miRNA expression. Indeed, a copy number change of DICER1 and Ago2 is frequently observed in melanoma, breast and brain cancer (88). Especially, TAR RNA-binding protein 2 (TARBP2), in the DICER-containing complex, showed frameshift mutations and caused a destabilization of DICER1 protein, resulting in global downregulation of mature miRNA in colorectal and gastric tumors (89). According to one estimate, the widespread downregulation of the miRNA expression levels is prevalent in several cancer types (90,91). In contrast, a kind of multi-functional polyphenolic compound, resveratrol, which is present in red wine, induced widespread upregulation of miRNAs and inhibited tumor growth through the acceleration of the expression and activity of Ago2 (92). Thus, the observation and management of the total balance of miRNAs are important for cancer diagnosis and treatment.

INHIBITION OF MIRNA EXPRESSION AND FUNCTIONS

For the therapeutic applications of miRNA, the intracellular expression levels of miRNAs have to be artificially controlled. Although it is relatively easy to upregulate miRNAs, the strategy for the downregulation of miRNAs requires a refined miRNA inhibitor such as a chemically modified antisense oligonucleotide. As the inhibitor against endogenous miRNA, locked nucleic acid (LNA), which has a methylene bridge connecting 2′ and 4′ carbons, is one of the most widely used platforms. LNA nucleotide organizes the phosphate backbone in the *N*-type (C3′-endo) conformation, whereas, in general, the conformations of DNA or RNA duplexes are flexible between *N*-type and S-type (C2′-endo). This conformational change contributes to a more efficient stacking of the nucleobases and functional inhibition of target miRNAs (93). In therapeutic applications, LNA against the liver-expressed miR-122, which is a potential therapeutic target in the hepatitis C virus (HCV), accomplished the long-lasting reduction of mature miR-122 and suppression of HCV viremia (94,95). Furthermore, LNA against hypoxia inducible factor 1α, the primary transcription factor activated by hypoxia that allows glycolysis and angiogenesis to progress, provides significant lowering of the expression of HIF1-α and suppression of tumor growth. Clinical trials of these LNA against miR-122 (SPC3649) and HIF1-α (EZN-2968) have progressed to Phases I and II by Santaris Pharma.

In addition to this, as competitive inhibitors of miRNAs, the miRNA sponge (96), the tough decoy (TuD) RNA (97), antagomirs (98), peptide nucleic acids (PNAs) (99) and anti-miRNA oligonucleotides (AMOs) (100) have also been developed toward medical practice targeting onco-miRNA as well as LNA. Antagomirs composed of 2′-*O*-Me, PS and cholesterol modification were the first miRNA inhibitors that provided a significant reduction in mammals (98,101). However, antagomirs were excluded as clinical candidates because they were less effective than other miRNA inhibitors. PNAs are replaced its sugar-phosphate backbone to

Table 4. Programs of clinical/pre-clinical study in miRNA therapeutics

miRNA	Therapy	Disease	Phase	Company
miR-208/499	Inhibitor	Chronic heart failure	Pre-clinical	MiRagen
miR-15/195	Inhibitor	Post-MI remodeling	Pre-clinical	Therapeutics
miR-451	Inhibitor	Polycythemia vera	Pre-clinical	
miR-122	Inhibitor	HCV	Pre-clinical	
miR-21	Inhibitor	HCC, cancer, fibrosis	Pre-clinical	
miR-10b	Inhibitor	Glioblastoma	Pre-clinical	Regulus
miR-33a/b	Inhibitor	Atherosclerosis	Pre-clinical	Therapeutics
miR-155	Inhibitor	Immuno-inflammatory diseases	Pre-clinical	
miR-122	Inhibitor	HCV	Phase II	Santaris Pharma
miR-29	Mimic	Cardiac fibrosis	Pre-clinical	MiRagen Therapeutics
let-7	Mimic	Lung cancer	Pre-clinical	Mirna Therapeutics
miR-34a	Mimic	Solid tumors	Pre-clinical	
miR-16	Mimic	Cancer	Pre-clinical	
miR-34a	Mimic	Hepatocellular carcinoma	Pre-clinical	Regulus
miR-146a	Mimic	Autoimmunity, cancer	Pre-clinical	Therapeutics

From MiRagen Therapeutics (http://www.miragentherapeutics.com), Regulus Therapeutics (http://www.regulusrx.com), Santaris Pharma (http://www.santaris.com), Mirna Therapeutics (http://www.mirnatherapeutics.com).

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N-(2-aminoethyl)glycine units, also have a potential to inhibit miRNA activities. Reports indicate that PNA-DNA chimeras have the potential to inhibit miRNA *in vitro* and *in vivo* (99). On the other hand, unlike chemically modified ASOs, a miRNA decoy can be stably integrated into the chromosomes and degrade miRNA targets. The stable suppression of miR-301a by a miRNA decoy was reported to have inhibited tumor growth by the upregulation of NF-κB-repressing factor in pancreatic cancer (102), and TuD-RNA against miR-122a showed a significant suppression of the HCV replication in liver hepatocytes (103).

PIPELINE OF MIRNA IN CANCER TREATMENT

In a recent study, onco-miRs or tumor-suppressive miRs that work as master regulators in cellular processes have been identified, and a number of pre-clinical trials have been conducted by firms such as MiRagen Therapeutics, Regulus Therapeutics, Santaris Pharma and Mirna Therapeutics (Table 4). For example, miR-34a, which is one of the best-studied tumor-suppressive miRNAs, was a therapeutic target in solid tumor treatment by Mirna Therapeutics and Regulus Therapeutics. miR-34a is commonly downregulated in human cancer, such as prostate, breast, lung, kidney, bladder, ovary and skin cancer (104–106), and was identified as a target of the tumor suppressor gene p53. The reduction of miR-34a by CpG methylation is observed in multiple types of cancer. The restoration of miR-34s has the potential to cause cell cycle arrest, senescence and apoptosis (107). Mirna Therapeutics has also been conducting pre-clinical trials with miR-16 and let-7 mimics, which are potent tumor-suppressive miRNAs (47,108,109). Furthermore, pre-clinical trials of miRNA inhibitors against miR-21 and miR-10b, which are targeted as onco-miRs in hepatocellular carcinoma and glioblastoma, are being conducted. In addition to these developments, a number of non-public candidates for miRNA therapy are being considered by Mirna Therapeutics; they include miR-Rx01, 02, 03, 06 and 07. Thus, miRNA therapeutics using miRNA mimics or inhibitors has been growing in pre-clinical studies and might appear in clinical trials over the next several years.

CONCLUSION

RNAi is one of the most versatile knockdown tools in recent biotechnology, and the potential of RNAi therapeutics using miRNA for cancer treatment has been rapidly expanding. In particular, unlike siRNAs as a tool that specifically impairs the function of a target gene, miRNAs work as key regulators that control target genes and establish balanced cellular organization. Indeed, the disruption of such a balance leads to the possibility of a tumor to become malignant (110,111). To utilize these discoveries of cellular biological basic research for clinical investigation, further innovations in the field of the delivery systemic and chemical modifying strategies are desired. Indeed, although chemically modified ASOs and ss-siRNAs are potentially promising nucleic acid drugs that can efficiently manage RNAi in animals, immeasurable synthesis costs and technical difficulties for bulk production remain. In addition, safer and more effective delivery systems, including a viral approach, are needed. However, the progression of RNAi technology over the past decade has been remarkable, and the hope is that ongoing investigations will result in the use of RNAi therapeutics as a prominent cancer treatment.

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Conflict of interest statement

None declared.

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microRNA 阻害法の開発：「TuD RNA 発現ベクター」と「S-TuD」

1 東京大学医科学研究所宿主寄生体学分野
2 岐阜大学応用生物科学部
原口　健[1]・上野　義仁[2]・伊庭　英夫[1]

1．はじめに

miRNA (microRNA)とは 20-25nt 程度の短い Non-coding RNA で、相補な配列を持つ mRNA を認識し、その分解や翻訳抑制を行うことにより遺伝子発現を負に制御する[1)-3)]。miRNA は多くの生物種に存在し、またヒトにおいては現在 2000 近く見つかっている。１種類の miRNA が標的とする mRNA は１種類ではなく多数に及ぶため、全遺伝子の 60%以上が miRNA の標的であるとの予測もなされている[4)]。そのため miRNA は数多くの遺伝子と発現制御ネットワークを形成し、発生・分化や癌などの疾患といった様々な生命現象において機能を果たしている。以上のことから生命現象を理解するためには、これまでの遺伝子機能解析に加え、miRNA の包括的な機能解析も必要である。RNAi 技術の登場により遺伝子機能解析が格段に進んだように、miRNA の機能解析にも高効率に阻害する手法が不可欠である。我々はこれまでに標的 miRNA を特異的に阻害する miRNA 阻害ベクターである「TuD RNA (Tough Decoy RNA)発現ベクター」および TuD RNA の構造を模した合成修飾核酸「S-TuD (Synthetic TuD)」を開発してきた[5),6)]。これらは従来法と比べ高い miRNA 阻害活性を有しているので、本稿においてご紹介する。

2．microRNA 阻害ベクター「TuD RNA 発現ベクター」

我々が開発した miRNA 阻害デコイ RNA である TuD RNA は図１に示した構造をしており、Pol III 系のプロモーターにより転写することができる[5)]。TuD RNA は独特の二次構造を有しており、４つの機能的構造によって構成される。MBS (miRNA binding site)は標的とする miRNA と相補な配列で、実際に標的 miRNA と結合する部位である。MBS を挟む位置にあるステムＩ構造およびステムＩＩ構造は、強固な二本鎖構造を形成しており、各種ヌクレアーゼ等への耐性を付与している。さらにステムＩ構造は長さが 18bp あり、miRNA の核外輸送タンパク質である XPO5 により認識されることにより細胞質への輸送能を担っている[7)]。また MBS の両側にリンカー配列を配することにより MBS どうしの結合を抑えて、標的 miRNA と MBS の結合しやすさを高めている。以上の機能的構造が協

調的に働くことによりTuD RNAは高いmiRNA阻害活性を示すものと考えられる。

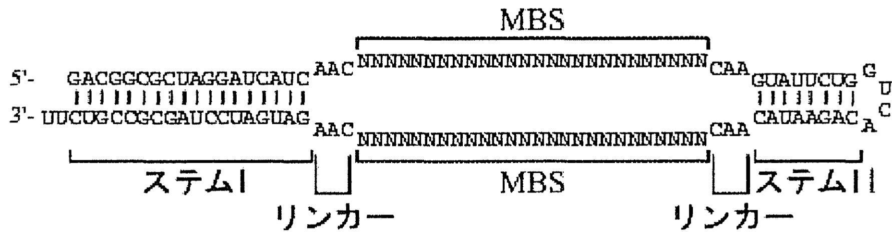

図1. TuD RNAの構造
MBS (miRNA binding site)配列、ステムＩ構造、ステムＩＩ構造、リンカー配列の４つの機能的構造により構成される。

TuD RNA発現ベクターのmiRNA阻害活性についてHCT116細胞内のmiR-21を標的として検討した結果が図２である。miR-21に対するTuD RNAのMBSを２種類比較するとともに「miRNA sponge」、「Antagomir」、「miRNA eraser」といった他のmiRNA阻害ベクターとも比較した。検討したMBSはmiR-21に完全相補な配列および完全相補な配列に４塩基のバルジ配列を挿入した配列である。挿入部位はRISC複合体が標的RNAを切断するmiRNAの5’端から10番目と11番目の塩基に相補な塩基の間である[8]。これにより標的miRNAを含むRISC複合体にTuD RNAが切断されるのを避けることができると考えられる。４塩基挿入したMBSを

(A) TuD-miR21-pf

MBS 5'- UCAACAUCAGUCUGAUAAGCUA -3'
miR21 3'- AGUUGUAGUCAGACUAUUCGAU -5'

TuD-miR21-4ntin

AU
A G
MBS 5'- UCAACAUCAGUC UGAUAAGCUA -3'
miR21 3'- AGUUGUAGUCAG--ACUAUUCGAU -5'

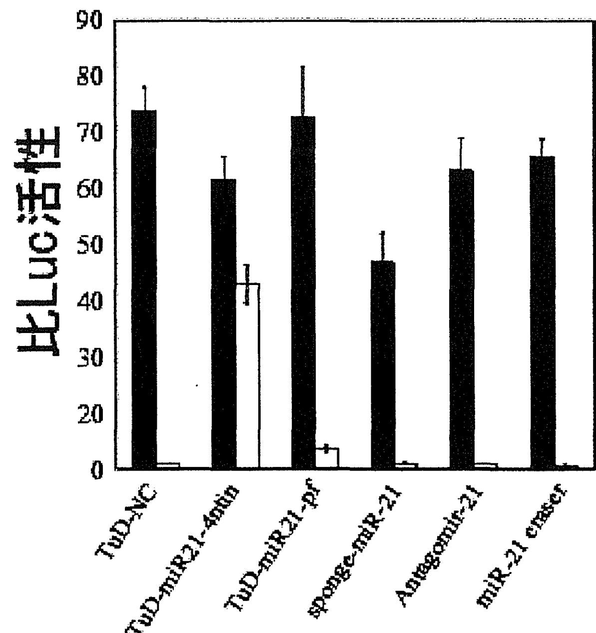

図2. TuD RNAのmiR-21阻害活性（文献5より）
(A)比較検討した２種類のTuD-miR21のMBS配列とmiR-21の結合パターン。(B) HCT-116細胞においてmiR-21と完全相補配列を3'-UTRに持つウミシイタケ・ルシフェラーゼレポーターを用いてアッセイを行った (n = 3) (ホタル・ルシフェラーゼをコントロールとして補正した) 。またmiR-21との相補配列を3'-UTRに持たないレポーターをコントロールレポーターとして用いた。対照 (TuD-NC) ベクターをトランスフェクションした時のmiR-21レポーターの値に対して正規化して表す。

持つ TuD RNA (TuD-miR21-4ntin)は極めて高い miR-21 阻害効果を示した。一方、完全相補な MBS を持つ TuD RNA(TuD-miR21-pf)は TuD-miR21-4ntin と比べるとかなり低下しているものの、他のベクターと比べ高い阻害効果を示した。

次に HCT116 細胞に TuD-miR200c 発現レンチウイルスベクターを導入し、miR-200c 阻害活性の持続性について検討したところ、100 日以上に渡って miR-200c 阻害効果が持続していた (図３Ａ)。さらに miR-200c が上皮-間充織転換に関わっていることが報告されていたので[9]、この TuD-miR200c 発現細胞において上皮-間充織転換が誘導されているかどうかを調べるために、上皮細胞マーカー遺伝子の E-cadherin と間充織細胞マーカー遺伝子の Vimentin の発現量および細胞形態を観察した (図３ＢＣ)。TuD-miR200c 発現レンチウイルスベクター導入から１１日以降の細胞において E-cadherin の減少、Vimentin の発現上昇が見られ、また細胞の形態も間充織様に変化していた。このように TuD RNA 発現カセットを搭載するベクターを選択することにより長期間阻害効果を持続させることができ、さらにその効果は biological effect を誘導するのに十分に高いことが示された。

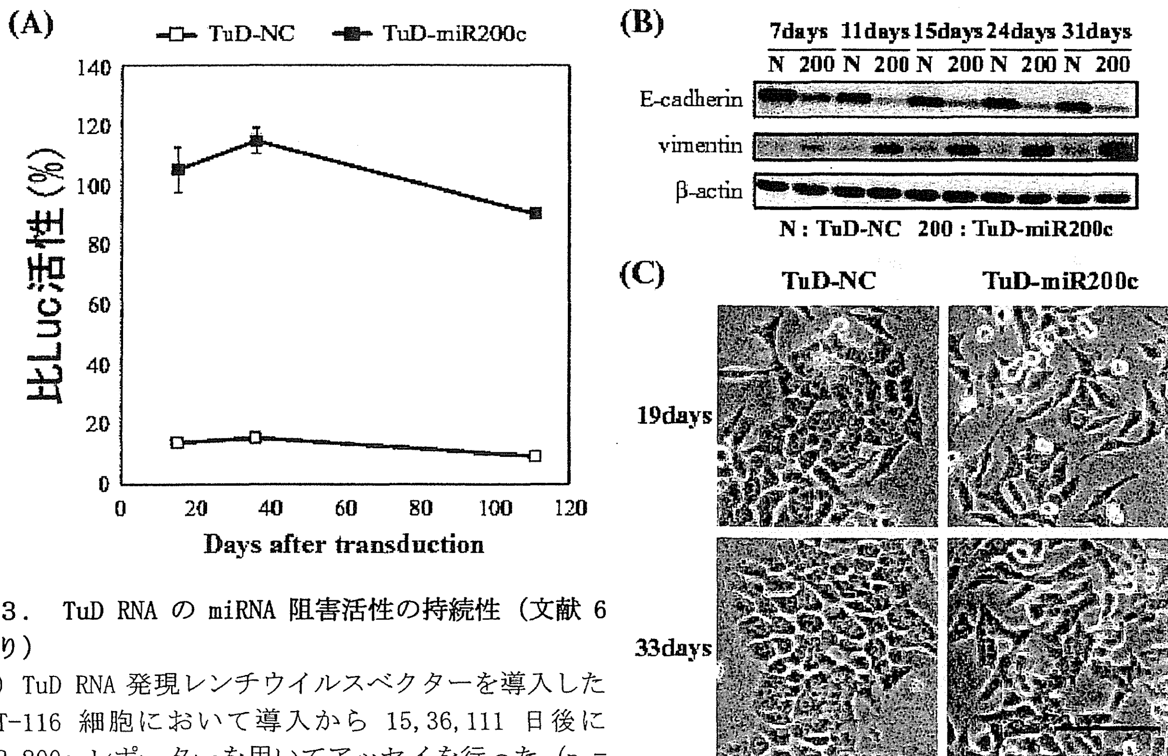

図３. TuD RNA の miRNA 阻害活性の持続性（文献 6 より）

(A) TuD RNA 発現レンチウイルスベクターを導入した HCT-116 細胞において導入から 15,36,111 日後に miR-200c レポーターを用いてアッセイを行った (n = 3)。コントロールレポーターの値に対する百分率比で表す。(B) TuD RNA 発現レンチウイルスベクターを導入した HCT-116 細胞において E-cadherin および Vimentin の発現量をウェスタンブロットにより測定した。β-actin をローディングコントロールとして用いた。(C) TuD RNA 発現レンチウイルスベクター導入から 19,33 日後の細胞形態。黒のバーは 10um を示す。

TuD RNA 発現ベクターは、我々以外の研究グループによる使用例も多く報告されているので、特にTuD RNA 発現ベクターの性能を示すのに適した例についてご紹介する。Zamore 博士らのグループは TuD-miR122 を発現するカセットを搭載したアデノ随伴ウイルスベクターをマウスに導入し、肝臓内の miR-122 の抑制を試みている[10)]。miR-122 は肝臓細胞内の全 miRNA 量の8割を占めるほど発現の高い miRNA であるが、TuD-miR122 搭載アデノ随伴ウイルスベクターにより 20%程度にまで抑制されていた。miR-122 は脂質代謝に関わっているため、抑制によりコレステロール量の減少が導入から 25 週に渡って確認された。また Mikkelsen 博士らのグループは TuD RNA 発現ベクターと既存の7種類の miRNA 阻害ベクターとの miRNA 阻害活性の比較実験を行い、TuD RNA 発現ベクターが最も高い miRNA 阻害活性を有していたことを報告している[11)]。本稿ではご紹介しなかったが、TuD RNA 発現ベクターが高い miRNA 阻害活性を発揮するには適切な MBS 配列・リンカー配列の設計が必要である。TuD RNA 発現ベクターの設計・作製方法については別書にて詳解してあるので参考にされたい[12)]。

3．合成修飾核酸「S-TuD」による miRNA の活性阻害

miRNA は様々な生命現象に関与しており、疾患においても miRNA の発現異常が認められる例が数多く報告されている。このような疾患においては miRNA を標的とした治療法が有望であると考えられる。miRNA を対象とした核酸医薬治療法としては、miRNA の発現が減少している疾患に対して miRNA を補う補充療法と、miRNA の発現が亢進している疾患に対して miRNA を阻害する阻害療法が挙げられる。そこで我々は TuD RNA の技術を基盤とした miRNA 阻害活性を持つ核酸医薬の開発を目指して、TuD RNA と同様の二次構造を有する分子「S-TuD」を開発した（図4）[6)]。

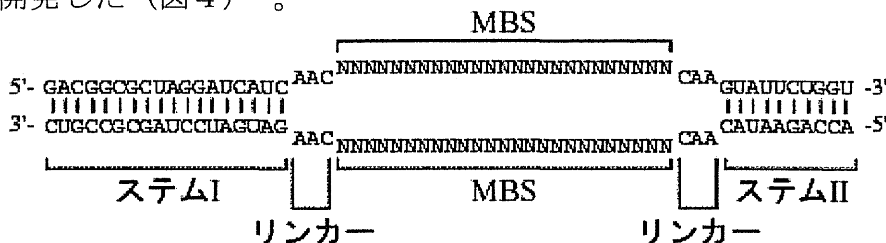

図4. S-TuD の構造
TuD RNA と同様の構造をしており、2本の 55 塩基長程度の 2'-OME-RNA で構成される。

図4に示すように、S-TuD は 55nt 程度の長さの2本のオリゴをアニールすることにより作製できる。120nt 程度の TuD RNA をそのまま合成するのは困難であるので、2本に分けて合

成することとした。さらに RNase に対する耐性を高めるために、S-TuD は全ての塩基を 2’-OMe RNA で構成することとした。

TuD RNA においては MBS 配列に 4nt 挿入することにより、標的 miRNA を含む RISC 複合体による切断を避け、その結果阻害活性が高まることが分かっているが、S-TuD は 2’-OME RNA オリゴで構成されているため RISC 複合体による切断を受けにくいものと考えられる。そこで S-TuD の MBS 配列について検討した。TuD RNA で検討した MBS 配列の「pf」、「4ntin」および、「pf」の標的 miRNA の 5’端から 10 番目の塩基に結合する塩基にミスマッチを入れた「10mut」の 3 種類について miR-21、miR-200c、miR-16 それぞれを標的として試験した。S-TuD-miR21 については「10mut」、「4ntin」、「pf」の順に miR-21 阻害活性が高かった（図５A）。そして S-TuD-miR200c と S-TuD-miR16 については「pf」、「10mut」、「4ntin」の順に miR-200c 阻害活性が高かった（図５ＢＣ）。このように S-TuD-miR21 と S-TuD-miR200c、S-TuD-miR16 とで最適な MBS 配列が異なっていた。この原因がそれぞれの S-TuD の二次構造にあると考え、S-TuD-miR21、S-TuD-miR200c、S-TuD-miR16 それぞれの二次構造を CentroidFold (http://www.ncrna.org/centroidfold/) により予測することとした [13]。CentroidFold は 1 本鎖の RNA の二次構造予測プログラムであるので、２本の 2’-OME RNA オリゴで構成されている S-TuD の代替として、同じ MBS 配列を持つ TuD RNA の二次構造を予測した。その結果 S-TuD-miR21-pf および S-TuD-miR200c-4ntin は MBS どうしが強く結合していると予測された。そこで S-TuD-miR21-pf、S-TuD-miR21-4ntin、S-TuD-miR21-10mut についてそれぞれの二本の 2’-OME RNA オリゴの結合強度を UV 融解曲線により調べた（図５Ｄ）。S-TuD-miR21-pf では 50℃付近および 70℃付近に、S-TuD-miR21-4ntin と S-TuD-miR21-10mut では 30℃付近および 70℃付近に変曲点が見られた。３種類の S-TuD-miR21 全てに共通している 70℃付近の変曲点はステムＩＩ構造の解離を反映しているものと考えられる。一方 S-TuD-miR21-pf の 50℃付近、S-TuD-miR21-4ntin と S-TuD-miR21-10mut の 30℃付近の変曲点はステムＩ構造の解離を反映しているものと考えられる。S-TuD-miR21-pf のステムＩ構造の解離温度が S-TuD-miR21-4ntin と S-TuD-miR21-10mut よりも高かったのは S-TuD-miR21-pf の MBS どうしが強く結合していることを示唆している。以上の結果から標的 miRNA との結合親和性が高い MBS 配列の方がより効果的であること、S-TuD 分子内での、MBS 間の結合が強い場合は S-TuD は効果が大きく減弱することが分かった。MBS どうしが強く結合していると標的 miRNA との結合が妨げられ、その結果 miRNA 阻害活性が低下してしまうものと考えられる。

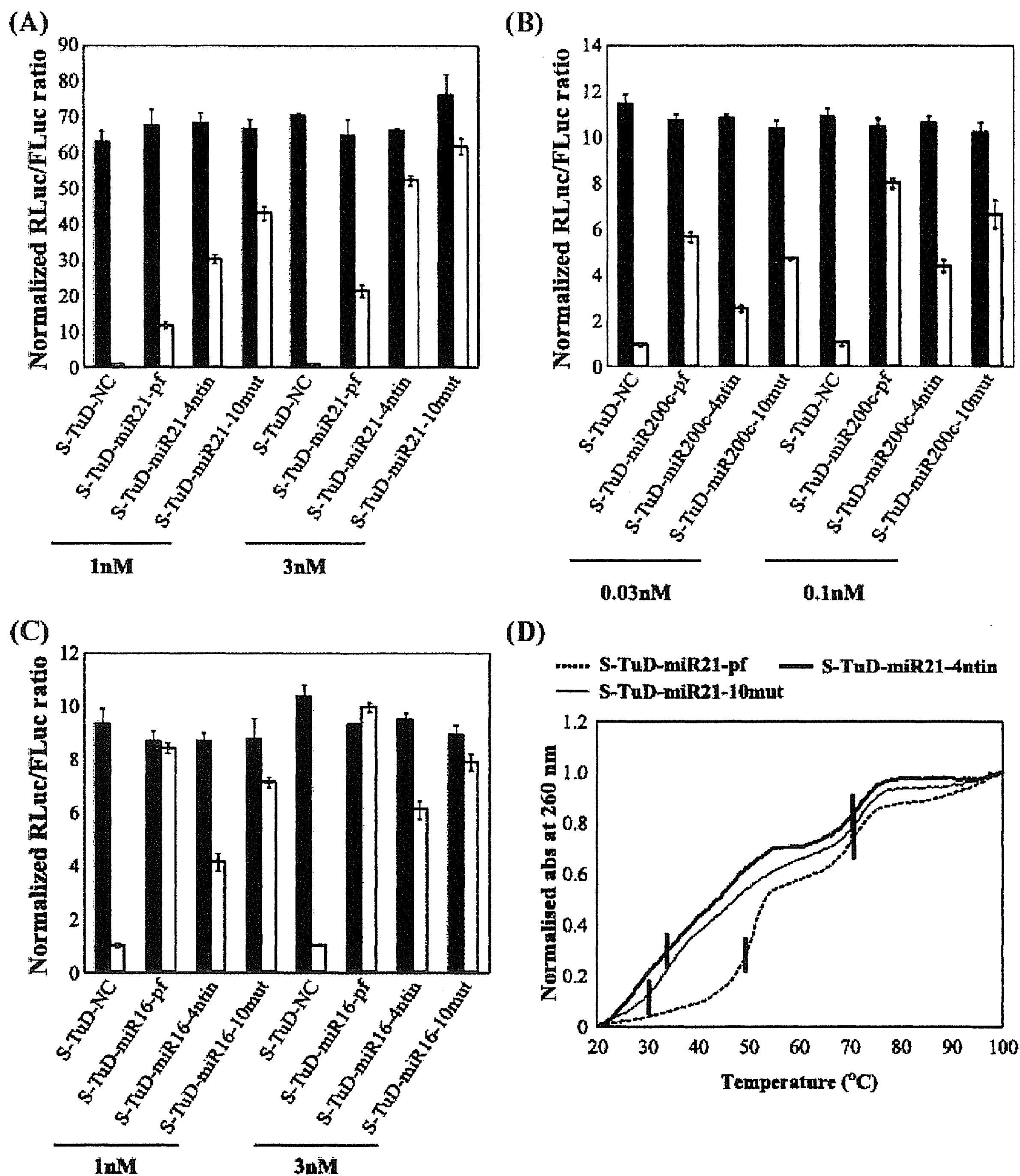

図５． S-TuD の MBS 配列の検討（文献６より）
HCT-116 細胞において(A)miR-21、(B)miR-200c、(C)miR-16 をそれぞれ標的として３種類の異なる MBS 配列(pf, 4ntin, 10mut)を持つ S-TuD の miRNA 阻害活性をそれぞれルシフェラーゼレポーターアッセイにより測定した(n=3)。(D)S-TuD-miR21-pf、S-TuD-miR21-4ntin、S-TuD-miR21-10mut の UV 融解曲線。黒のバーは変曲点を示す。

S-TuD の miRNA 阻害活性の濃度依存性を miR-21 および miR-200c について検討した。S-TuD-miR21-10mut について 0.04nM-0.2nM-1nM-5nM-25nM の濃度での miR-21 阻害活性を検討するとともに、2'-OMe RNA オリゴ miRNA 阻害剤 miRIDIAN (Thermo Scientific 社)との比較を行った（図６Ａ)。S-TuD-miR21-10mut は 0.2nM で阻害効果を示し、5nM で阻害効果が飽和した。これに対し、miRIDIAN-miR21 は 1nM で阻害効果を示し、25nM で阻害効果が飽和した。次に S-TuD-miR200c-pf について 0.003nM-0.03nM-0.3nM-3nM の濃度での miR-200c 阻害活性を検討するとともに、LNA/DNA キメラオリゴ miRNA 阻害剤 miRCURY (Exiqon 社)との比較を行った（図６Ｂ)。S-TuD-miR200c-pf が 0.003nM で阻害効果を示し、0.3nM で阻害効果が飽和点に達する一方、miRCURY-miR200c は 3nM においても阻害効果が飽和しなかった。このように S-TuD は極めて低い濃度でも高い阻害活性を有していることが示された。

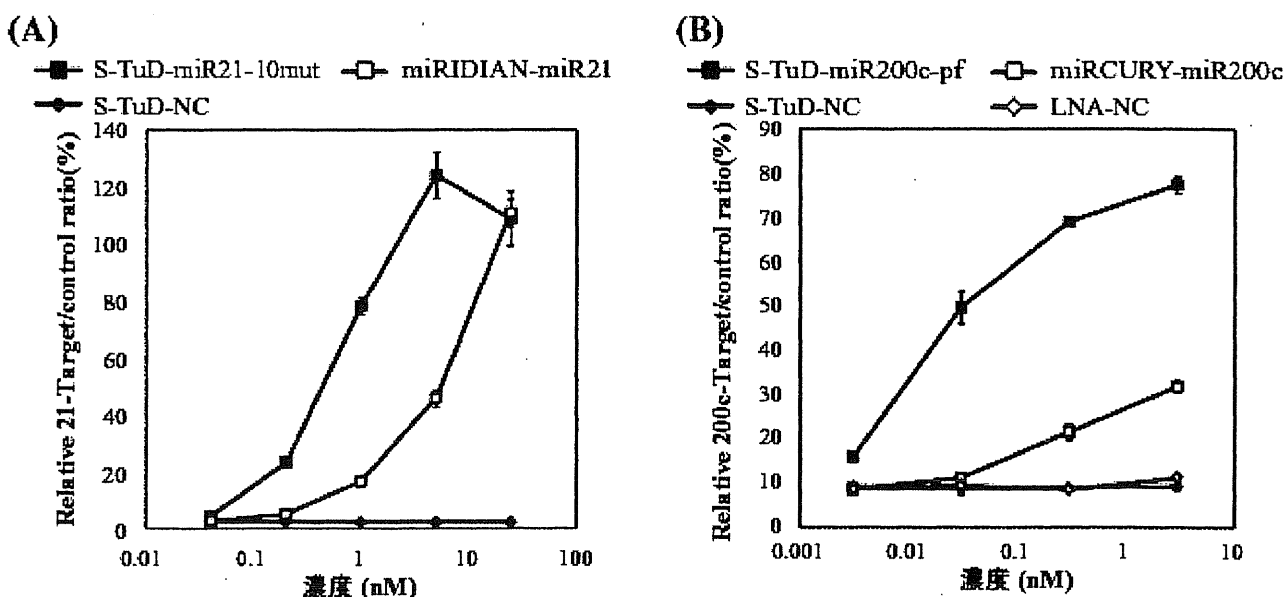

図６.　S-TuD の濃度依存性および既存の miRNA 阻害剤との比較（文献６より）
(A)HCT-116 細胞における S-TuD-miR21-10mut および miRIDIAN-miR21 の miR-21 阻害活性を 0.04nM, 0.2nM, 1nM, 5nM, 25nM でルシフェラーゼレポーターアッセイにより測定した。(B) HCT-116 細胞における S-TuD-miR200c-pf および miRCURY-miR200c の miR-200c 阻害活性を 0.003nM, 0.03nM, 0.3nM, 3nM でルシフェラーゼレポーターアッセイにより測定した。

最後に S-TuD の miRNA 阻害活性の持続性について miR-200c を標的として検討した例についてご紹介する。まず S-TuD の片鎖の 5' 端を 5-FAM 修飾した 5-FAM-S-TuD を HCT-116 細胞にトランスフェクションし、２日後に 5-FAM 陽性細胞を FACS にて分取した。このようにして S-TuD がトランスフェクションされた細胞のみを培養を続け、miR-200c 阻害活性を

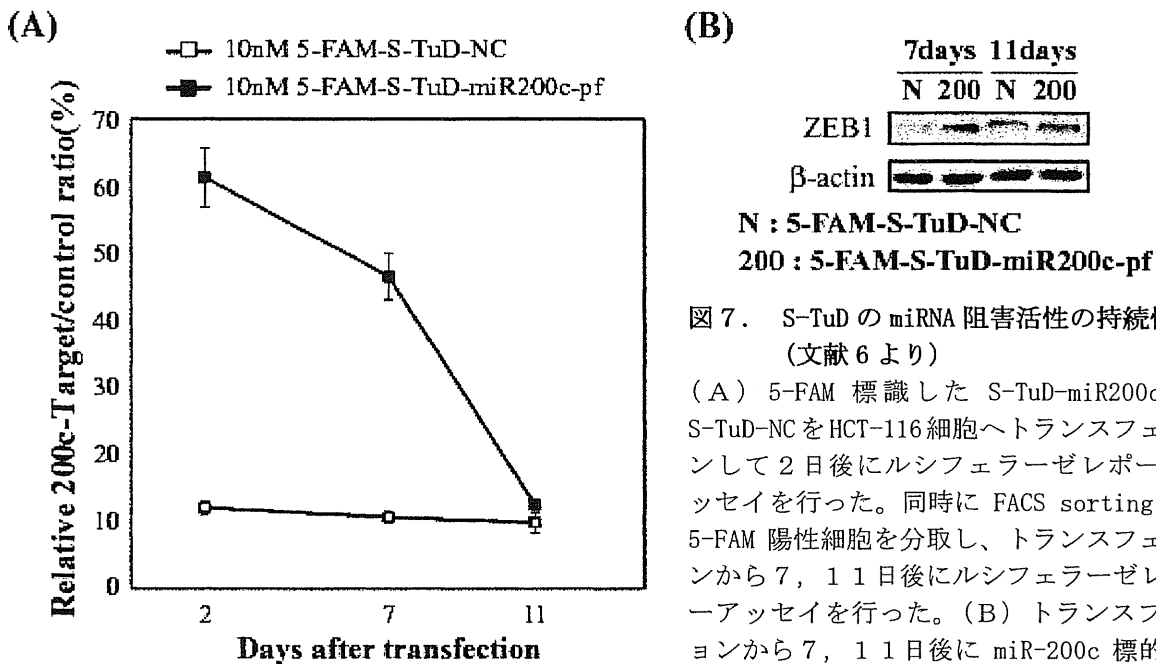

図７．　S-TuD の miRNA 阻害活性の持続性（文献６より）
（Ａ）5-FAM 標識した S-TuD-miR200c-pf と S-TuD-NC をHCT-116 細胞へトランスフェクションして２日後にルシフェラーゼレポーターアッセイを行った。同時に FACS sorting を行い 5-FAM 陽性細胞を分取し、トランスフェクションから７，１１日後にルシフェラーゼレポーターアッセイを行った。（B）トランスフェクションから７，１１日後に miR-200c 標的遺伝子である ZEB1 の発現量をウェスタンブロットにより測定した。β-actin をローディングコントロールとして用いた。

レポーターアッセイにより評価した（図７Ａ）。その結果、トランスフェクションから 7 日後までは高い阻害活性を維持していた。さらに miR-200c の標的遺伝子である ZEB1 の発現量をトランスフェクションから 7、11 日後に測定したところ（図７Ｂ）、7 日後においては ZEB1 の発現上昇が見られたが、11 日後においてはその効果は消失していた。HCT-116 細胞は倍加時間が 16～18 時間と細胞分裂速度の速い細胞であるので [14]、細胞分裂によって S-TuD はかなり希釈されてしまっていると考えられるが、このような細胞においても 7 日程度阻害効果が持続することが分かった。分裂速度の遅い細胞やほとんど分裂しない細胞においては S-TuD の miRNA 阻害活性はさらに長期間持続するものと考えられる。

４．おわりに

「miRNA」と命名されてからおよそ 10 年が経過した現在、様々な研究分野において miRNA に関する研究が報告されるようになった。今後もますます miRNA 研究は行われていくものと期待される。siRNA や shRNA ベクターが現在の遺伝子機能解析研究において不可欠なように、S-TuD および TuD RNA 発現ベクターにより今後の miRNA 研究が大いに進むことが期待される。また miRNA は疾患の治療標的としても有望であるので、現在は個体への導入方

法の検討など、S-TuD を核酸医薬として応用できるよう開発を進めている。TuD RNA 発現ベクターおよび S-TuD の構造と用法については日本・米国・中国においてすでに特許取得・許可通知受領しているので（欧州は審査中）[15)-17)]、日本発の核酸医薬とできるよう開発を進めていきたい。

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7SK small nuclear ribonucleoprotein complex is recruited to the HIV-1 promoter via short viral transcripts

Taketoshi Mizutani[1,2,3,*], Aya Ishizaka[1,2], Yutaka Suzuki[4], and Hideo Iba[1]

[1]Division of Host-Parasite Interaction, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

[2]Laboratory of Basic Science, Institute of Microbial Chemistry ;BIKAKEN, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan

[3]RNA and Biofunctions, Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

[4]Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa-shi, Chiba 277-8568, Japan

[*]Corresponding Author: Taketoshi Mizutani
Laboratory of Basic Science, Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan;
Tel: +81-3-3441-4173; Fax: +81-3-3441-7589;
E-mail: mizutanit@bikaken.or.jp.

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Abstract

In this study, we demonstrate that the 7SK small nuclear ribonucleoprotein (snRNP) complex is recruited to the HIV-1 promoter via just-synthesized HIV-1 nascent transcripts (short transcripts) in an hnRNP A1-dependent manner and negatively regulates viral elongation. Our deep-sequence analysis showed these short transcripts were mainly arrested at approximately +50 to +70 nucleotides from the transcriptional start site in HIV-1 latent model, U1 cells. TNF-α treatment promptly disrupted the 7SK snRNP complex on the nascent transcripts and viral elongated transcripts were increased. This report provides insight into how 7SK snRNP complex is recruited to HIV-1 promoter in the absence of Tat.

Abbreviations

antiretroviral therapy (ART), RNA polymerase II (RNAPII), long terminal repeat (LTR), transactivation responsive element (TAR), small nuclear ribonucleoprotein complex (snRNP), positive elongation factor b (p-TEFb), cyclin dependent kinase 9 (CDK9), La-related protein (LARP7), hexamethylene bis-acetamide inducible 1 (HEXIM1), methylphosphate capping enzyme (MePCE), heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1)

Highlights

1) HnRNP A1 negatively regulates HIV-1 transcripts

2) Viral short transcripts aborted at approximately +50 to +70 nucleotides from the TSS in U1 cells

3) 7SK snRNP is recruited to viral abortive transcripts in an hnRNP A1-dependent manner

4) TNF-α treatment disrupted the 7SK snRNP complex on the early viral transcripts

5) HEXIM1 is constantly present at the HIV-1 promoter via interaction with viral short transcripts

1. Introduction

Under ART, latent infections of HIV-1 are often caused by repression of transcriptional initiation of the viral genome and elongation of nascent transcripts. A useful clue in the study of latent infection is the presence of abortive transcripts, approximately 60 nucleotides long, in resting T cells of patients undergoing ART, even though latently infected T cells may not produce virions and are difficult to distinguish from uninfected cells (1,2). Generally, these observations are not viral-specific, as genome-wide studies have suggested that cellular RNAPII generates many abortive transcripts (3,4).

The transcription of the HIV-1 provirus is characterized by early Tat-independent and late Tat-dependent phases. In the early Tat-independent phase, HIV-1 transcription depends upon the interaction of host transcription factors with cis-regulatory DNA elements within the viral 5′ LTR as well as the assembly of the transcription apparatus, including RNAPII, on these sequences. In the steady state, Tat-independent HIV-1 basal promoter activity is weak, because some host factors, which are reported to function as negative regulators of viral LTRs, are known to restrict HIV-1 basal promoter activity (5). One of these host factors, the 7SK snRNP, functions as a negative regulator of HIV-1 transcription by interacting with the p-TEFb, which is composed of CDK9 and Cyclin T1 or Cyclin T2, to mask RNAPII-directed kinase activity. Main components of the 7SK snRNP complex are 7SK snRNA, HEXIM1, LARP7 and MePCE. LARP7 is a crucial component of the 7SK snRNP complex and acts as an integral molecule in 7SK-mediated negative regulation (6,7). However viral accessory protein, Tat interacts with viral promoter proximal TAR RNA element and it recruits p-TEFb from nucleoplasm or 7SK snRNP complex to transcriptional active RNAPII. After loss of p-TEFb, 7SK snRNP complex is known to release HEXIM1 by changing its conformation (8). Previous report showed that 7SK snRNP complex with or without Tat are recruited to the HIV-1 cis regulatory enhancer element (SP1) before initial transcription, although it has been unclear how HIV-1 transcription is selectively negative regulated by 7SK snRNP complex (9).

HnRNP A1 is a known as an RNA-binding protein that associate with pre-mRNA and functions in viral and host mRNA splicing or metabolism, including the splicing machinery involved in Tat transcription in HIV-1 (10,11). HnRNP A1 shuttles from nuclear to cytoplasm and is involved in HIV-1 mRNA transport (12). HnRNP A1 also play a role in stabilizing the 7SK snRNP complex through interacting with 7SK snRNA lacking p-TEFb and HEXIM1 (8).

In this study, we report that hnRNP A1 facilitates recruitment of the 7SK snRNP complex to the

HIV-1 promoter region through short viral transcripts generated by a promoter-paused RNAPII.

2. Materials and methods

2.1. Plasmid construction and cell culture

A 0.7-kb PCR fragment of the 5′ LTR region of the NL43 HIV-1 molecular clone was inserted into a pWLG plasmid (13) to generate pHIV-LTR-GFP. The region from the 5′ LTR to the splicing acceptor region (0.9 kb) of pHIV-LTR-GFP was inserted into the *ClaI*-*Bam*HI minimal promoter region of the pNF-κB-MinP-Luc plasmid to generate pLTR-Luc-pA (HLpA6), which has been previously described in detail (14). Transiently transfected shRNA expressing plasmid (pmU6) was driven by mouse U6 RNA polymerase III promoter. Control shGFP and sh-hnRNP A1 transduced U1 derived cells were generated by stably infecting with pSSSP (SIN type-shRNA expressing SV40-puro) retrovirus vectors and puromycin drug selection in 2 weeks. The shRNA sequence used in this study are listed in Supplemental Table S1.

2.2 Quantitation of HIV-1 viral production by TNF-α stimulation

U1-derived cells (1×10^6/24-well plate) were stimulated by TNF-α (10 ng/ml; R&D Systems), and culture supernatants were then collected by centrifugation at 0, 0.5, 1, 3, 6, 12, or 24 h after stimulation. Viral transcript and particle production were monitored by qRT-PCR and HIV-1 p24 antigen ELISA (ZeptoMetrix) according to the manufacturer's instructions.

2.3 Antibodies

HEXIM1 [ab25388] (Abcam), CDK9 [C12F7], hnRNP A1 [R196] (Cell Signaling Technology), LARP7 [A303-723A] (Bethyl Laboratories), MePCE [14917-1-AP] (proteintech), CyclinT1 [SC10750], GAPDH [SC25778] (Santa Cruz) (BD Transduction Laboratories), β-actin [017-24551] (Wako).

2.4 Chromatin immunoprecipitation (ChIP) assay

Cells were cross-linked with 1% formaldehyde. The lysates were then sonicated on ice to shear the DNA into small fragments with an average length of less than 0.5 kb by using

ELESTAIN035SD (ELECON Science. Corp). The lysates were incubated overnight on a rotating platform at 4°C with the respective antibodies (5 mg each), which were previously bound to Dynabeads Protein G (Invitrogen). After washing, the DNA was purified and quantified according to the same protocol described in the RT-PCR section.

2.5 RNA preparation and quantitative RT-PCR

Total RNA was prepared from cells using the Isogen II isolation kit (Wako). For short transcripts, the small RNA fraction (<200 nt) was purified using the same kit. These isolated RNA were treated with Turbo DNase (Ambion) in accordance with the manufacturer's instructions. For long transcripts (>200 nt), cDNA was synthesized from total RNA by first-strand cDNA synthesis using the PrimeScript RT Master Mix (Takara Bio). Quantitative PCR was performed with a 7300 Real-Time PCR System (Applied Biosystems) using Premix Ex Taq (Probe qPCR) or SYBR Premix Ex Taq (Takara Bio). For short transcripts, cDNA was synthesized using the miScript Reverse Transcription Kit (Qiagen). Quantitative PCR was performed with a 7300 Real-Time PCR System (Applied Biosystems) using Premix Ex Taq (Probe qPCR; Takara Bio). The elongated transcript was amplified as the region between the 5′ LTR and splicing donor site. Threshold values (Ct) were calculated, and all reactions were run in triplicate. The specific primer pairs and probes used in this study are listed in Supplemental Table S1.

2.6 RNA immunoprecipitation (RIP) assay

RIP assays were performed and modified using the RiboCluster Profiler™/RIP-Assay Kit (MBL), according to the manufacturer's instructions. RNA was purified from the precipitates with Isogen II (Nippon Gene) reagent. These isolated RNAs were treated with Turbo DNase (Ambion) in accordance with the manufacturer's instructions. The cDNA was synthesized and quantified according to the same protocol described in the RT-PCR section.

2.7 Deep-sequence analysis of viral short transcripts

We prepared a small RNA fraction (<200 nt) from U1 cells and amplified viral short transcripts. Deep sequencing was performed using the Illumina GAIIx genome analyzer (Illumina) according to the manufacturer's instructions. Generated sequences were mapped to the reference HIV proviral genomic sequence (HIVNL43; GenBank accession No.

M19921-2). Sequence data that met the criteria of a quality value greater than 30 (100,000 sequences) were analyzed. Only the sequences that perfectly matched the first 20 nt (GGU CUC UCU GGU CCA UAG GA) of the reference HIV genomic sequence were used, and we then analyzed the following sequence of the short transcripts (total 82,000 reads). All sequence data are deposited at the DNA Databank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) (accession number DRA000547).

2.8 Statistical analyses

We performed t test. All statistical tests were two-sided. We considered *P* values less than 0.05 are statistically significant.

3. Results

3.1 HnRNP A1-knockdown releases suppression of HIV production in U1 cells

To elucidate the role of hnRNP A1 in the transcriptional regulation of HIV-1 proviral expression, retroviral vectors mediating either hnRNP A1 shRNA or control (GFP) shRNA expression vectors were stably transduced into U1 cells. This cell line harbors 2 copies of intact latent HIV-1 with defective Tat genes, and are often used as a cell model of latent HIV-1-infection (15). The expression level of hnRNP A1 was reduced in U1 cells transduced with the hnRNP A1 shRNA vector (Fig. 1A). Interestingly, we found that, when hnRNP A1 was knocked down, albeit in partial efficiency, the basal viral transcription level was 1.4-fold increased in comparison with the control (Fig. 1B and 1C). Since it has been shown that viral transcriptional initiation and elongation is regulated by some host factors, we thought that hnRNP A1 is likely to play a role in regulation of HIV-1 transcriptional initiation or elongation.

After 96hr TNF-α stimulation, the level of elongated viral transcription was 8-fold upregulated in cells in which hnRNP A1 was knocked down as compared to that in the control (Fig. 1D). Furthermore, a p24 ELISA demonstrated that, after TNF-α stimulation, production of HIV-1 particles from the hnRNP A1 shRNA-transduced U1 cells was significantly increased compared with that of U1 cells expressing control shRNA (Fig. 1E). In contrast some pTEFb-dependent cellular genes (Myc and Fos) were no affect by knockdown of hnRNP A1 in U1 cells (Fig. 1C) (16). Together, these results indicated that hnRNP A1 functions as a negative regulator of proviral transcription in U1 cells.