の関連性を調べた。S18Y 存在下では、Wild-Type (WT) に比べ huntingtin タンパク質の凝集体形成に変化は見られず、huntingtin タンパク質量の劇的な変化も観察されなかった。また、ATP assay、LDH assay により、huntingtin の polyQ の長さ依存的に細胞死が引き起こされることがわかったが、WT あるいは S18Y 発現での差は観察されなかった。

（2）ハンチントン病の治療法開発

ハンチントン病原因遺伝子である huntingtin に特異的な siRNA の直接投与が、疾患モデル動物の延命に効果があることが報告されている。その siRNA を元に shRNA 発現ベクターを構築し、培養細胞や出生直後のマウス脳内に直接投与することで、その治療効果の有無を検討した。培養細胞に shRNA 発現ベクターを導入した 48 時間後では、対照群に比べ正常 huntingtin タンパク質量は変化せず、異常 huntingtin タンパク質量が減少した。一方、導入１２０時間後では、正常 huntingtin タンパク質、異常 huntingtin タンパク質が共に減少した細胞群が観察された。また、shRNA を脳内に投与した疾患モデルマウスは、対照群に比べ、発症時期の遅延、緩やかな体重減少、運動機能の回復、生存期間の延長が観察された。

（3）ハンチントン病における細胞死のメカニズム解明

伸長したポリグルタミンを含む Huntingtin の発現が UCH-L1 の存在によって変化するかを調査した。今回の実験から huntingtin の発現レベルが UCH-L1 の存在によって減少することが明らかとなった。Huntingtin の発現によりポリユビキチン量が増加することが言われているが、今回の実験でもポリユビキチン量は huntingtin タンパク質に比例して減少していた。

（4）ハンチントン病における細胞死のメカニズム解明

オートファジーは細胞内の浄化メカニズムとしてユビキチン- プロテアソーム分解と並んで重要である。オートファジーはハンチントン病を始めとする神経変性疾患等の原因異常タンパク質の蓄積を低下させ、これらの発症に対して抑制的に働くと知られている。そこで伸長したポリグルタミンを含む Huntingtin タンパク質に相互作用するタンパク質について、オートファジー関連因子を中心にスクリーニングを行なった。その結果、microtubule associated protein 1A light chain3/LC3-I が同定された。この結合は通常のポリグルタミン (Q22) を含む Huntingtin タンパク質では確認されなかった。

D．考察

パーキンソン病、ハンチントン病など神経変性疾患については近年の分子遺伝学的解析から家族性疾患を中心にいくつもの病因遺伝子が同定された。数多くの孤発性については病因の特定はいまだなされていないが家族性の成果を発展させることで、対症療法の高度化だけでなく根本的治療法開発も展望できるとの期待が高まっている。成因に関しては国内外における研究から、蛋白質の構造変化、凝集、蓄積と神経細胞死・神経変性との関連が示されており conformation 病の概念確立とともに、アポトーシスに加えて神経細胞機能不全も神経変性の主因として位置づけられるようになってきた。このような世界の潮流の中で、神経変性疾患の分子機序を明らかにし、よりヒトに近いモデル動物を使い、先端的かつ臨床応用が十分可能な治療法を開発することが重要である。その達成にむけハンチントン病に焦点を当て、その発症機序を培養細胞で調査し、また有効な治療法を培養細胞やハンチントン病モデルマウス (B6CBA-TgN (Hdexon1) 62Gpb/　J) を用いて検討を行った。

神経変性疾患に認められる種々のユビキチン陽性封入体にUCH-L1が存在することから、UCH-L1は様々な神経変性疾患との関連性が示唆されている。またS18Y多型がハンチントン病やアルツハイマー病の感受性因子であると言う報告もある。我々の研究結果からは、野生型存在下でもS18Y存在下でも異常型huntingtinタンパク質量に差は観察されなかった。またpolyQ依存的な細胞死においても野生型そしてS18Y存在下で差異は見られなかった。これらの結果から、UCH-L1(S18Y)はhuntingtinタンパク質の凝集や分解、huntingtin遺伝子による細胞死に直接的に関与していない可能性が考えられる。そこで野生型UCH-L1の存在下、非存在下でhuntingtinタンパク質の量を比較すると、UCH-L1存在下でhuntingtinタンパク質量の減少が観察された。UCH-L1は、脱ユビキチン化酵素、ユビキチンリガーゼ、ユビキチンキャリアタンパク質としての機能を有し、その多機能性が神経発生、アポトーシス、神経伝達などの制御に密接に関与していることが明らかになっている。UCH-L1の持つ多機能性から、今回の結果について２つの可能性が考えられる。１つはhuntingtinタンパク質分解の亢進と、もう１つはタンパク質合成の阻害である。

ハンチントン病を含む多くの神経変性疾患では異常タンパク質の蓄積とそれによる細胞死が観察される。それらの現象は細胞内にあるタンパク質管理機構の障害によるものと考えられている。細胞内タンパク質品質管理機構にはユビキチンープロテアソームやオートファジーなどがある。UCH-L1はモノユビキチンを安定化させる働きを持ち、またUCH-L1を発現させた細胞ではプロテアソーム阻害時にポリユビキチンの蓄積が増加するという報告がある。これらのことからUCH-L1はユビキチンープロテアソーム系のタンパク質分解システムに深く関与していると考えられる。すなわち、UCH-L1が発現した細胞ではより積極的に細胞内の不要なタンパク質が分解されるのではないかと予想される。

もう１つの可能性であるタンパク質合成の阻害については、転写レベル又は翻訳レベルで制御されているかは不明であるが、これらの制御に関与するタンパク質の中にユビキチンープロテアソーム系によって分解されるタンパク質も多いと考えられることから、それらがUCH-L1によって分解されることが原因になっているかもしれない。Huntingtinタンパク質の合成を制御するターゲットの探索として、UCH-L1に注目することでハンチントン病の新しい治療法開発に結び付けられるのではないかと考えている。

さらに我々は前述のユビキチンープロテアソーム系と並んでタンパク質管理機構に重要とされるオートファジーについて、huntingtinタンパク質との関連性を調査した。オートファジーはマクロオートファジー、ミクロオートファジー、シャペロン介在性オートファジー（CMA）に分類できる。通常オートファジーと呼ばれるバルクなタンパク質分解システムはマクロオートファジーのことを指す。神経細胞でのマクロオートファジーの欠損は異常タンパク質の蓄積と神経脱落を引き起こすことや、マクロオートファジーにより神経変性疾患の原因となる異常タンパク質の蓄積を低下させて発症に対して抑制的に働くことが明らかとなっている。

Huntingtinタンパク質と相互作用するタンパク質のスクリーニングの結果、microtubule associated protein 1A light

chain3/LC3-Iが同定された。LC3-Iは通常細胞質に存在するタンパク質で、オートファゴソーム膜に結合した後、リン脂質による修飾を受けLC3-IIとなってオートファゴソーム膜形成に関わる。おもしろいことに伸長したポリグルタミンを含むhungtintinタンパク質は非修飾型のLC3-Iとのみ結合し、リン脂質修飾型のLC3-IIとの結合は認められなかった。今回の結果は伸長したポリグルタミンを含むhuntingtinがLC3-Iのオートファゴソーム膜結合を阻害し、マクロオートファジーの機能抑制をひきおこしたという可能性を示唆する。今後はLC3に焦点を当て、伸長したポリグルタミンを含むhuntingtinによるマクロオートファジーの機能変化を調査し、それが治療ターゲットとして適格となるか検討することが必要だと考える。また、最近の報告でマクロオートファジーとCMAが相互に補填し合うことが示された。さらに以前我々はUCH-L1がCMAによって分解されることを示した。以上のことより、我々はハンチントン病におけるhuntingtin、UCH-L1、マクロオートファジー、CMAとの間には相関性があるのではないかと推測しており、その解明を今後の課題としている。

ハンチントン病の治療法開発については、培養細胞において発現抑制効果のあるハンチントン病遺伝子特異的siRNAが開発できたのに続き、当該siRNAを元にshRNAを開発し、その脳内直接投与がハンチントン病モデルマウスの延命にsiRNAよりもより効果のあることを見出した。ハンチントン病に限らずgain of toxicityで発症する神経変性疾患の根本治療にRNAi法は極めて有望であると考えられる。しかし、一部の細胞群では、shRNA投与により正常型huntingtinタンパク質量が減少することが観察された。正常huntingtinタンパク質は正常発達に必須で、成長後の正常脳では大脳皮質一線条体系に含まれる脳由来神経栄養因子（BDNF）の軸索輸送に関わっていると考えられている。正常huntingtin遺伝子を抑制した場合の副作用や、それがどの程度正常huntingtin遺伝子を抑制した場合に起きるかなどはまだ不明のままである。今後、RNAi法を実践的な治療法に近づける為には、正常huntingtin遺伝子抑制時の基礎的データの収集は重要であり、さらに、よりヒト病態に近い臨床像を呈することが予想される中型動物や小型霊長類で将来疾患モデルを作出する基盤を築きあげ、先端的治療法の開発に向けて応用することをめざすことが必要であると考える。これらの研究はハンチントン病の根本的治療法開発をめざす上で必須のものであり、その研究計画の達成は他の神経変性疾患にも応用可能な治療技術を提供し、広く神経難病の克服に貢献する。

E．結論

1）ハンチントン病原因遺伝子に対するshRNA発現ベクターを構築し、疾患モデル動物個体においてその効果を確認した。

2）UCH-L1-S18Y多型はhuntingtinタンパク質の凝集や分解、huntingtin遺伝子による細胞死に直接関与していない可能性が示唆された。しかしながら、野生型UCH-L1はhuntingtinタンパク質の発現量を調節している可能性が示唆された。

3）伸長したポリグルタミン鎖を含むhuntingtinタンパク質に結合するタンパク質のスクリーニングの結果、マクロオートファジー関連因子であるLC3-Iが同定された。

F．健康危険情報

特になし

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H. 知的所有権の出願・登録状況（予定を含む）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

NEW METHODS AND TECHNOLOGIES

Assessment of allele-specific gene silencing by RNA interference with mutant and wild-type reporter alleles

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ABSTRACT

Allele-specific gene silencing by RNA interference (RNAi) is therapeutically useful for specifically suppressing the expression of alleles associated with disease. To realize such allele-specific RNAi (ASP-RNAi), the design and assessment of small interfering RNA (siRNA) duplexes conferring ASP-RNAi is vital, but is also difficult. Here, we show ASP-RNAi against the Swedish- and London-type amyloid precursor protein (*APP*) variants related to familial Alzheimer's disease using two reporter alleles encoding the *Photinus* and *Renilla* luciferase genes and carrying mutant and wild-type allelic sequences in their 3'-untranslated regions. We examined the effects of siRNA duplexes against the mutant alleles in allele-specific gene silencing and off-target silencing against the wild-type allele under heterozygous conditions, which were generated by cotransfecting the reporter alleles and siRNA duplexes into cultured human cells. Consistently, the siRNA duplexes determined to confer ASP-RNAi also inhibited the expression of the *bona fide* mutant APP and the production of either amyloid β 40- or 42-peptide in Cos-7 cells expressing both the full-length Swedish- and wild-type *APP* alleles. The present data suggest that the system with reporter alleles may permit the preclinical assessment of siRNA duplexes conferring ASP-RNAi, and thus contribute to the design and selection of the most suitable of such siRNA duplexes.

KEYWORDS: *RNAi, allele-specific gene silencing, amyloid precursor protein, Swedish mutation, London mutation, reporter allele*

INTRODUCTION

RNA interference (RNAi) is a powerful tool for suppressing the expression of a gene of interest (Dykxhoorn et al, 2003; Meister and Tuschl, 2004; Mello and Conte, 2004). In mammals, RNAi can be induced by direct introduction of synthetic small interfering RNA (siRNA) duplexes into cells or generation of siRNA duplexes using short-hairpin RNA expression vectors and its application is expanding to various fields of science; therapeutic use of RNAi in medical science and pharmacogenesis is particularly promising (Caplen, 2004; Dykxhoorn et al, 2003; Hannon and Rossi, 2004; Karagiannis and El-Osta, 2005; Wood et al, 2003). Allele-specific gene silencing by RNAi (allele-specific RNAi: ASP-RNAi) is an advanced application of RNAi techniques, by which the expression of an allele of interest can be inhibited (Victor et al, 2002). Accordingly, ASP-RNAi is thought to be therapeutically useful, i.e., it can specifically suppress the expression of alleles causing disease without inhibiting the expression of corresponding wild-type alleles. To realize and control such ASP-RNAi, the following issues must be addressed: selection of competent siRNA duplexes that strongly induce ASP-RNAi; and qualitative and quantitative evaluation of allele-specific gene silencing.

In this article, we describe an easy assay system for assessment of ASP-RNAi with mutant and wild-type reporter alleles encoding the *Photinus* and *Renilla luciferase* genes. Using the amyloid precursor protein

(*APP*) variants (the Swedish- and London-type variants) related to familial Alzheimer's disease (Goate et al, 1991; Mullan et al, 1992) as model mutant alleles, we determined the effects of siRNA duplexes against the mutant *APP* on allele-specific silencing as well as off-target silencing against the wild-type allele. The siRNA duplexes having the potential to specifically suppress the expression of the mutant reporter allele consistently inhibited the expression of the *bona fide* mutant APP as well as amyloid β 40- and 42-peptides in Cos-7 cells expressing both the full-length Swedish- and wild-type *APP* alleles. These observations suggest that the present system could permit the selection of siRNA duplexes having the potential to confer ASP-RNAi.

MATERIALS AND METHODS

Preparation of oligonucleotides

DNA and RNA oligonucleotides were obtained from INVITROGEN and TAKARA, respectively. For preparation of duplexes, sense- and antisense-stranded oligonucleotides (20 μM each) were mixed and annealed as described previously (Hohjoh, 2002). The sequences of synthesized oligonucleotides are shown in Tables 1 and 2. Non-silencing siRNA duplex (siControl; Qiagen) was used as a negative control.

Cell culture

HeLa, T98G and Cos-7 cells were grown at 37°C in Dulbecco's modified Eagle's medium (Wako) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma) in 5% CO_2-humidified chamber. T98G cells (Registry No. IFO50295) were obtained from the Health Science Research Resources Bank.

Construction of reporter and expression plasmids

In order to construct plasmids carrying reporter alleles, the phRL-TK (Promega) and pGL3-TK (Ohnishi et al., 2005) plasmids encoding the *Renilla* and *Photinus* luciferase genes, respectively, both of which were driven by the same herpes simplex virus thymidine kinase (TK) promoter, were digested with Xba I and Not I, and were subjected to ligation with synthetic oligonucleotide duplexes corresponding to the Swedish-, London- and wild-type *APP* alleles (sequences of the oligonucleotides used are indicated in Table 1). The resultant plasmids carry allelic *APP* sequences in the 3'-untranslated regions (UTRs) of the luciferase genes (Figure 1A). Expression plasmids, $pAPP695_{WT}$ and $pAPP695_{SWE}$ encoding full-length cDNAs of the wild- and Swedish-type *APP* alleles, respectively, were kindly provided by Dr Tanahashi (Tanahashi and Tabira, 2001).

Transfection and reporter assay

The day before transfection, cells were trypsinized, diluted with fresh medium without antibiotics, and seeded into 24-well culture plates (approximately 0.5×10^5 cells/well). Cotransfection of synthetic siRNA duplexes with reporter plasmids was carried out using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions, and to each well, 0.24 μg (40 nM) of siRNA duplexes, 0.2 μg of pGL3-TK-backbone plasmid, 0.05 μg of phRL-TK-backbone plasmid and 0.1 μg of pSV-β-Galactosidase control vector (Promega) were applied. Twenty-four hours after transfection, cell lysate was prepared and expression levels of luciferase and β-Galactosidase were examined by the Dual-Luciferase reporter assay system (Promega) and Beta-Glo assay system (Promega), respectively, according to the manufacturer's instructions. In the case of transfection of siRNA duplexes and expression plasmids ($pAPP695_{WT}$ and $pAPP695_{SWE}$) into Cos-7 cells, 0.4 μg of each plasmid and 0.24 μg of siRNA duplexes were applied. Forty-eight hours after transfection, culture media was collected and cell lysate was prepared.

Western blotting and ELISA

Culture media and cell lysate prepared from transfected Cos-7 cells were examined by western blotting as described previously (Lesne et al., 2003). Equal amounts of proteins were separated by SDS-PAGE and electrophoretically blotted onto PVDF membranes (Millipore). Membranes were blocked for 1 h in blocking solution (5 % (v/w) fat-free milk and 0.05 % (v/v) Tween-20 in PBS) and

Table 1. Synthetic DNA oligonucleotides

Name	Sequence (5'----------3')
ssAPPwt(Sw)	CTAGCATGCAGGAGATCTCTGAAGTGAAGATGGATGCAGAATTCCGACA
asAPPwt(Sw)	GGCCTGTCGGAATTCTGCATCCATCTTCACTTCAGAGATCTCCTGCATG
ssAPP(K670N-M671L)	CTAGCATGCAGGAGATCTCTGAAGTGAATCTGGATGCAGAATTCCGACA
asAPP(K670N-M671L)	GGCCTGTCGGAATTCTGCATCCAGATTCACTTCAGAGATCTCCTGCATG
ssAPPwt(Lo)	CTAGCATGCTGTCATAGCGACAGTGATCGTCATCACCTTGGTGATGCTGA
asAPPwt(Lo)	GGCCTCAGCATCACCAAGGTGATGACGATCACTGTCGCTATGACAGCATG
ssAPP(V717I)	CTAGCATGCTGTCATAGCGACAGTGATCATCATCACCTTGGTGATGCTGA
asAPP(V717I)	GGCCTCAGCATCACCAAGGTGATGATGATCACTGTCGCTATGACAGCATG
ssAPP(V717F)	CTAGCATGCTGTCATAGCGACAGTGATCTTCATCACCTTGGTGATGCTGA
asAPP(V717F)	GGCCTCAGCATCACCAAGGTGATGAAGATCACTGTCGCTATGACAGCATG
ssAPP(V717G)	CTAGCATGCTGTCATAGCGACAGTGATCGGCATCACCTTGGTGATGCTGA
asAPP(V717G)	GGCCTCAGCATCACCAAGGTGATGCCGATCACTGTCGCTATGACAGCATG

Table 2. Synthetic siRNAs used in this study. Sense- and antisense-stranded siRNA elements are indicated by '-ss' and '-as', respectively.

siRNAs against the Swedish *APP* mutant	
Name	**Sequence (5'----------3')**
si(T7/C8)-ss	AGUGAAUCUGGAUGCAGAAUUU
si(T7/C8)-as	AUUCUGCAUCCAGAUUCACUUU
si(T8/C9)-ss	AAGUGAAUCUGGAUGCAGAAUU
si(T8/C9)-as	UUCUGCAUCCAGAUUCACUUUU
si(T9/C10)-ss	GAAGUGAAUCUGGAUGCAGAUU
si(T9/C10)-as	UCUGCAUCCAGAUUCACUUCUU
si(T10/C11)-ss	UGAAGUGAAUCUGGAUGCAGUU
si(T10/C11)-as	CUGCAUCCAGAUUCACUUCAUU
si(T11/C12)-ss	CUGAAGUGAAUCUGGAUGCAUU
si(T11/C12)-as	UGCAUCCAGAUUCACUUCAGUU
si(T12/C13)-ss	UCUGAAGUGAAUCUGGAUGCUU
si(T12/C13)-as	GCAUCCAGAUUCACUUCAGAUU
siRNAs against the London *APP* mutants	
Name	**Sequence (5'----------3')**
si(A8)-ss	AGUGAUCAUCAUCACCUUGUU
si(A8)-as	CAAGGUGAUGAUGAUCACUUU
si(A9)-ss	CAGUGAUCAUCAUCACCUUUU
si(A9)-as	AAGGUGAUGAUGAUCACUGUU
si(A10)-ss	ACAGUGAUCAUCAUCACCUUU
si(A10)-as	AGGUGAUGAUGAUCACUGUUU
si(A11)-ss	GACAGUGAUCAUCAUCACCUU
si(A11)-as	GGUGAUGAUGAUCACUGUCUU
si(A12)-ss	CGACAGUGAUCAUCAUCACUU
si(A12)-as	GUGAUGAUGAUCACUGUCGUU
si(T8)-ss	AGUGAUCUUCAUCACCUUGUU
si(T8)-as	CAAGGUGAUGAAGAUCACUUU
si(T9)-ss	CAGUGAUCUUCAUCACCUUUU
si(T9)-as	AAGGUGAUGAAGAUCACUGUU
si(T10)-ss	ACAGUGAUCUUCAUCACCUUU
si(T10)-as	AGGUGAUGAAGAUCACUGUUU
si(T11)-ss	GACAGUGAUCUUCAUCACCUU
si(T11)-as	GGUGAUGAAGAUCACUGUCUU
si(T12)-ss	CGACAGUGAUCUUCAUCACUU
si(T12)-as	GUGAUGAAGAUCACUGUCGUU
si(G8)-ss	GUGAUCGGCAUCACCUUGGUU
si(G8)-as	CCAAGGUGAUGCCGAUCACUU
si(G9)-ss	AGUGAUCGGCAUCACCUUGUU
si(G9)-as	CAAGGUGAUGCCGAUCACUUU
si(G10)-ss	CAGUGAUCGGCAUCACCUUUU
si(G10)-as	AAGGUGAUGCCGAUCACUGUU
si(G11)-ss	ACAGUGAUCGGCAUCACCUUU
si(G11)-as	AGGUGAUGCCGAUCACUGUUU
si(G12)-ss	GACAGUGAUCGGCAUCACCUU
si(G12)-as	GGUGAUGCCGAUCACUGUCUU

were incubated with anti-APP antibody 22C11 (Chemicon) or anti-α-tubulin antibody DM1A (Sigma) followed by washing in PBS and further incubation with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). Antigen-antibody complexes were visualized using ECL chemiluminescent reagent (Amersham). Levels of Aβ40 and Aβ42 production in culture media were examined by human/rat β amyloid 40 and 42 ELISA kits (Wako) according to the manufacturer's instructions.

RT-PCR

Total RNA extraction, including treatment with DNase I (Ambion) twice followed by reverse transcription, were carried out as described previously (Sago et al., 2004). The resultant cDNAs were examined by real-time (RT)-PCR using the ABI PRISM 7300 sequence detection system (Applied Biosystems) with a SYBER green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. PCR primers used were as follows:

For detection of the *Renilla luciferase* transcript:

renilla-F; 5'-GTTCTTTTCCAACGCTATTG-3'
renilla-R; 5'-GAAGCTCTTGATGTACTTAC-3'

For detection of the *Photinus luciferase* transcript:

photinus-F; 5'-TTTGATATGTGGATTTCGAG-3'
photinus-R; 5'-ATCGTATTTGTCAATCAGAG-3'

RESULTS

Assessment of siRNAs in heterozygous model system

In this study, the Swedish- and London-type mutants of the *APP* gene, which are involved in familial Alzheimer's disease, were used as model mutant alleles. The Swedish- and London-type *APP* mutants carry double and single nucleotide substitutions, respectively, which are followed by amino acid substitutions (K670N-M671L in the Swedish APP; V717I, V717F or V717G in the London APP) (Goate et al, 1991; Mullan et al, 1992). The resultant amino acid sequences in the Swedish and London-type APPs are preferably digested by β- and γ-secretase, respectively, resulting in accumulation of Aβ40 and Aβ42 peptides, which are the key factors of Alzheimer's disease (Cai et al, 1993; Citron et al, 1992; Mattson, 2004; Suzuki et al, 1994).

Mutant and wild-type reporter alleles were constructed as described in Materials and Methods. The resultant reporter alleles (Figure 1A), synthetic siRNA duplex against the mutant allele and the *β-galactosidase* gene (control) were cotransfected into human cells. Note that the transfected cells are artificially heterozygous with the mutant and wild-type *APP* reporter alleles; thus, the effects of test siRNA duplexes on suppression of both the mutant and wild-type alleles can be simultaneously examined.

ASP-RNAi against the Swedish-type APP allele

When the *Renilla* and *Photinus luciferase* genes were regarded as the Swedish and wild-type reporter alleles, respectively, the effects of the si(T7/C8) - si(T12/C13) duplexes against the Swedish mutant on allele-specific gene silencing were examined in HeLa cells. The results

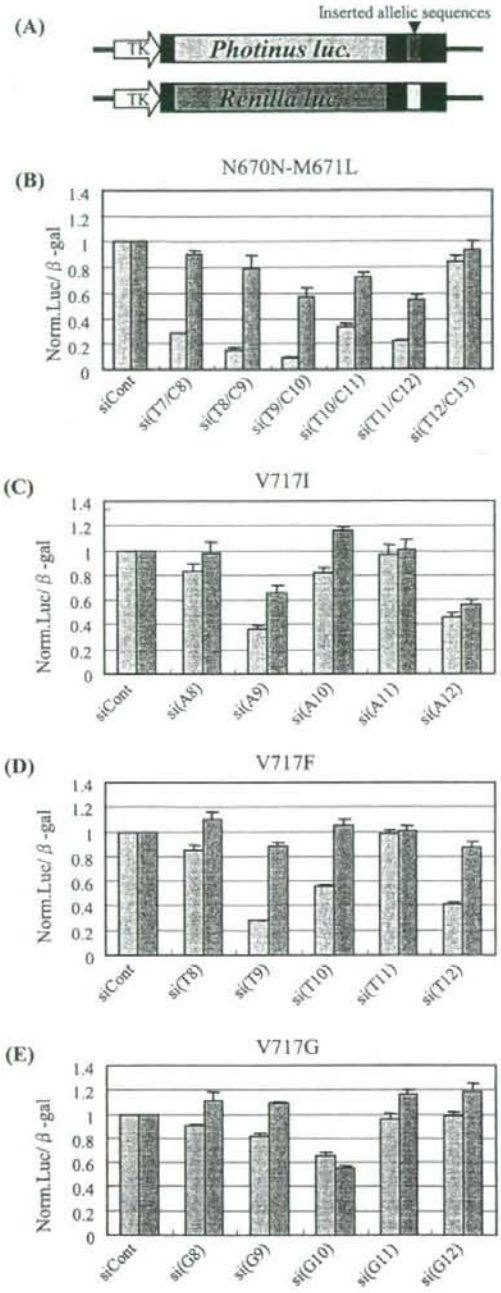

Figure 1. Assessment of ASP-RNAi with reporter alleles. (**A**) Schematic drawing of reporter alleles. Reporter alleles were constructed based on the *Photinus* and *Renilla luciferase* reporter genes driven by the same TK promoter, and allelic sequences of wild-type and mutant (synthetic oligonucleotides) were inserted into the 3'-UTRs of the reporter genes, i.e., the reporter alleles encode *luciferase* reporter genes carrying artificially inserted allele sequences of interest. Assessment of siRNA duplexes on the induction of ASP-RNAi against the Swedish *APP* mutant (**B**) and against the London APP mutants (**C-E**) was carried out. Synthetic siRNA duplexes against the mutants indicated were cotransfected with the mutant and wild-type reporter alleles and the *β-galactosidase* gene (control) into HeLa cells. The *Photinus* and *Renilla luciferase* genes carry the mutant and wild-type allelic sequences, respectively. Twenty-four hours after transfection, dual-luciferase and β-galactosidase assays were carried out. The levels of either *Photinus* (blue boxes) or *Renilla* (pink boxes) luciferase activity was normalized against the levels of β-galactosidase activity, and the ratios of mutant and wild-type luciferase activities in the presence of siRNA duplexes were normalized against the control ratio obtained in the presence of the siControl duplex (siCont). Data are averages of at least three independent determinations. Error bars represent standard deviations.

are shown in Figure 1B. The siRNA duplexes, except for the si(T12/C13) duplex, appeared to induce inhibition of mutant (*Photinus*) allele expression, while little or moderate inhibition of wild-type (*Renilla*) allele expression was seen, suggesting that the siRNA duplexes were able to discriminate the mutant reporter allele from the wild-type reporter allele. The si(T12/C13) duplex appeared to yield little or no RNAi activity. Considering the influence of the siRNA duplexes on the expression of the wild-type allele, the si(T8/C9) duplex appears to be the most suitable siRNA duplex conferring ASP-RNAi against the mutant allele. As for the si(T9/C10) and si(T11/12) duplexes inducing moderate levels of inhibition of wild-type allele expression, further analyses were carried out (Figure 4). Similar results were also obtained when the luciferase genes were exchanged between the mutant and wild-type reporter alleles, i.e., the *Photinus* and *Renilla luciferase* genes carried the wild-type and Swedish allele sequences, respectively (data not shown). In addition, when T98G cells, a human glioblastoma cell line, and Cos-7 cells were used instead of HeLa cells, results similar to those obtained in HeLa cells were observed (data not shown).

ASP-RNAi against London-type APP alleles

Because the London-type mutant possesses three types of single nucleotide change involved in amino acid substitution at position 717 (V717I, V717F and V717G), three mutant reporter alleles and corresponding wild-type reporter allele were constructed, and the effects of synthetic siRNA duplexes against the London-type mutants on suppression of the expression of either the target mutant allele or wild-type allele were examined under the present system. As shown in Figure 1C-E, various levels of gene silencing were observed and some of the siRNA duplexes, si(T9) and si(T12) (Figure 1D), appeared to discriminate the mutant alleles from the wild-type allele to some degree, resulting in ASP-RNAi; however, the other siRNA duplexes examined yielded less significant ASP-RNAi. Compared with the results for ASP-RNAi against the Swedish allele (Figure 1B), the induction and activation of ASP-RNAi against the London alleles appeared to be inferior to those against the Swedish mutant.

Western blot analyses of wild-type and Swedish APP in ASP-RNAi

We further investigated ASP-RNAi of siAPP duplexes against the Swedish mutant with full-length cDNAs of the Swedish and wild-type *APP* alleles, which were transiently

expressed in Cos-7 cells. The pAPP695$_{SWE}$ and/or pAPP695$_{WT}$ expression plasmids encoding full-length cDNAs of the Swedish and wild-type *APP* alleles, respectively, and siRNA duplexes targeting the Swedish mutant were cotransfected into Cos-7 cells, and expression of wild-type APP (APP$_{WT}$) and Swedish APP (APP$_{SWE}$) was examined by Western blotting. As shown in Figure 2, under homo(or hemi)zygous-like conditions, in which either APP$_{WT}$ or APP$_{SWE}$ was expressed, the signal intensity of sAPP$_{SWE}$ (secreted APP) and cAPP$_{SWE}$ (celluar APP) was apparently decreased in the presence of the si(T8/C9), si(T9/C10) and si(T11/C12) duplexes. In contrast, signals for either sAPP$_{WT}$ or cAPP$_{WT}$ were detected in the presence of any of the siRNA duplexes examined, which is consistent with the data for the reporter alleles described above. When APP$_{SWE}$ and APP$_{WT}$ were both expressed in the cells (heterozygous-like conditions), signals for APP were seen in the presence of any of the siRNA duplexes. Based on the results under homozygous-like conditions, the signals for APP in the presence of the si(T8/C9), si(T9/C10) and si(T11/C12) duplexes were most likely derived from APP$_{WT}$.

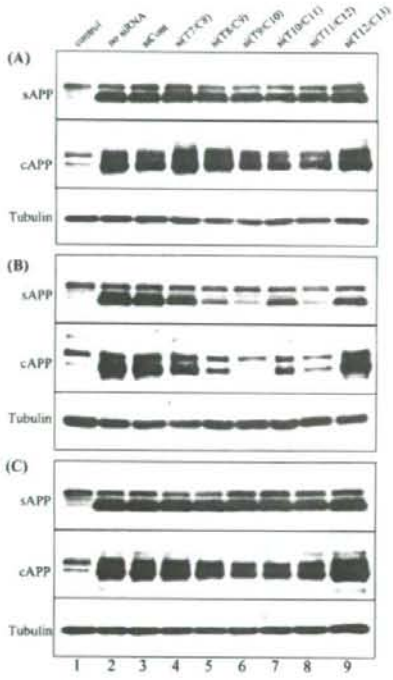

Figure 2. Expression of APP$_{WT}$ and APP$_{SWE}$ polypeptides under ASP-RNAi. Either the pAPP695$_{WT}$ (**A**) and pAPP695$_{SWE}$ (**B**) expression plasmids or the plasmids (**C**) together with the indicated siRNA duplexes against the Swedish mutant were introduced into Cos-7 cells, and expressed APP polypeptides in culture media (secreted APP: sAPP) and in cells (cellar APP: cAPP) were examined by Western blotting. Lane 1 (control) shows no transfected Cos-7 cells, in which endogenous APP is detectable. Lanes 2-9 are cells transfected with expression plasmid(s), and lanes 3-9 are cotransfected cells with the indicated siRNA duplexes. Expression of α-tubulin (control) is also shown.

The utility of ASP-RNAi using the siRNA duplexes assessed here in medical treatment can be demonstrated by confirming a significant decrease in Aβ peptides, which are a key factor in the development of Alzheimer's disease under heterozygous conditions expressing both APP$_{SWE}$ and APP$_{WT}$. We thus determined the production levels of Aβ40 and Aβ42 peptides by means of ELISA. As shown in Figure 3, significant decreases in the production of either Aβ40 or Aβ42 peptide by RNAi (Figure 3A-C) and ASP-RNAi (Figure 3D-F) with the evaluated siRNA duplexes, particularly si(T8/C9), si(T9/C10) and si(T11/C12), was confirmed under homozygous and heterozygous conditions, respectively. Therefore, these results suggest the potential utility of such siRNA duplexes as therapeutic agents.

DISCUSSION

While ASP-RNAi is believed to be a useful technique, to realize and control ASP-RNAi, it is vital to design and select competent siRNA duplexes conferring ASP-RNAi; however, this is rather difficult without a procedure for assessing such siRNA duplexes. The system we present here could allow assessment, if designed siRNA duplexes have the potential for specifically inhibiting the expression of target alleles without suppressing the expression of other alleles. From a series of experiments with the Swedish- and London-type APP variants as model mutant alleles, we were able to determine potential siRNA duplexes for inducing ASP-RNAi. With regard to siRNA duplexes targeting the Swedish mutant, we further demonstrated that the si(T8/C9), si(T9/C10) and si(T11/C12) siRNA duplexes were able to significantly decrease the production of either Aβ40 or Aβ42 peptide in Cos-7 cells expressing both the full-length Swedish- and wild-type *APP* alleles. Accordingly, such competent siRNA duplexes conferring ASP-RNAi against mutant alleles likely hold utility as therapeutic agents.

In contrast to the Swedish mutant, there were difficulties in suppressing the London-type mutants carrying single nucleotide substitutions from the wild-type allele by ASP-RNAi. The difference between ASP-RNAi activities against the Swedish- and London-type mutants may have been caused by the number of base substitutions: the former and latter mutants carry double and single base substitutions, respectively. Another important point to note in the results for the London-type mutant is that different substitutions showed different ASP-RNAi activities, suggesting that the type of base change between the mutant and wild-type alleles could influence ASP-RNAi. With regard to the V717I (Figure 1C) and V717G (Figure 1E) mutants, a possible wobble base pair between siRNA and the wild-type mRNA (Du et al, 2005) and high GC content of siRNA used(Ui-Tei et al, 2004), respectively, might have negatively influenced the induction of ASP-RNAi; these possibilities require further examination in the future.

To further progress ASP-RNAi, it is necessary to design competent siRNA duplexes conferring strong allele-specific gene silencing. Chemical modifications (Chiu and Rana, 2003; Hall et al, 2004) and structural devices in siRNAs are considered to be applicable for improving

ASP-RNAi, and assessment of such siRNAs is feasible using the system we presented here. Altogether, it is suggested that the present assay system may contribute to the design and selection of the most suitable of siRNA duplexes conferring ASP-RNAi.

Finally, we add data indicating the possible inhibition of wild-type allele translation by the present siRNA duplexes. Because si(T9/C10) and si(T11/C12) exhibited moderate levels of inhibition of the expression of wild-type reporter allele (Figure 1B), we further investigated RNA levels of the wild-type allele by RT (real-time)-PCR. As shown in Figure 4, the levels of RNA expression of the wild-type allele in the presence of si(T9/C10) were similar to those in the presence of siControl, suggesting the possible inhibition of translation of the wild-type allele by the si(T9/C10) duplex. This may be due to a microRNA-like effect (Poy et al, 2004; Tang, 2005), and further study into this possibility remains necessary. With regard to the si(T11/C12) duplex, because a decrease trend in the levels of wild-type allele transcript was seen, it is possible that off-target gene silencing (Jackson et al, 2003) of the wild-type allele may occur in the presence of the duplex. Consequently, it is conceivable that the present system could further contribute to studies into off-target gene silencing and the function of microRNAs.

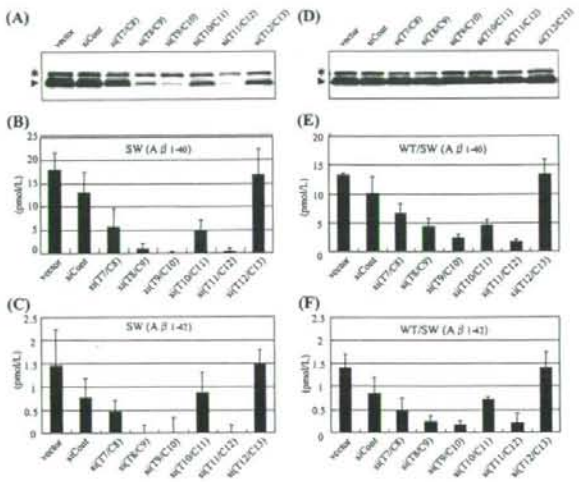

Figure 3. Production of Aβ40 and Aβ42 peptides under ASP-RNAi. The pAPP695$_{SWE}$ (**A-C**) plasmid and both the pAPP695$_{SWE}$ and pAPP695$_{WT}$ (**D-F**) plasmids together with the indicated siRNA duplexes against the Swedish mutant were cotransfected into Cos-7 cells, and expressed sAPP polypeptide and Aβ40 and Aβ42 peptides in culture media were examined by western blotting (**A, D**) and ELISA (**B, C, E, F**), respectively. "Vector" indicates cells transfected with only plasmid(s). Endogenous and exogenous (expressed) sAPPs are indicated by asterisks and arrow heads, respectively. ELISA data are averages of three independent determinations. Error bars represent standard deviations.

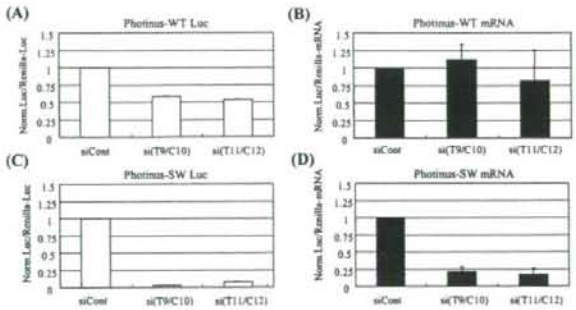

Figure 4. Possible translation inhibition and off-target silencing of wild-type reporter allele by siAPP duplexes. The si(T9/C10) or si(T11/C12) duplexes against the Swedish mutant allele together with either wild or mutant reporter allele plasmid carrying *Photinus luciferase* and the phRL-TK plasmid encoding *Renilla luciferase* (control) were introduced into HeLa cells. Twenty-four hours after transfection, dual-luciferase assay and isolation of total RNA were carried out. Off-target (to wild-type reporter allele) (**A**) and on-target (RNAi; to mutant reporter allele) (**C**) gene silencing were assessed based on luciferase activities. Ratios of normalized target (Photinus) luciferase activity to control (Renilla) luciferase activity are indicated: the ratios of luciferase activity determined in the presence of the si(T9/C10) or si(T11/C12) duplexes were normalized against the ratios obtained in the presence of the siControl duplex (siCont). Isolated RNAs in (**B**) and (**D**) corresponding to (**A**) and (**C**), respectively, were subjected to reverse transcription to