighboring tumors *in vivo*.

DISCUSSION

In this study we documented that miR-143 derived from non-cancerous cells had the ability to suppress the growth of cancer cell proliferation not only *in vitro* but also *in vivo*. These observations suggest that tumor-suppressive miRNAs can be implicated in cell competition between cancer cells and non-cancer cells. In this context, normal cells attempt to prevent the outgrowth of precancerous cells by secreting anti-proliferative miRNAs and maintain a healthy condition; however, the abnormal cells can circumvent this inhibitory machinery, finally resulting in a tumor expansion (Fig. 2*A*). Cell competition could be a homeostatic mechanism that tumor cells need to overcome (1).

Here, we discuss two possible mechanisms by which cancer cells can gain resistance to secretory tumor-suppressive miRNAs. One is a blockade for the uptake of miRNAs, and the other is a cancellation of silencing activity of the incorporated miRNAs. As previously reported, miRNAs are loaded into exosomes and then secreted from living cells (7, 22, 23). If exosomes enriched in miRNAs are actively incorporated by recipient cells, cancer cells can impair the uptake mechanism to escape from the attack of secretory tumor-suppressive miRNAs. This scenario is supported by a recent publication regarding a Tim4 expected for an exosome receptor (24).

In the latter case cancer cells need to specifically compromise the incorporated tumor-suppressive miRNAs because there are some types of miRNAs that are indispensable for the expansion of cancer cells. A RISC assembly is composed of many protein families, such as the mammalian AGO family, GW182, and heat shock proteins (25). Moreover, each gene family also consists of many members, thereby generating diversity of RISC assemblies. The heterogeneity of RISC assemblies allows tumor-suppressive miRNAs to selectively bind with a RISC and silence their target genes on the complex. If cancer cells can exclusively destroy the tumor-suppressive RISC assembly, they can safely

grow in a limited niche full of anti-proliferative miRNAs. The detailed mechanism of the resistance to cell competition remains unknown.

In addition to the acquired resistance, there is another possibility that normal cells will lose secretory capacity of exosomal miRNAs. p53 was shown to enhance exosome production in cells undergoing a p53 response to stress (26). In other words, dysfunction of p53 will result in decreased miRNA secretion. The tumor-suppressive ability of p53 can partly depend on the control of miRNA release from normal cells.

Numerous studies show a broad variety of reasons for tumor initiation, including gene amplification, cellular stress, metabolic alteration, and epigenetic changes. This work suggests that the disruption of the cell competitive process mediated by secretory miRNAs will result in the occurrence of neoplasm. Understanding the mechanism by which homeostasis is impaired leads to a novel therapeutic approach for cancer progression.

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REVIEW ARTICLE
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Unraveling the mystery of cancer by secretory microRNA: horizontal microRNA transfer between living cells

Nobuyoshi Kosaka and Takahiro Ochiya*

Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo, Japan

Edited by:
Michael Rossbach, Genome Institute of Singapore, Singapore

Reviewed by:
Lauren Averett Byers, UT MD Anderson Cancer Center, USA
Leonard Lipovich, Wayne State University, USA
Raffaele A. Calogero, University of Torino, Italy

****Correspondence:***
Takahiro Ochiya, Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
e-mail: tochiya@ncc.go.jp

microRNAs (miRNAs) have been identified as a fine-tuner in a wide array of biological processes, including development, organogenesis, metabolism, and homeostasis. Deregulation of miRNAs causes diseases, especially cancer. This occurs through a variety of mechanisms, such as genetic alterations, epigenetic regulation, or altered expression of transcription factors, which target miRNAs. Recently, it was discovered that extracellular miRNAs circulate in the blood of both healthy and diseased patients. Since RNase is abundant in the bloodstream, most of the secretory miRNAs are contained in apoptotic bodies, microvesicles, and exosomes or bound to the RNA-binding proteins. However, the secretory mechanism and biological function, as well as the significance of extracellular miRNAs, remain largely unclear. In this article, we summarize the latest and most significant discoveries in recent peer-reviewed research on secretory miRNA involvement in many aspects of physiological and pathological conditions, with a special focus on cancer. In addition, we discuss a new aspect of cancer research that is revealed by the emergence of "secretory miRNA."

Keywords: secretory microRNA, microRNA, exosome, cell–cell communication, cancer

INTRODUCTION

microRNAs (miRNAs) are small non-coding RNA that repress a wide variety of target genes expression at the post-transcriptional level by sequence-specific base pairing to the 3′ untranslated region of multiple target mRNAs. They are conserved through species, and form an important class of regulators that participate in multiple biological phenomena, including development, organogenesis, and homeostasis. Because of their ability to bind to many target mRNAs (Kwak et al., 2011), once their expression is altered, disease could occur through the deregulation of their target gene networks, particularly that leading to cancer. For this reason, many recent studies have focused on the development of novel diagnosis and therapeutics in the field of oncology. Current studies have revealed that miRNAs are secreted outside of the cells, and their biological significance is beginning to be recognized (Zernecke et al., 2009; Kosaka et al., 2010b; Pegtel et al., 2010). This article is a summary of the latest and most significant findings of original studies on the involvement of secretory miRNAs in cancer, with a special focus on the potential of secretory miRNAs as a humoral factor for cancer biology.

RNA IS NOT ONLY THE MEDIATOR IN THE CENTRAL DOGMA BUT ALSO A SECRETORY FUNCTIONAL MOLECULE

Before Watson and Crick (1953) described the double-helical structure of the DNA molecule, Mandel and Metais (1947) had found that DNA is present in plasma and serum in 1947. They showed the presence of nucleic acids in healthy subjects as well as in ill patients. After that, many researchers have tried to examine the circulating nucleic acid to develop them as a potential biomarker, especially in the research field of cancer (Fleischhacker and Schmidt, 2007). It is now well documented that RNA can also be detected in plasma, serum, and other body fluids as well as from cell-free supernatants of *in vitro* cultivated cells. One of the first papers demonstrating the presence of extracellular RNA was published by Stroun et al. (1978). They reported the presence of an RNA form in a nucleoprotein complex spontaneously released from human blood lymphocytes and frog cell systems from auricle cultures. They also showed that the RNA from this complex has a stimulating effect on DNA synthesis *in vitro*, suggesting the function of secretory RNA in recipient cells.

Meanwhile, the uptake of RNA by recipient cells was also observed. More than 40 years ago, RNAs were reported to be readily taken up by ascites tumor cells (Galand and Ledoux, 1966). In addition, during a study of co-cultured cells that were previously incubated with or without tantalum particles, intact labeled RNA was found to be transferred into the non-labeled recipient cells from labeled donor cells (Kolodny, 1971). Namely, cell–cell communication was mediated not only by proteins, such as cytokines, chemokines, and hormones, but also by secretory RNA.

Given that the concentration of RNA-degrading enzymes, RNase, is high in normal people and even higher in cancer patients (Reddi and Holland, 1976; Tsui et al., 2002) and that RNase is extremely stable, it was reasoned that the RNA released from the cells into the extracellular space must be complexed and in a form that is resistant against RNases. The first study of associating circulating RNA, as RNA–proteolipid complexes, in serum was reported in 1987 (Wieczorek et al., 1987). This study reported a relationship between the presence of RNA–proteolipid complexes and tumor mass/response to therapy. These complexes disappeared ~48 h after tumor removal and were undetected in benign disorders. Another study demonstrated that the release of a macromolecular substance containing ^{32}P and ^{3}H was found when prelabeled

Chinese hamster ovary cells were treated with trypsin under conditions in which cells remain fully viable (Rieber and Bacalao, 1974). In contrast, a ribonuclease treatment affected neither the ^{32}P nor the ^{3}H radioactivity. The authors concluded from these experiments that RNA together with glycoproteins is released from the external cell surface.

FUNCTIONAL IMPORTANCE OF SECRETORY miRNA IN VARIOUS KINDS OF LIFE PHENOMENA

miRNAs, a class of post-transcriptional gene expression regulators, play critical roles in various kinds of biological phenomena, including development, organogenesis, and homeostasis. Dysregulation of miRNA leads to cancer development and progression and has different expression profiles in normal tissues and cancers (Garzon et al., 2010). For this reason, miRNAs have been investigated for their potential use in the diagnosis, prognosis, and treatment of cancer. miRNAs have recently been detected in human body fluids, including peripheral blood plasma as extracellular nuclease-resistant entities (Kosaka et al., 2010a). Reports in two landmark papers noted that not only mRNAs but also miRNAs were secreted outside of the cells and circulated in human body fluid (Chim et al., 2008; Lawrie et al., 2008). Chim et al. (2008) reported the existence of placental miRNAs in maternal plasma. Interestingly, they showed that the four most abundant placental miRNAs (miR-141, miR-149, miR-299-5p, and miR-135b) were detectable in maternal plasma during pregnancy and showed reduced detection rates in post-delivery plasma. Furthermore, Lawrie et al. (2008) investigated whether miRNAs have diagnostic utility by comparing the levels of tumor-associated miR-155, miR-210, and miR-21 in serum from diffuse large B-cell lymphoma patients with healthy controls and showed that the levels were higher in patients than in control sera. These observations support the idea that circulating miRNAs can be used as biomarkers to monitor an individual's health. In addition, these reports also suggest the possibility that secretory miRNA must be contained in or attached to something that could protect RNA from RNase-mediated degradation.

One breakthrough about circulating RNA was the discovery of mRNA and miRNA in exosomes (Valadi et al., 2007). Valadi et al. (2007) showed that mouse and human mast cell-derived exosomes, which are vesicles of endocytic origin released by many kinds of cells that can mediate communication between cells, contain RNA and miRNA. The RNA from mast cell exosomes is transferable to other mouse and human mast cells. After the transfer of mouse exosomal RNA to human mast cells, new mouse proteins were found in the recipient human cells, indicating that transferred exosomal mRNA can be translated after entering another cell. Observations from these three reports indicated one important fact, namely, that miRNA could be existent in the outer space of the cells, where the RNase is present, and could be functional in this new location.

After the discovery of miRNA in exosome, many researchers attempted to identify the function of secretory miRNA because the report from Valadi et al. (2007) had not clarified it in the exosomal miRNA in recipient cells. One of the earliest studies to prove the function of secretory miRNA was revealed by an apoptotic body (Zernecke et al., 2009). They demonstrated that CXCL12 production was mediated by miR-126, which was enriched in apoptotic bodies and repressed the function of the regulator of G protein signaling 16. This enabled CXCR4 to trigger an autoregulatory feedback loop that increased the production of CXCL12, leading to the recruitment of progenitor cells. This study strongly indicated the importance of a "dying message" for the regulating homeostasis of a healthy status and highlights the functions of miRNAs in health and disease that may extend to the recruitment of progenitor cells during other forms of tissue repair or homeostasis.

After the study of miRNA in apoptotic bodies, three reports showed the function and transfer of secretory miRNAs contained inside the exosome. Pegtel et al. (2010) showed that mature EBV-encoded miRNAs are secreted by EBV-infected B cells through exosomes. These EBV-miRNAs repress the EBV target immunoregulatory genes, and these target genes are down-regulated in primary EBV-associated lymphomas. Interestingly, using peripheral blood mononuclear cells from patients with an increased EBV load, these researchers also showed that, although EBV DNA is restricted to the circulating B-cell population, EBV BART miRNAs are present in both B-cell and non-B-cell fractions, suggestive of miRNA transfer *in vivo*. Zhang et al. (2010) reported that miR-150 is contained inside the exosomes and is secreted from a cultured human monocyte/macrophage cell line and that this exosome delivers miR-150 into human microvascular endothelial cells. Then, elevated exogenous miR-150 effectively reduced c-Myb expression and enhanced cell migration in human microvascular endothelial cells. Our group also demonstrated that a secreted tumor-suppressive miRNA, which is miR-146a down-regulated in prostate cancer, was transported to cancer cells and exerted gene silencing in the recipient prostate cancer cells through the suppression of its target gene, thereby leading to cell growth inhibition (Kosaka et al., 2010b). This suggested that secreted miRNA could function as a cell–cell communication tool between the cancer cells and their microenvironmental cells.

These three reports clarified a variety of physiological and pathological phenomena, including virus infection, vascular disease, and cancer. The variety of research fields highlights the importance of secretory miRNAs in phenomena vital to life. Indeed, recent reports have pointed to various functions of secretory miRNA in many aspects of life, such as cellular communication involving antigen-dependent, unidirectional intercellular transfer of miRNAs by exosomes during immune synapsis (Mittelbrunn et al., 2011), nasopharyngeal carcinoma-mediated transfer of EBV-encoded BART miRNA (Gourzones et al., 2010), hepatocellular carcinoma (Kogure et al., 2011), and cardiovascular diseases (Kuwabara et al., 2011). These reports mainly described the importance of exosomes as an miRNA carrier; however, it is not always the exosome that is important in secretory miRNA-mediated cell–cell communication.

High-density lipoprotein (HDL) transports endogenous miRNAs and delivers them to recipient cells with functional targeting capabilities (Vickers et al., 2011). The human HDL-miRNA profile of normal subjects is significantly different from that of familial hypercholesterolemia subjects. Interestingly, a recent report showed that the mechanism of horizontal transfer of miRNAs is not only dependent on vesicle transfer, such as exosomes, but

also intercellular connection tools, such as gap junction and RNA-binding protein. Lim et al. (2011) clarified that miRNA was transmitted from bone marrow stroma to breast cancer cells via gap junctions and exosomes in tumor cell quiescence. Arroyo et al. (2011) employed a technique, differential centrifugation and size-exclusion chromatography, to characterize circulating miRNA complexes in human plasma and serum and found that the majority of circulating miRNAs cofractionated with Argonaute2 (Ago2, the key effector protein of miRNA-mediated silencing) protein complexes rather than within vesicles. This study was also confirmed by other groups which have shown Ago2 (Turchinovich et al., 2011) or nucleophosmin 1 as secretory miRNA carriers (Wang et al., 2010). Further biological studies are required to understand the function of miRNAs secreted with an RNA-binding protein, such as Ago2 or nucleophosmin 1, in a variety of research fields.

To certify the significance of secretory miRNAs in variety of life phenomena, it is also essential to understand the secretion mechanism of miRNA from cells. Previously, we found in HEK293 and COS-7 cells that the secretion of miRNAs was regulated by neutral sphingomyelinase 2 (nSMase 2; Kosaka et al., 2010b), which is the catalytic enzyme of ceramide biosynthesis and is known as an exosome regulatory protein (Trajkovic et al., 2008). The decreased activity of nSMase 2 with a chemical inhibitor, GW4869, and a specific siRNA resulted in the reduced secretion of miRNAs. Complementarily, overexpression of nSMase 2 increased the extracellular amounts of miRNAs. This observation was also confirmed using other cells including T-cells (Mittelbrunn et al., 2011) and hepatocellular carcinoma cells (Kogure et al., 2011). Contrary to our results, inhibition of nSMase 2 significantly increased the amount of miRNAs exported to HDL from macrophages (Vickers et al., 2011).

It remains necessary to elucidate how miRNA is sorted into exosomes or other vesicles, such as microvesicles. Microvesicles, also known as microparticles or shedding vesicles, represent a heterogeneous population of vesicles with a diameter of 100–1000 nm that are released by budding of the plasma (Muralidharan-Chari et al., 2010). It has been shown that microvesicles isolated from embryonic stem cells increase pluripotency of hematopoietic stem cells after horizontal transfer of embryonic stem cell-derived mRNA. Although the functions of microvesicles were recently elucidated, unlike exosome, not only the function but also the sorting mechanisms of miRNAs into microvesicles have not been clarified yet. Furthermore, it has not been shown yet what kind of protein bind to miRNAs in the vesicles such as exosomes, microvesicles, and apoptotic bodies, although Arroyo et al. (2011) clearly showed that circulating Ago2-binding miRNAs were not contained inside vesicles. Gibbings et al. (2009) detected some AGO2 in the purified exosomes, albeit less than in whole-cell lysates, on the contrary, GW182, which required for miRNA function through its binding to AGO2, was dramatically enriched in exosomes. Detecting the proteins, which bind to miRNAs in vesicles, might lead to revealing the sorting mechanism of miRNAs in vesicles. Clarifying the details of the molecular mechanisms of secretory miRNA, such as the manner of cell–cell transfer or secretion mechanisms, will help us understand a variety of diseases, especially cancer (**Figure 1**).

SECRETORY miRNA AS A HUMORAL FACTOR IN CANCER CELLS

As shown in this report, secretory miRNAs are functional molecules that modulate many aspects of the biological process. In addition, destruction of the secretion of miRNA from cells might lead to disease, such as cardiovascular diseases, virus infections, deterioration of the immune system, and cancer. From the field of cancer research, we would like to propose two hypotheses regarding secretory miRNA-mediated cancer progression (**Figure 2**).

One is the function of secretory miRNA in a metastatic niche (**Figure 2A**). As already shown in several reports, various types of the cells have been shown to have the capability to take up exosomes. The tumor microenvironment is a complex tissue comprising variable numbers of tumor cells, epithelial cells which originated cancer cells, fibroblasts, endothelial cells, and infiltrating leukocytes. Recent reports have explained the mechanism of controlling the cancer cell-mediated phenotypical change of microenvironmental cells through cytokines (Hanahan and Weinberg, 2011). Cytokines are considered as key molecules controlling autocrine or paracrine communications within and between these individual cell types. However, considering the existence of secretory miRNA within these environments, their influence to the cancer niche should be reconsidered. An exosome contains nearly 300 proteins (Atay et al., 2011) with the potential to modulate the state of microenvironment cells. In addition, miRNAs are known to regulate hundreds of target mRNA expressions. Thus, not only exosomal miRNAs but also other types of secretory miRNAs could control the state of cellular phenotypes to the benefit of cancer cells within their niche.

Another hypothesis deals with the function of secretory miRNAs in distant organs (**Figure 2B**). Recently, Hood et al. (2011) provided evidence of exosome-mediated conditioning of lymph nodes and defined microanatomic responses that enable the metastasis of melanoma cells. Homing of melanoma exosomes to sentinel lymph nodes imposes synchronized molecular signals that affect melanoma cell recruitment, extracellular matrix deposition, and vascular proliferation in the lymph nodes. They showed the physiological importance of exosomes for distal metastasis; however, they have not provided evidence of the molecules species that take part in the modulation of the distal site of metastasis. To reveal the exact function of miRNA targeting sites that are distant from the primary organ, we should identify the molecular mechanisms of the tropism of secretory miRNA transported by carriers.

SECRETORY miRNA AS A HUMORAL FACTOR IN ORGANISMS

In this study, systemic transfer of miRNAs has been introduced. However, an active mechanism for the transport of double strand RNA (dsRNA) across tissues and cellular boundaries was found in other organisms, such as nematode and plant. Transmembrane channel-forming protein SID-1 has been shown to mediate passive cellular uptake and cell-to-cell distribution of dsRNA in the nematode *C. elegans* (Feinberg and Hunter, 2003). In addition, recent report showed that mammalian SID-1 homolog localized to the cell membrane of human cells enhances their uptake of small interfering RNA, resulting in increased siRNA-mediated gene silencing efficacy (Duxbury et al., 2005). Furthermore, although RNA molecules have been implicated in systemic cell-to-cell communication