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創薬基盤推進研究事業

高速シークエンサーを用いたnon-coding RNAまで包括された トランスクリプトーム解析による新規安全性バイオマーカーの同定

平成２１年度　総括研究報告書

研究代表者　土屋　創健

平成２２（２０１０）年４月

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総括研究報告書

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トランスクリプトーム解析による新規安全性バイオマーカーの同定

研究代表者　土屋　創健　京都大学大学院薬学研究科助教

研究要旨

本研究の目的は、ギガシークエンサーを用いて、non-coding RNA まで包括されたデジタルトランスクリプトーム解析を世界に先駆けてトキシコゲノミクスに適用し、従来よりも高感度・高精度な薬物毒性予測を実現する肝毒性の新規安全性バイオマーカーを同定することである。

近年、生理作用や病態発現に重要な役割を果たす機能性non-coding RNAの存在が明らかとなり、non-coding RNA に着目した新たな生命科学研究に注目が集まっているが、現在までにnon-coding RNAに着目したトキシコゲノミクスの研究報告は皆無である。そこで、non-coding RNA まで包括された網羅的なデジタル発現情報を用いることにより、従来までの限局された解析では発見できなかった従来よりも高感度・高精度な薬物毒性予測を実現する肝毒性の新規安全性バイオマーカーの同定を試みるとともに、non-coding RNA の関与する薬物毒性の発症・分子メカニズムの解明、さらには肝細胞の細胞障害・細胞死を抑制するための治療標的分子の発見を目指す。

平成２１年度は、従来では不可能であった転写方向性情報を有するnon-coding RNA 包括デジタルトランスクリプトームデータ取得法を新規に開発し、トログリタゾン肝毒性モデルにおける経時的データを取得・評価した。

A. 研究目的

近年、transfer RNA や ribosomal RNA 以外のタンパク質をコードしないRNA、non-coding RNA が messenger RNA (mRNA) と同等レベルの２万種類以上転写されていることが明らかとなり、とりわけ、短鎖の non-coding RNA、microRNA が生理作用や病態発現に重要な役割を果たすことが見いだされ、non-coding RNA に着目した新たな生命科学研究が注目・展開されている。しかしながら、現在までにnon-coding

RNA に着目したトキシコゲノミクスの研究報告は皆無である。そこで本課題では、次世代型高速シークエンサーであるギガシークエンサーを用いて non-coding RNA まで包括したデジタルトランスクリプトーム解析を世界に先駆けて行い、従来よりも高感度・高精度な薬物毒性予測を実現する肝毒性の新規安全性バイオマーカーを同定することを目的とする。本研究課題の特色はギガシークエンサーによるデジタルトランスクリプトームデータ解析をトキシコゲノミクスに初めて適用する点であり、それにより mRNA と non-coding RNA の発現情報をデジタル情報として同時に取得でき、マイクロアレイを用いた解析では困難であった未同定の RNA の検出と各 RNA 内・間に渡る高精度なバイオインフォマティクス解析が可能となる。その結果、従来の解析では見いだせなかった新規安全性バイオマーカーの同定、薬物毒性の新たな発症・分子メカニズムの解明、さらには肝細胞の細胞障害・細胞死を抑制するための治療標的分子の発見が可能となり、早期毒性予測による医薬品開発期間・コストの軽減化と、国民における医薬品使用の安全性の向上に繋がるものと期待される。具体的には、平成２０年度にトログリタゾンによる肝毒性モデルの最適化を行い、平成２１年度に、従来では不可能であった転写方向性情報を有する non-coding RNA 包括デジタルトランスクリプトームデータ取得法を新規に開発し、トログリタゾン肝毒性モデルにおける経時的データを取得・評価した。平成２２年度には、肝毒性を有する他の薬物による肝毒性データを重層し、個々の薬物に特異的または共通な肝毒性発現メカニズムと新規安全性バイオマーカーの同定を目指す予定である。

B. 研究方法

細胞培養：非動化（５６℃、３０分）された 10% (v/v) fetal bovine serum 含有の Dulbecco’s Modified Eagle Medium (Sigma) を用い、コラーゲンコートディッシュにて 37℃、5% CO_2 の条件で HepG2 細胞を培養した。

WST-1 細胞増殖測定：WST-1 を基質とした Nicotinamide adenine dinucleotide (NADH)還元酵素の活性を指標に、細胞増殖を測定した。HepG2 細胞をトログリタゾン(vehicle (0.1% dimethylsulfoxide; DMSO), 1 µM、100 µM)で刺激し、各０，６，１２，２４時間後に回収した。$1x10^4$ cells の HepG2 細胞に飽和 WST-1 溶液 (Roche) を加え、37℃、5 % CO_2 の条件下で 1 時間反応させた後、還元型 WST-1 (Formazan) の 460nm とバックグランド補正用の 650nm の吸光度をマイクロ

プレートリーダーで測定した。
ギガシークエンサーを用いたnon-coding RNA包括トランスクリプトーム解析：vehicle及び各濃度（1 μM, 100 μM）のトログリタゾンで刺激したHepG2細胞を、刺激後０，６，２４時間にそれぞれ回収し、acid guanidiniumthiocyanate-phenol-chloroform法を用いてtotal RNAを抽出した。次にribosomal RNAのanti-senseプローブを用いてribosomal RNAの除去を行い、アルコール沈殿による精製後、アルカリ加水分解法によりRNAを断片化した。それらをRneasy micro kit (Qiagen)を用いて精製し、RNAアダプターを5'側と3'側にそれぞれ特異的に連結させた。アダプター部位の配列を用いて逆転写反応を行い、さらにアダプター部位のプライマー配列を用いてPCR増幅を行って、ギガシークエンサーによる解析を行うために必要な約200 bpの２本鎖DNAを調製した。そこでギガシークエンサーを用いて、PCR反応によりin situで個々のクラスターを形成させ、蛍光標識塩基を用いた１塩基伸長反応を用いることにより、76塩基の配列を約2,000万種同時に解読した。その中から、transfer RNAやribosomal RNAおよびミトコンドリアゲノムにアライメントされるものを除去し、さらにヒトゲノムにアライメントされたもののみを更なる解析に使用した。
網羅的な遺伝子発現解析：UniGeneにアライメントされたリードに関して、Rを用いて階層的クラスタリング解析を行った。さらに同じくRを用いて、相関係数の計算、principal component analysisを行った。

（倫理面への配慮）
本課題の包括的デジタルトランスクリプトーム解析は商業的に販売されているヒト培養細胞を対象としていることから、倫理面での問題は特にないものと判断される。また、本課題を遂行するにあたり、組換えＤＮＡ実験を行うが、文部科学省並びに京都大学大学院薬学研究科施設の取り扱い規定に準拠し、組換えＤＮＡ実験安全委員会の承認を得るとともに、研究機関等における組換えＤＮＡ実験等の実施に関する基本指針に従う。

C. 研究結果

本研究プロジェクトではnon-coding RNAをトキシコゲノミクス解析に適用し、その有用性を評価するとともに新たな肝薬物毒性の安全性バイオマーカーを同定し、さらには薬物毒性の分子メカニズムを明らかにすることを主眼とする。そこで、薬物毒性を評価する上で最も重要な組

織の一つである肝臓における薬物毒性発現の分子メカニズムを明らかにするため、肝毒性を有する薬物として劇症肝炎などの肝障害を引き起こしたため臨床での使用が中止されたトログリタゾン（チアゾリジン骨格を有するインスリン抵抗性改善薬、peroxisome-proliferator activated receptor γ のリガンド）をモデルとしてヒト肝癌由来細胞株HepG2を用いて解析を行った。

まず、トログリタゾンのHepG2細胞に対する毒性用量を確認するため、WST-1 法を用いてトログリタゾンがHepG2 細胞の増殖に及ぼす影響を調べた。その結果、vehicle と比較して、トログリタゾン 100 μM 刺激後１２時間と２４時間において有意な増殖の抑制が検出された。一方、EC50 をこえるトログリタゾン 1 μM で刺激してもvehicle と有意な差は検出されなかった。以上のことから、HepG2 細胞に対するトログリタゾンの毒性用量としては 100 μM、薬効(非毒性)用量としては 1 μM と定義し、vehicle 及び各濃度（1 μM, 100 μM）のトログリタゾンで刺激した HepG2 細胞の０，６(毒性が現れる直前)，２４時間における経時的かつ用量依存的な non-coding RNA 包括トランスクリプトームデータを取得した。

次にアライメント解析を行った。ゲノムにアライメントされたリードの内、７割が UniGene にヒットし、残りの３割は未同定の新規転写産物に由来することが示唆された。UniGene にヒットしたリードの内、その８割以上が Refseq 配列の転写開始からの終了までの領域のゲノムにアライメントされ、そのうちエクソン領域にアライメントされたのはさらにその８割だった。従って、最終的にゲノムにアライメントされたリードの内 45%が従来までのマイクロアレイを用いたトキシコゲノミクスの解析対象であった mRNA に由来し、残りの 55%のリードは non-coding RNA に由来する可能性が示唆された。アライメントされる 76 塩基の配列情報を持つリードの割合はいずれのサンプルおいても差がなかった。そこで uniGene のカテゴリーにおいて vehicle に対して毒性（100 μM）もしくは薬効 dose（1 μM）で２倍以上発現変化した UniGene の数を調べるとそれぞれ 4,755、365 種類あり、そのうち mRNA に附属する UniGene はそれぞれ 2,311、99 種であった。従って、発現変動した UniGene の半分以上が non-coding RNA に由来している可能性が示唆された。

この Unigene のカテゴリーにおいて、non-coding RNA 包括トランスクリプトーム解析の再現性評価も含めたさらなる検討を行った。まず階層的クラ

スタリング解析を行った結果、non-coding RNA 包括トランスクリプトームデータは毒性と非毒性のデータを明確に分類した。また、再現性における相関係数はいずれのサンプルにおいても 0.99 であり、本法は高い再現性を有することが示唆された。そこで、これらの principal component analysis を行ったところ、第二軸において毒性のサンプル群と非毒性のサンプル群のベクトルが分離され、この第二軸コンポーネントとトログリタゾンによる薬物毒性との強い相関が示唆された。そこで、この第二軸コンポーネントに強い影響力を有する 11 個の UniGene を選出したところ、そのうち５つのみが mRNA に附属し、残りは non-coding RNA であると考えられた。

D. 考察

近年、数万種に及ぶ non-coding RNA の存在が見いだされたことから、これをトキシコゲノミクスに適用することでより高精度な毒性予測及び毒性発現分子メカニズムの解明が可能となることが予測されたが、現在までにトキシコゲノミクス分野における non-coding RNA の適用事例は皆無であった。そこで、本研究では、トログリタゾンで刺激した HepG2 細胞を肝毒性のモデルとして non-coding RNA 包括トランスクリプトーム解析を行い、その結果、薬物毒性刺激により mRNA と同等数以上の non-coding RNA の発現量が変動していることを初めて明らかにした。さらに non-coding RNA 包括トランスクリプトームデータは毒性と非毒性のサンプルを明確に分類し、principal component analysis により６つの non-coding RNA がトログリタゾンによる薬物毒性に関与している可能性が強く示唆された。実際、この principal component analysis で選出されてきた５つの mRNA には肝障害のマーカーとして報告されている遺伝子や、細胞外マトリックス成分で肝細胞のアポトーシスを抑制することが報告されている遺伝子、さらには脂質代謝に関わる遺伝子などが含まれていた。同様の手法により選出された６種類の non-coding RNA がトログリタゾンによる薬物毒性に実際に関与しているかどうかはたいへん興味深い点であり、今後、トログリタゾンの細胞増殖や細胞毒性に対する機能解析を行うことで新たな薬物肝毒性発現メカニズムの解明が期待される。

non-coding RNA という新たな領域にトキシコゲノミクス解析を適用することによって得られた本研究成果は、まさに世界に先駆けるものであり、今後、他の肝毒性を有する薬物や、薬

品の種類を問わず発現し、現れる臨床症状も多様である腎毒性、薬物の毒性評価を進める上で最も予測し難い要因であるアレルギー毒性への本解析手法の適用によってさらなる薬物毒性メカニズムの解明や高精度な毒性予測手法の確立が可能になるものと期待される。

E. 結論

トログリタゾンを用いた肝毒性モデルにおいて、non-coding RNA が薬物肝毒性に強く関与している可能性が示唆された。

F. 健康危険情報

特記事項無し

G. 研究発表

1. 論文発表

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第32回日本分子生物学会年会（横浜）2009年12月

H. 知的財産権の出願・登録状況

1. 特許取得

無し

2. 実用新案登録

無し

3. その他

無し

研究成果の刊行に関する一覧表

雑誌

発表者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tsuchiya S., Oku M., Imanaka Y., Kunimoto R., Okuno Y., Terasawa K., Sato F., Tsujimoto G. and Shimizu K.	MicroRNA-338-3p and microRNA-451 contribute to the formation of basolateral polarity in epithelial cells.	***Nucleic Acids Res.***	37	3821-3827	2009
Sato F., **Tsuchiya S.**, Terasawa K. and Tsujimoto G.	Intra-platform Repeatability and Inter-platform Comparability of MicroRNA Microarray Technology.	***PLoS One***	4	e5540	2009

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MicroRNA-338-3p and microRNA-451 contribute to the formation of basolateral polarity in epithelial cells

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ABSTRACT

MicroRNAs are small noncoding RNA species, some of which are playing important roles in cell differentiation. However, the level of participations of microRNAs in epithelial cell differentiation is largely unknown. Here, utilizing an epithelial differentiation model with T84 cells, we demonstrate that miR-338-3p and miR-451 contribute to the formation of epithelial basolateral polarity by facilitating translocalization of β1 integrin to the basolateral membrane. Among 250 microRNAs screened in this study, the expression levels of four microRNAs (miR-33a, 210, 338-3p and 451) were significantly elevated in the differentiated stage of T84 cells, when epithelial cell polarity was established. To investigate the involvement of these microRNAs in terms of epithelial cell polarity, we executed loss-of- and gain-of-function analyses of these microRNAs. The blockade of endogenous miR-338-3p or miR-451 via each microRNA-specific antisense oligonucleotides inhibited the translocalization of β1 integrin to the basolateral membrane, whereas inhibition of miR-210 or miR-33a had no effect on it. On the other hand, simultaneous transfection of synthetic miR-338-3p and miR-451 accelerated the translocalization of β1 integrin to the basolateral membrane, although the introduction of individual synthetic microRNAs exhibited no effect. Therefore, we concluded that both miR-338-3p and miR-451 are necessary for the development of epithelial cell polarity.

INTRODUCTION

Epithelial cells carry out key directional functions such as absorption, secretion and protection against the surrounding environment for host multicellular organisms, and these functions depend upon tight junctions (TJs) and the polarized distribution of plasma membrane molecules. The TJs, which are rigid paracellular permeability barriers between the outside and the inside of an organism, divide the plasma membrane of epithelial cells into two domains: an apical domain facing the external environment and a basolateral domain in contact with the internal environment. These plasma membrane domains have different protein and lipid compositions (1–6).

Recent studies suggested that three major polarization complexes contribute to the formation of TJs, apical domains and basolateral domains, respectively (7,8). The differentiation process of epithelial cells is initiated in response to cell–cell and cell–matrix adhesion. These adhesion stimulations form a complex of partition-defective (PAR) 3, PAR6 and atypical protein kinase C (aPKC), and this complex subsequently stabilizes belt-like adherens junctions (AJs), cortical actin bundles and TJs. Thereafter, the PAR3/PAR6/aPKC complex and a Crb/Pals1/PATJ complex (Crumbs, proteins associated with Lin seven and protein-associated with tight protein 102) contribute to formation of the apical domain. On the other hand, the formation of the basolateral domain was accelerated by PAR1b and an Lgl/Scrib/Dlg complex (Lethal giant larvae, Scribble and Discs large). Additionally, membrane trafficking of specific molecules to apical or basolateral membranes play key roles in maturation of the polarized membrane (9). *N*-/*O*-glycans, glycosylphosphatidylinositol-anchored proteins (GPI-APs) and lipid rafts are known to be involved in the transport

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to the apical domain, whereas clathrin, adaptor protein 1B (AP1B), and CD98 play important roles in the trafficking to the basolateral membrane (1,9–11). Basolateral sorting signals of basolateral membrane-specific proteins are mainly found in the cytoplasmic domain, consisting of tyrosine or leucine, while apical sorting signals of apical proteins have not been discovered yet (9). Currently, mechanisms underlying construction and maintenance of the TJs and epithelial cell polarity are not fully understood.

Recently, it has been shown that expression of some microRNAs (miRNAs) was altered during the differentiation process of epithelial cells, although roles of their miRNAs in the differentiation process remained unknown (12). MiRNAs are evolutionarily conserved small noncoding RNAs (20–23 nt), which regulate gene expression by translational inhibition or cleavage of target mRNAs. The miRNAs play important roles in the development, differentiation and function of various cell types, and in the pathogenesis of various human diseases, e.g., cancer (13,14). Currently, over 800 human miRNAs have been identified and registered in the miRNA database, miRBase (15). Strikingly, about 30% of protein-coding human transcripts are predicted to be regulated by these miRNAs (16,17). Here, we investigated the potential roles of miRNAs in the epithelial cell differentiation.

MATERIALS AND METHODS

Cell culture

T84 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM)/Ham's F-12 mixture (Invitrogen, San Diego, CA) containing 10% heat-inactivated (56°C for 30 min) fetal bovine serum at 37°C in a fully humidified 5% CO_2 atmosphere. To differentiate into polarized epithelial cells, T84 cells were seeded onto polycarbonate filters with 24-mm diameter and 0.4 µm pores in transwell chambers (Coster, Cambridge, MA) at a density of 5×10^5 cells per well, and incubated for 7 days. Cultured cell media were changed into fresh medium on alternate days.

RNA extraction and qRT-PCR

T84 cells were harvested at the indicated periods after plating in transwell chambers, and total RNA was extracted by the acid guanidinium thiocyanate-phenol–chloroform method (18), followed by qRT-PCR. For quantification of intestinal alkaline phosphatase (*ALPI*) mRNA expression levels, total RNA was transcribed to cDNA using random primer and SuperScript II (Invitrogen), and quantitative PCR was performed in 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems). The gene expression was quantified using standard curves generated by serially diluted reference samples, and normalized by the expression level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The specificity of the PCR products was confirmed by gel electrophoresis and a dissociation curve analysis. Primer sequences were shown as follow (*ALPI* forward: 5′-tcagctcatctccaacatgg-3′, reverse: 5′-tgagatgggtc acagactgg-3′; *GAPDH* forward: 5′-gaaggtgaaggtcggagt c-3′, reverse: 5′-ggaagatggtgatgggatttc-3′). For quantification of miRNAs and RNA U6 small nuclear 2 (*RNU6-2*), we use TaqMan MicroRNA Assays (Applied Biosystems) following the manufacturer's protocol, which detects specifically mature miRNAs (19,20). The miRNA expression was normalized by the expression level of *RNU6-2*.

Transfection and immunostaining

Just after plating 5×10^5 cells on a polycarbonate filter, cells were transfected with 15 pmol of oligonucleotides for miR-210, miR-338-3p, miR-33a, miR-451 or RNA which sequence has minimal homology with human miRNAs (denoted as 'NC' negative control) (Pre-miR miRNA Precursor Molecule; Ambion, Austin, TX) using Hiperfect (Qiagen, Valencia, CA) for overexpression. For inhibition of miRNAs function, 150 pmol of specific microRNA Hairpin Inhibitors or NC (cel-miR-239b; minimal sequence identity with miRNAs in human, mouse and rat) (Dharmacon, Chicago, IL) was transfected. Filters with T84 cells were harvested at 5 or 7 days after transfection, and were fixed in 4% formaldehyde. For ZO1 and β1 integrin staining, filters were incubated in can get signal (Toyobo, Osaka, Japan) with a rabbit anti-ZO1 antibody (1:100) (Zymed Laboratories, South San Francisco, CA) and a mouse anti-β1 integrin antibody (1:100) (BD Bioscience, Bedford, MA), and then were incubated with a Alexa 488-conjugated goat anti-mouse IgG and a Alexa 594-conjugated donkey anti-rabbit IgG (1:1000) (Molecular Probes, Leiden, Netherlands). No significant staining was observed when cells were incubated without primary antibody. All experiments were repeated at least three times. F-actin was stained by Alexa 647-conjugated phalloidin to investigate formation of cortical actin bundles. The signal area was calculated with ImageJ software.

Microarray analysis

Total RNA of T84 cells was labeled and prepared for hybridization to GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) using standard methods. The GEO database accession code of this microarray data is GSE15385. We used the robust multi-array average (RMA) expression measure for log transformation ($\log_2$) and normalization of the GeneChip data (21). The RMA measures were computed using the R package program, which is freely available on the web site (http://www.bioconductor.org).

Statistics

Results are expressed as mean ± SE. Student's *t*-test or Welch test was used to compare data between two groups. *P*-values < 0.05 were considered as statistically significant. Individual experiments were performed in triplicate, and each experiment was independently performed three times.

RESULTS

A differentiation model of epithelial cells

To investigate a role of miRNAs in the epithelial cell differentiation, we utilized an epithelial cell differentiation model using T84 cells, a human colon cancer cell line. T84 cells can transform into a differentiated state with TJs and cell polarity, and are frequently used in the research of epithelial cells (2). To confirm the degree of epithelial cell differentiation from Day 1 to Day 7 after plating undifferentiated T84 cells (day 0), localization of ZO1 and β1 integrin, a TJ marker and a basolateral membrane marker, respectively, was assessed by immunostaining and a confocal microscopy (2–4). In horizontal sections at a relatively higher level of T84 cells, ZO1 was gradually localized in a chicken wire pattern from Day 1 to Day 5, which was consistent with the distribution of TJs (Figure 1A). On the other hand, signals of β1 integrin were gradually disappeared in horizontal sections at the height where the ZO1 protein localized. Additionally, in vertical sections, β1 integrin stayed in intracellular regions (presumably Golgi apparatus and granules) until Day 5, and β1 integrin was translocalized into the basolateral membrane at Day 7 (Figure 1A). Accordingly, the expression level of *ALPI* mRNA, a marker of epithelial cell differentiation and apical membrane (22,23), was significantly increased by Day 7 (Figure 1B). These findings demonstrated that T84 cells successfully gained morphological characteristics of differentiated epithelial cells during the 7-day culture. To be more exact, T84 cells formed TJs by Day 5, and showed the apical and basolateral polarization from Day 5 to Day 7.

Screening of miRNAs related with differentiation

In order to identify miRNAs involved in epithelial cell differentiation, we assumed that these miRNAs were differentially expressed between undifferentiated and differentiated cells. Firstly, utilizing qRT-PCR, we measured expression levels of 250 miRNAs on Day 0, Day 1 and Day 7 in the T84 cells (Supplementary Table 1). To screen the differentially expressed miRNAs, we set a criterion that cycle threshold (Ct) values (in Day 0 or 7 samples) <32, and Ct value difference >2.5, where Ct value is defined as the number of cycles required for accumulation of the fluorescent signal to cross the threshold. Thus, we selected four candidate miRNAs (miR-210, miR-338-3p, miR-33a and miR-451) for further study. These miRNAs were upregulated in T84 cells on Day 7, compared to Day 0 and/or 1. On the other hand, among examined 250 miRNAs, we could not find any downregulated miRNAs that met the criterion. We confirmed that there was no significant change in the expression level of *RNU6-2* as a negative control.

Next, we performed more detailed time course analysis on the expression of these four miRNAs (Figure 2). The expression levels of three miRNAs, miRNA-210, miRNA-338-3p and miRNA-451, were significantly elevated between Day 5 and Day 7 (P-values < 0.05). *RNU6-2* was not changed in the detailed time course of epithelial differentiation. Thus, the elevation of these miRNA expression levels were closely linked with translocalization of β1 integrin (Figure 1A) and the elevation of *ALPI* mRNA levels (Figure 1B). Therefore, this finding suggested that these three miRNAs would be correlated to the cellular polarization after the TJ formation.

Furthermore, we validated this finding using a different model. The expression levels of these three miRNAs were also significantly upregulated in differentiated Caco-2 cells (Supplementary Figure 1), which is a human colon cancer cell line and was applied to an epithelial cell differentiation model.

Roles of miRNAs in basolateral polarity

The marked elevation of miRNA-210, miRNA-338-3p and miRNA-451 expression between Day 5 and Day 7 strongly suggested that these miRNAs would be involved in the establishment of epithelial cell polarity, such as apical and basolateral domains, after the formation of TJs, because kinetics of these miRNA expressions matched those of *ALPI* mRNA expression levels and polarized localization of β1 integrin (Figures 1 and 2). Thus, we performed gain- and loss-of-function analyses for these miRNAs to determine whether these miRNAs are related with phenotypes of the epithelial polarity.

We initially assessed transfection efficiency and stability of exogenous RNA oligonucleotides in T84 cells. According to the observation by confocal microscopy, cy3-labeled synthetic control miRNA appeared to be transfected in almost all cells (Supplementary Figure 2A). Additionally, sequential quantification of transfected synthetic miR-210, miR-338-3p, miR-33a and miR-451 by qRT-PCR indicated that amount of these four miRNAs introduced into T84 cells was maintained more than endogenous expression levels of each miRNA for 7 days, although the introduced amount of miR-33a was less than those of other three miRNAs (Supplementary Figure 2B–F). There was little difference in the stability of four synthetic miRNA oligonucleotides. These results suggested that this miRNA transfection protocol is sufficient to evaluate the functions of miRNAs.

As a loss-of-function analysis, 2′-*O*-methylated antisense RNA oligonucleotides of each miRNA were transfected individually into T84 cells to inhibit each endogenous miRNA. The blockage of either miR-338-3p or miR-451 inhibited translocalization of β1 integrin in basolateral membrane, whereas inhibition of either miR-210 or miR-33a did not alter the translocalization of β1 integrin to the basolateral membrane (Figure 3). Moreover, the inhibition of these four miRNAs did not affect formation of TJs with a dense chicken wire pattern (data not shown), values of transepithelial electrical resistance (TER) as a function of TJs in Caco-2 cells at Day 7 (when TER value reaches a plateau) (Supplementary Figure 3A and B) and the *ALPI* mRNA expression level (Supplementary Figure 4A). Taken together, these findings suggested that miR-338-3p or miR-451 could regulate basolateral polarity of the T84 cells, but not the formation of TJ and apical polarity.

As a gain-of-function analysis, at first, we individually transfected miR-210, miR-338-3p, miR-33a or miR-451

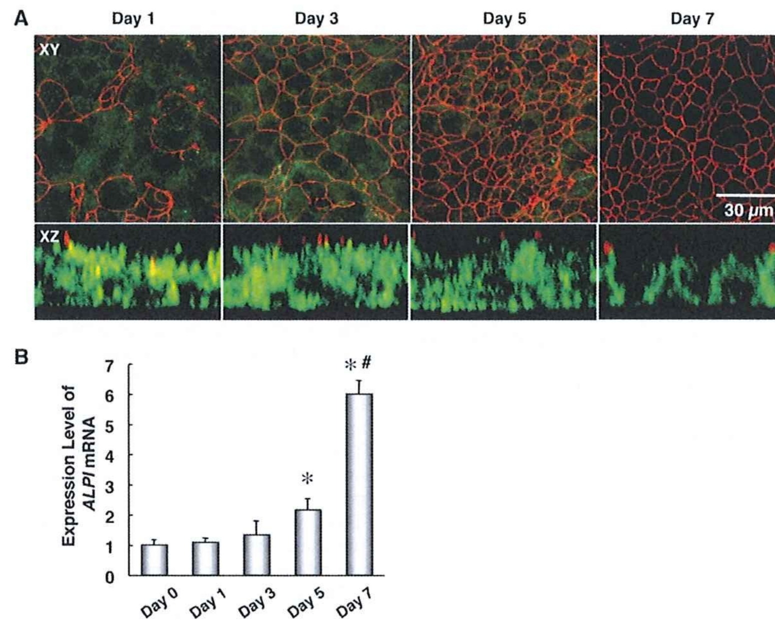

Figure 1. Time course of cellular distribution of ZO1, β1 integrin and ALPI mRNA accumulation in differentiation-induced T84 cells. T84 cells were cultured onto polycarbonate filters in transwell chambers at confluence for the indicated periods. (**A**) Confocal sections of T84 cells were immuno-labeled for ZO1 (red), β1 integrin (green) to detect endogenous proteins by immunofluorescence. Upper panels show horizontal (XY-crossed) sections of T84 cells. Scale bars, 30 μm. Lower panels show vertical (XZ-crossed) sections. (**B**) The amounts of ALPI mRNA were analyzed by qRT-PCR as described in the 'Materials and Methods' section. T84 cells before plating in transwell chamber were used for the value at Day 0, and the values are shown as the fold of values obtained from the sample at Day 0 (Student's t-test: $*P < 0.01$ for cells plated in transwell chamber versus cells at Day 0; $\#P < 0.01$ for Day 7 versus Day 5), and are represented as mean ± SE ($n = 3$). The data are a representative of three independent experiments.

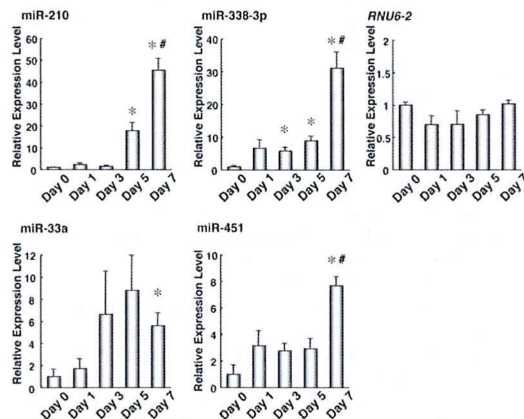

Figure 2. Increased expression levels of miR-210, miR-338-3p, miR-33a and miR-451 along with the epithelial cell differentiation of T84 cells. The expression levels of miR-210, miR-338-3p, miR-33a and miR-451 in T84 cells cultured in transwell chambers for the indicated periods were determined by the qRT-PCR. The changes in expression levels are shown as fold of the value for Day 0. The values are shown as mean ± SE ($n = 3$). The data are a representative of three independent experiments. $*P < 0.01$ is regard as significant by the Welch test (versus Day 0), $\#P < 0.05$ is regard as significant by the Welch test (Day 7 versus Day 5).

into T84 cells at Day 0. Each transfected miRNA did not alter the pattern of β1 integrin localization in the basolateral membrane as compared with cells transfected with none or NC (Figure 4A). However, when these four miRNAs were transfected together, translocalization of β1 integrin was observed in the basolateral membrane at Day 5. As a result of the quantification of areas with a signal of β1 integrin, the extent of the relocation of β1 integrin was significantly altered when four miRNAs were transfected simultaneously (Figure 4B). The degree of this change was nearly equaled to that in nontransfected T84 cells at Day 7 to 5 (Figure 4B and C). On the other hand, transfection of individual miRNAs or even these four miRNAs together did not affect the formation (data not shown) and function (Supplementary Figure 3C) of TJs, and the expression levels of *ALPI* mRNA (Supplementary Figure 4B). Lastly, we investigated whether both miR-338-3p and miR-451 are sufficient for promotional formation of basolateral polarity (Figure 4). When both miR-338-3p and miR-451 were transfected, translocalization of β1 integrin as well as at Day 7 of nontransfected T84 cells was observed in the basolateral membrane at Day 5 (Figure 4). Transfection of miR-210 together with either miR-338-3p or miR-451 had no effect on the translocalization of β1 integrin in the basolateral membrane. Accordingly, our experiments showed that both of miR-338-3p and miR-451 are required for acceleration

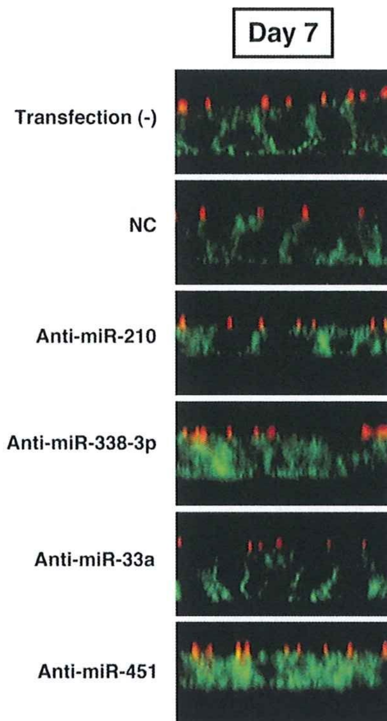

Figure 3. Effects of functional inhibition of endogenous miR-210, miR-338-3p, miR-33a and miR-451 on the localization of β1 integrin to the basolateral domain. Fluorescence images of T84 cells transfected with microRNA Hairpin Inhibitors of miR-210, miR-338-3p, miR-33a, miR-451 or NC were stained for ZO1 (red) and β1 integrin (green). For immunostaining studies, T84 cells at the Day 7 were fixed, permeabilized and incubated with the specific antibodies against ZO1 and β1 integrin. The data are a representative of three independent experiments.

of basolateral polarity formation, and that miR-210 and miR-33a are not necessary for it.

Alternation of gene expression patterns by miR-338-3p and miR-451

These results suggested that both miR-338-3p and miR-451 were essential for the acquisition of basolateral polarity, although neither miRNA was sufficient on its own (Figures 3 and 4). To understand the role of these miRNAs and their mechanism of action in the formation of basolateral polarity, we performed a comprehensive analysis of gene expression. NC-transfected T84 cells ('NC') at Day 5 were used as undifferentiated cells without basolateral polarity. Samples of (i) 'NC' at Day 7, (ii) T84 cells transfected with the four miRNAs ('4 miRNAs') at Day 5 and (iii) T84 cells transfected with both miR-338-3p and miR-451 ('miR-338-3p and miR-451') at Day 5 were assayed as differentiated cells. Total RNA from these cells was hybridized to a microarray.

Candidate target genes of miR-338-3p and miR-451 may show similar expression patterns in the three classes of differentiated T84 cells. Hence, we selected genes whose expression was decreased or increased by more than 2-fold in the three types of differentiated T84 cells compared with 'NC' at Day 5, and Venn diagrams of these downregulated and upregulated genes are shown (Figure 5A and B, respectively). Four genes (*CLDN2*, *EEF1DP3*, *PRKAR2B* and *SMOC2*) were downregulated in all three classes, and none of them contained candidate target sites for either miR-338-3p or miR-451 within their 3′UTRs. In contrast, 154 genes were upregulated in all three classes of differentiated cells, and these included genes such as *RAB11a*, which accelerates the formation of epithelial cell polarity (Supplementary Table 2) (24).

DISCUSSION

This study demonstrated for the first time that miRNAs contribute to the formation of basolateral polarity in epithelial cells. First, we found that the expression of four miRNAs (miR-210, miR-338-3p, miR-33a and miR-451) was upregulated significantly during the process of epithelial cell differentiation. Among these four miRNAs, we found that miR-338-3p and miR-451 play a fundamental role in the formation of epithelial cell polarity. In an effort to identify the target gene(s) for these miRNAs, we performed a series of microarray and functional studies, which showed that the gene expression profile was altered drastically when T84 cells were transfected with these mRNAs. However, we could not identify a direct link between the affected genes and the miRNAs. Hence, the molecular mechanism by which the two miRNAs induce the formation of epithelial cell polarity remains unclear. However, the results of this study highlight a potentially important role for miRNAs in the process of epithelial cell differentiation.

Among the four miRNAs, whose expression was found to be well correlated with the process of epithelial cell differentiation, we further determined that both miR-338-3p and miR-451 are essential for the translocalization of β1 integrin to the basolateral membrane, which contributes to the formation of basolateral polarity in epithelial cells. However, neither miR-338-3p nor miR-451 alone was sufficient to induce translocalization. In contrast, the other two miRNAs, miR-210 and miR-33a, were found to have little effect on epithelial cell differentiation, although miR-210 was the more highly expressed of the four miRNAs. To identify the functional roles of the two miRNAs in the formation of basolateral polarity in epithelial cells, we performed a series of gene expression studies in the presence and absence of exogenous miRNAs. The microarray analyses showed that the expression of many genes was altered dramatically during the process of epithelial cell differentiation; however, we could not identify a close link between the differentially regulated genes and miR-338-3p or miR-451. Our analysis of the transcriptome failed to identify candidate targets of these miRNAs, and the functional role of the miRNAs in the formation of basolateral polarity in

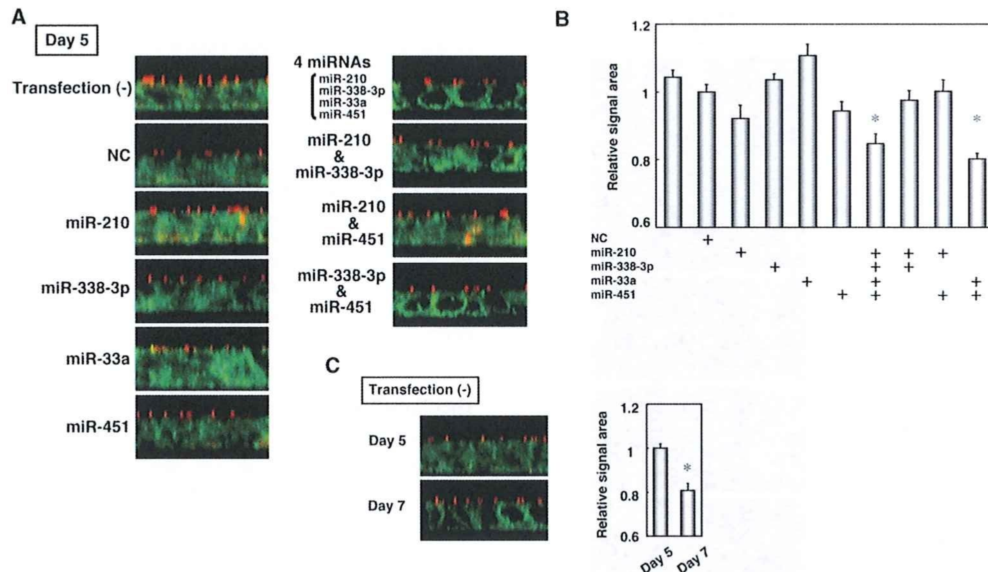

Figure 4. Effects of overexpression of miR-210, miR-338-3p, miR-33a and miR-451 on the distribution of β1 integrin in the basolateral membrane. (**A**) T84 cells were transfected with NC or combination of miR-210, miR-338-3p, miR-33a and miR-451 and T84 cells at the Day 5 were stained with the specific antibodies against ZO1 (red) and β1 integrin (green). (**B**) Quantification of areas with signal intensity of β1 integrin in (A). The values are shown as the fold of values obtained from the sample transfected with NC (Student's *t*-test: $^*P < 0.01$ versus NC-tansfected cells), and are represented as mean ± SE. (**C**) Representative fluorescence images of ZO1 (red) and β1 integrin (green) in T84 cells at Day 5 and 7, and quantification of areas with signal intensity of β1 integrin. The values are shown as the fold of values obtained from the sample at Day 5 (Student's *t*-test: $^*P < 0.01$ Day 7 versus Day 5), and are represented as mean ± SE. The data are a representative of three independent experiments.

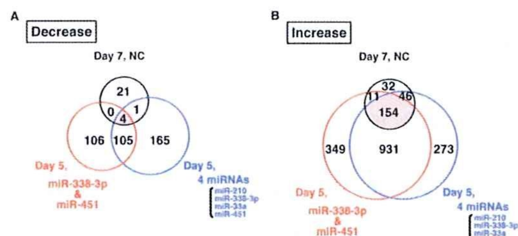

Figure 5. Overlapping sets of more than 2-fold decreased or increased genes in 'NC' at Day 7, '4 miRNAs' at Day 5 and 'miR-338-3p and miR-451' at Day 5 compared with 'NC' at Day 5. The number in the overlapping region of the Venn diagram represents shared genes. Genes were selected if the ratio of the relative expression level compared with 'NC' at Day 5 was smaller than 0.5 (**A**) or larger than 2.0 (**B**).

epithelial cells still remains unclear. However, it is known that miRNAs can regulate the expression of proteins associated with the biological change without producing a detectable change in the corresponding mRNA levels. In fact, very recently two groups reported that miRNAs can repress the production of hundreds of proteins without downregulating their mRNA levels (25,26).

Our observations should provide a deeper insight into the mechanisms that underlie the construction and maintenance of epithelial cell polarity. Given that the disruption of cell junctions and polarity is associated with poor prognosis for carcinomas that are derived from epithelial cells (27–29), this finding might be beneficial for the development of novel cancer therapies. Thus, miRNAs that promote epithelial cell differentiation may provide a novel therapeutic approach. In addition, given that miR-451 has been reported to negatively regulate the expression of multidrug resistance 1 (MDR1) (30,31), miR-451 may play a role in sensitivity toward anticancer drugs. In conclusion, our present study highlights a potentially important role for miRNAs in epithelial cell differentiation, and further suggests a novel regulatory mechanism for this fundamental phenomenon in cellular physiology and pathophysiology.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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MicroRNA-338-3p and microRNA-451 contribute to the formation of basolateral polarity in epithelial cells

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SUPPLEMENTARY DATA

SUPPLEMENTARY MATERIALS AND METHODS

Cell Culture

Caco-2 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) (Sigma, Saint Louis, MO) containing 0.1M non-essential amino acid (Invitrogen, San Diego, CA) and 10% heat-inactivated (56 °C for 30 min) fetal bovine serum at 37 °C in a fully-humidified 5% CO_2 atmosphere, and were seeded onto polycarbonate filters with 24 mm diameter and 0.4 μm pores in transwell chambers (Coster, Cambridge, MA) at a density of 3 x 10^5 cells per well, and incubated for 21 days. Cultured cell media were changed into fresh medium on every another days. Culture of T84 cells was described in the section of *"MATERIALS AND METHODS"*.

RNA extraction and quantification of microRNAs and intestinal alkaline phosphatase

Caco-2 and T84 cells were harvested at the indicated periods after plating in

transwell chambers, and total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method, followed by quantitative RT-PCR (qRT-PCR). For quantification of miR-210, miR-338-3p, miR-33a, miR-451 and RNA U6 small nuclear 2 (*RNU6-2*) in Cco-2 cells, we use TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA) following the manufacture's protocol in 7300 Real-Time PCR System (Applied Biosystems), which detects specifically mature microRNAs (miRNAs). The miRNA expression was normalized by the expression level of *RNU6-2*. Methods for quantification of intestinal alkaline phosphatase (*ALPI)* mRNA expression levels in T84 cells were previously described in the section of *"MATERIALS AND METHODS"*.

Transfection

Just after plating on a polycarbonate filter, Caco-2 and T84 cells were transfected with 15 pmol of oligonucleotides for miR-210, miR-338-3p, miR-33a, miR-451 or RNA which sequence has minimal homology with human miRNAs (denoted as "NC" negative control) (Pre-miR miRNA Precursor Molecule; Ambion, Austin, TX) using Hiperfect (Qiagen, Valencia, CA) for overexpression. For inhibition of miRNAs function, 150 pmol of specific microRNA Hairpin Inhibitors or NC (cel-miR-239b; minimal sequence identity with miRNAs in human, mouse and rat) (Dharmacon, Chicago, IL) was transfected.

Statistics