厚生労働科学研究費補助金

第3次対がん総合戦略研究事業

放射線感受性ナノバイオ・ウイルス製剤の開発と難治性固形癌に対する臨床応用の検討

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研究代表者　藤原　俊義

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研究代表者　藤原　俊義

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【研究要旨】

最近のゲノム科学やナノテクノロジーの進歩により、癌の悪性形質の発現に関わる分子機構が明らかとなり、癌に特異的な標的分子を定めることが可能となってきた。p53はヒト悪性腫瘍で最も高頻度に異常がみられる癌抑制遺伝子であり、その正常型の遺伝子導入により放射線感受性増強をはじめとする多彩な作用機序を介した抗腫瘍効果が認められる。しかし、非増殖型ウイルスベクターでは腫瘍内へのウイルス拡散や遺伝子導入効率に限界があり、根治を目指した治療には至っていない。ウイルスは本来、ヒトの細胞に感染して複製・増殖することで細胞を破壊する。その増殖機能に選択性を付加することにより、ウイルスを癌細胞のみを殺傷する抗癌剤として用いることが可能となる。本研究では、テロメラーゼ依存性に増殖し、細胞死を誘導するOBP-301（Telomelysin）をさらに武装化（arming）し、ウイルス増殖による細胞死とともにアポトーシス誘導分子p53を発現することで強力な抗腫瘍活性を発揮する新規ナノバイオ・ウイルス製剤OBP-702を開発する。平成19年度はOBP-301の構造を基盤として*p53*遺伝子を組み込む遺伝子改変を行い、作成したOBP-702ウイルスを用いて*in vitro*における機能解析と抗腫瘍活性の検討を行った。平成20年度は、OBP-702とOBP-301、さらに*p53*遺伝子を発現する非増殖型アデノウイルスAdvexinとの抗腫瘍活性の比較検討を行い、またOBP-702のOBP-301より強力な放射線増感作用を検証した。平成21年度には、OBP-702の作用機序として、細胞周期制御因子p21の発現を顕著に低下させることで、細胞周期を止めずに効率的にアポトーシス細胞死を引き起こしていることを明らかにし、OBP-702の放射線併用によるOBP-301より強力なアポトーシス誘導能を示した。

A．研究目的

難治固形癌に対する新たな抗癌剤開発は、分子標的薬剤の開発などにより積極的に進められており、その治療成績の向上も現実のものとなってきている。しかし、副作用や耐性の出現など解決すべき問題点は多く、新たな治療戦略の開発は必須と考えられる。本研究の目的は、ベクターとして多くの遺伝子治療で使用され、その安全性が確認されてきたアデノウイルスのゲノムを改変し、より強力な抗腫瘍活性を有する武装化（armed）ナノバイオ・ウイルス製剤を開発することである。

ウイルスは本来ヒトの細胞に感染して、その構造蛋白質を産生することで複製・増殖する。その増殖機能に選択性を付加することにより、ウイルスを癌細胞のみを殺傷する抗癌剤として用いることが可能となる。「かぜ」症状の原因となるアデノウイルス5型を基本骨格とし、80-90%のヒト悪性腫瘍で極めて高い活性がみられる不死化関連酵素テロメラーゼの構成分子であるhTERT (human telomerase reverse transcriptase)遺伝子のプロモーターでウイルス増殖に必須の*E1A*および*E1B*遺伝子を制御することで、癌細胞のみで増殖する腫瘍融解アデノウイルス（Oncolytic adenovirus）を構築する。さらに、放射線感受性プロモーターEgr-1で強力なアポトーシス誘導機能を持つ*p53*癌抑制遺伝子を駆動する発現カセットを、ウイルスの*E3*遺伝子領域に搭載する。

このナノバイオ・ウイルス製剤OBP-702は、癌細胞で選択的に増殖することにより標的細胞死を引き起こす機能を有する。また、放射線によるアポトーシス誘導でもp53は重要なシグナル伝達経路であり、OBP-702によるp53の過剰発現により放射線感受性自体も増強されると考えられる。さらに、原発腫瘍内に局所投与されたOBP-702は、周辺のリンパ節にも到達してリンパ節転移巣でも増殖するため、臨床的には所属リンパ節を含めた放射線治療との強力な相乗効果が期待される。

平成19年度はウイルス構築と*in vitro*における抗腫瘍効果の確認を行い、平成20年度にはOBP-301および*p53*遺伝子を発現する非増殖型アデノウイルスAdvexinとの抗腫瘍効果の比較検討を行った。本年度は、OBP-702の強力な抗腫瘍効果の分子機構を明らかにし、さらにアポトーシス誘導能を指標としたOBP-702の放射線感受性増強作用を検証した。

B．研究方法

1）テロメラーゼ（hTERT）特異的p53遺伝子発現腫瘍融解ウイルス製剤（開発コード：OBP-702）の作成

テロメラーゼ構成分子であるhTERT遺伝子のプロモーターとIRES配列を間置したアデノウイルスE1A遺伝子およびE1B遺伝子から成る増殖カセット、および放射線感受性プロモーターEgr-1とヒト正常型p53遺伝子を有するp53遺伝子発現カセットを、アデノウイルス5型ゲノムのE1領域とE3領域にそれぞれ挿入した。このウイルスゲノムのプラスミドをE1遺伝子によるトランスフォームされたヒト腎臓293細胞にトランスフェクションし、上清中に産生されたウイルスを抽出精製、放射線感受性プロモーターEgr-1でp53遺伝子発現を制御するテロメラーゼ特異的ウイルス製剤OBP-702を作成した。

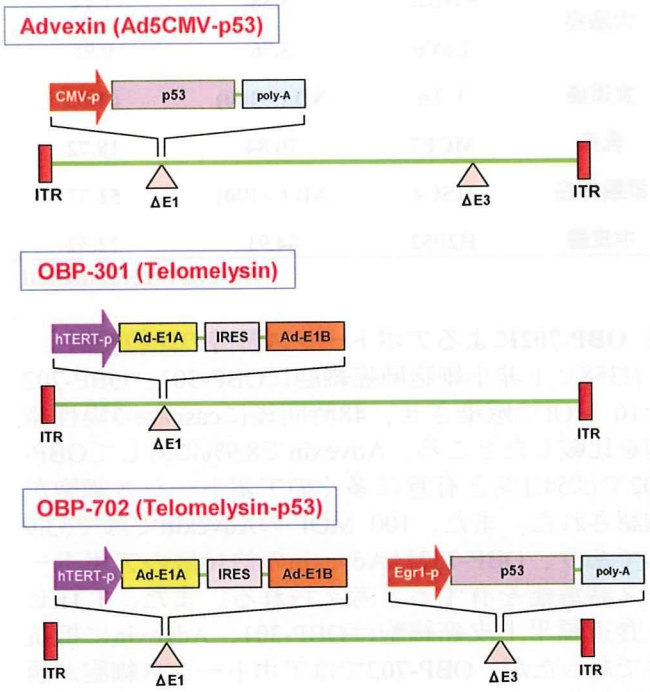


図１　ウイルス製剤の構造

2）OBP-702と放射線照射による*p53*遺伝子発現の検討

H1299ヒト肺癌細胞にOBP-702を感染させ、経時的にp53蛋白質発現をウエスタンブロット法にて確認した。また、各種ヒト癌細胞（H1299、H460、H358ヒト肺癌細胞、SW620、LoVoヒト大腸癌細胞、HepG2、Huh7ヒト肝癌細胞）にOBP-702を感染させ、24時間後に10Gyの放射線照射を行い、感染から48時間後にp53蛋白質発現を確認した。

3）*In vitro*におけるOBP-702の抗腫瘍効果の検討

上記細胞株を含む11種の癌細胞株において、OBP-702あるいはOBP-301感染後、経日的にXTTアッセイにて生細胞数比率を測定し、それぞれの抗腫瘍効果を比較検討した。

4）OBP-702によるアポトーシス誘導の比較検討

OBP-702、OBP-301、およびp53遺伝子を発現する非増殖型アデノウイルスAdvexinを用いて、ヒト非小細胞肺癌細胞株H358、ヒト食道扁平上皮癌細胞株T.Tnにおけるアポトーシス誘導能をBD FACSArray バイオアナライザーによる活性化caspase-3の発現比較にて解析した。

5）OBP-702によるアポトーシス誘導の分子機構の解析

*p53*遺伝子産物の標的分子であるp21の経時的発現変化をウエスタンブロット解析にて検討し、その分子機構を解析した。

また、その分子機構へのアデノウイルスE1Aタンパク質の関与を検証するために、E1Aを持たない非増殖型アデノウイルスdl312とE1Aを持つ野生型アデノウイルスAd-wt感染後のAdvexinによるp21発現変化を比較検討した。

6）*In vivo*におけるOBP-702の抗腫瘍効果の検討

H358ヒト非小細胞肺癌細胞をBALB/cヌードマウスの背部皮下に移植し、5-7 mm大の腫瘍形成がみられる14日目から10^8 plaque forming units (PFU)のAdvexin、OBP-301、OBP-702製剤を3日おきに3回の腫瘍内投与を行い、腫瘍サイズの変化を経日的に観察した。また、生存期間を比較検討した。

7）OBP-702と放射線照射による相乗効果の検討

H358ヒト非小細胞肺癌細胞およびT.Tnヒト食道扁平上皮癌細胞に0、0.1、1、5、10 multiplicity of infection (MOI)のOBP-301あるいはOBP-702を感染させ、24時間後に0、2、5、10、20 Gyの放射線を照射し、5日後にXTTアッセイにて生細胞数を比較検討した。また、Combination Index（CI）をCalcuSyn software（ver. 2）にて計算して、相乗効果の解析を行った。

8）OBP-702と放射線照射によるアポトーシス誘導効果の検討

H358ヒト非小細胞肺癌細胞およびT.Tnヒト食道扁平上皮癌細胞にそれぞれ5あるいは50 MOIのAdvexin、OBP-301、あるいはOBP-702を感染させ、24時間後に5Gyの放射線を照射した。アポトーシス誘導能をBD FACSArray バイオアナライザーによる活性化caspase-3の発現比較にて解析した。

9）OBP-702製剤の大量製造

遺伝子・細胞治療センターにWave 20バイオリアクターおよびAKTAカラムクロマトグラフィー装置を設置し、HeLa細胞を用いてOBP-702の大量製造を行った。

（倫理面への配慮）

本研究は「大臣確認実験」となるため、「第二種使用等拡散防止措置確認申請書」を作成、学内の担当部署での検討の後に文部科学省に申請し、研究計画実施の承認を得ている。

C．研究結果

1）テロメラーゼ（hTERT）特異的p53遺伝子発現腫瘍融解ウイルス製剤（開発コード：OBP-702）の作成

hTERT遺伝子のプロモーターとE1A／E1B遺伝子から成る増殖カセット、およびEgr-1プロモーターとヒト正常型p53遺伝子を有するp53発現カセットを、アデノウイルス5型ゲノムのE1領域とE3領域にそれぞれ挿入してOBP-702を作成した。

2）OBP-702と放射線照射によるp53遺伝子発現の検討

H1299ヒト肺癌細胞において、OBP-702感染後12時間からp53蛋白質発現がみられ、少なくとも72時間後まで発現は維持された。

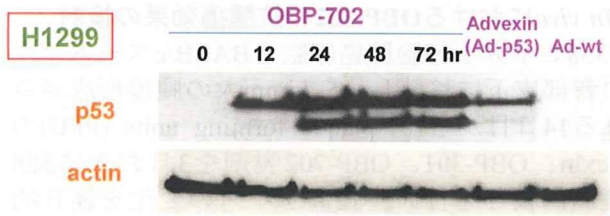


図2　OBP-702によるp53蛋白質発現

また、ヒト癌細胞（H1299、H460、H358ヒト肺癌細胞、SW620、LoVoヒト大腸癌細胞、HepG2、Huh7ヒト肝癌細胞）において、OBP-702を感染48時間後にp53蛋白質発現を確認されたが、放射線照射によってもp53蛋白質発現レベルの明らかな増強は認められなかった。これらの結果から、ウイルス増殖自体がストレスとしてEgr-1プロモーターを駆動したと考えられる。

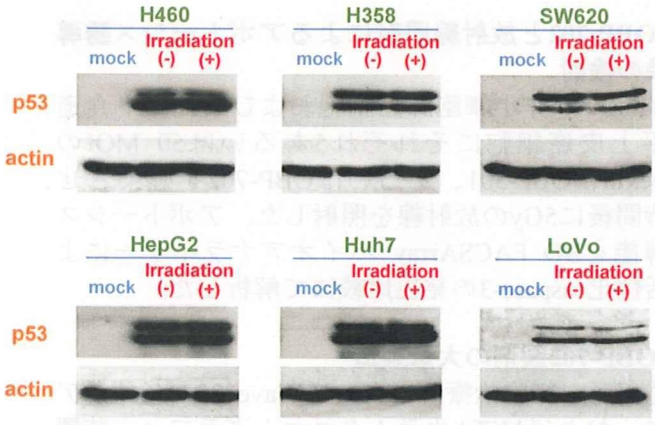


図3　放射線のOBP-702によるp53発現への影響

3）*In vitro*におけるOBP-702の抗腫瘍効果の検討

11種のすべての癌細胞株において、OBP-301よりもOBP-702で強い抗腫瘍活性がみられ、OBP-702の方が低いID50を示した。特に、OBP-301に完全に耐性のT.Tn食道癌細胞でOBP-702の抗腫瘍効果が認められ、その有用性が示唆された。

表1　ヒト癌細胞におけるOBP-702の抗腫瘍活性
(ID50 value on day 3)

		OBP-301	OBP-702
肺癌	H1299	4.24	1.49
	H460	13.13	2.41
	H358	21.51	4.19
肝臓癌	Huh7	10.35	0.93
	HepG2	1.8	0.47
大腸癌	SW620	5.58	1.14
	LoVo	3.76	0.98
食道癌	T.Tn	ND (>100)	61.05
乳癌	MCF7	70.84	18.72
頭頸部癌	HSC4	ND (>100)	51.77
中皮腫	H2052	64.91	22.32

ND: not determined

4）OBP-702によるアポトーシス誘導の比較検討

H358ヒト非小細胞肺癌細胞にOBP-301、OBP-702を10 MOIで感染させ、48時間後にcaspase-3陽性細胞を比較したところ、Advexinで8.9%に対してOBP-702では54.1％と有意に多くのアポトーシス細胞が確認された。また、100 MOI のAdvexinではで53.9％であり、OBP-702はAdvexinの約10倍のアポトーシス誘導能を有すると考えられる。また、T.Tnヒト食道扁平上皮癌細胞はOBP-301、Advexinに抵抗性であったが、OBP-702ではアポトーシス細胞が誘導された。

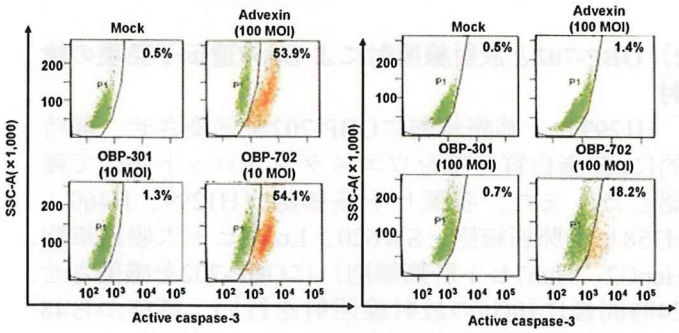


図4　OBP-702感染によるアポトーシス誘導能（1）
（左　H358細胞、右　T.Tn細胞）

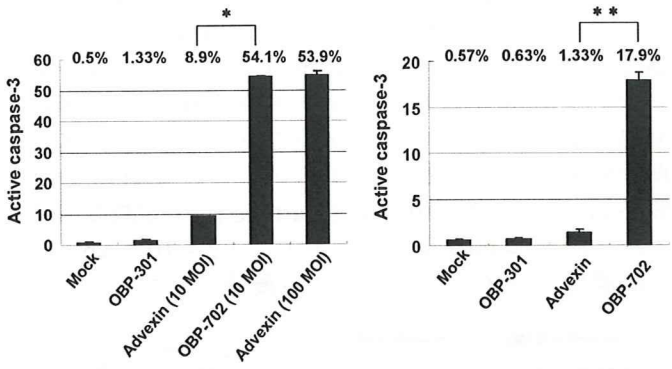


図5　OBP-702感染によるアポトーシス誘導能（2）
（左　H358細胞、右　T.Tn細胞）
（$*p < 0.05$　$**p < 0.01$）

5）OBP-702によるアポトーシス誘導の分子機構の解析

H358細胞では5 MOIのAdvexinで持続的なp21発現がみられたが、OBP-702 5 MOIの感染では24時間で一過性にp21の発現がみられた後に減弱した。T.Tn細胞では50 MOIのAdvexinでp21発現が経時的に増強したが、OBP-702感染ではp21発現は認められなかった。また、OBP-702の感染ではBaxの発現増強とともにpoly（ADP-ribose）polymerase (PARP)のcleavageが認められた。

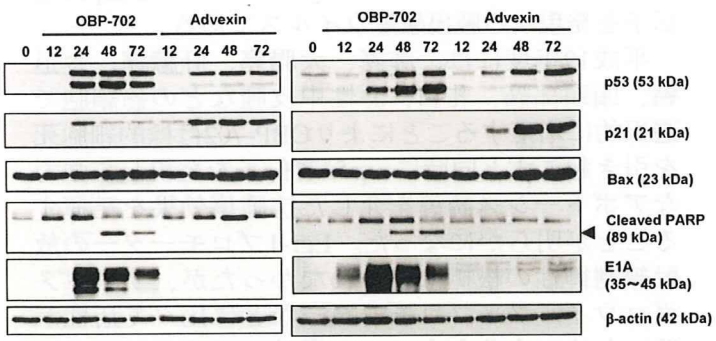


図6　OBP-702感染によるp21発現変化
（左　H358細胞、右　T.Tn細胞）

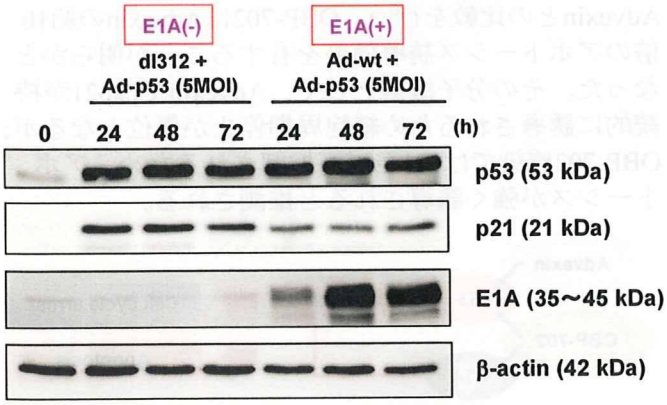
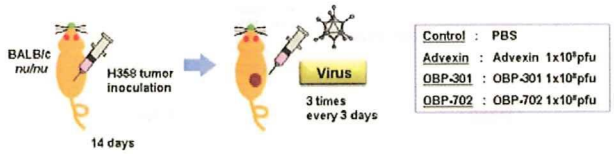


図7　アデノウイルスE1Aのp21発現への関与

さらに、H358細胞においてE1Aを持たない非増殖型アデノウイルスdl312の感染ではAdvexinによるp21発現は影響を受けなかったが、E1Aを持つ野生型アデノウイルスAd-wtの感染によってAdvexinによるp21発現は顕著に減弱した。

6）*In vivo*におけるOBP-702の抗腫瘍効果の検討

H358背部腫瘍へのAdvexinおよびOBP-301の腫瘍内投与はコントロール群に比べて有意な増殖抑制効果を示したが、OBP-702投与群ではさらに有意な抗腫瘍活性が認められた。



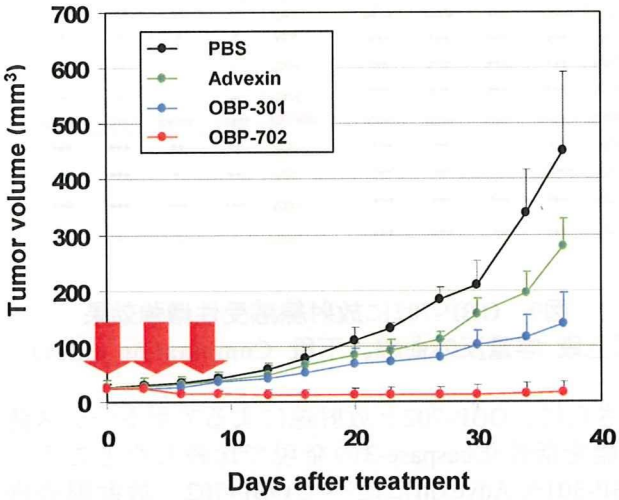


図7　*In vivo*でのOBP-702による抗腫瘍効果（1）

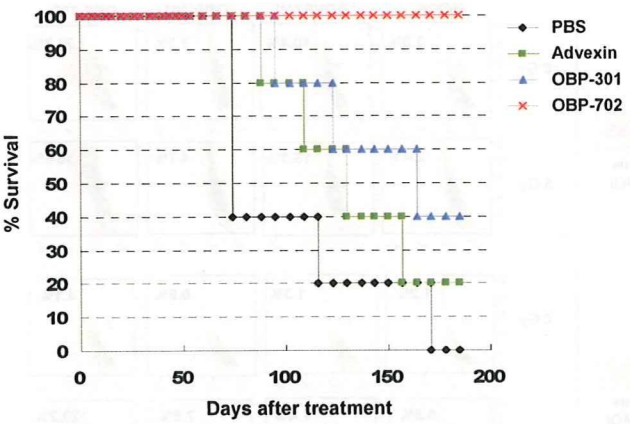


図8　*In vivo*でのOBP-702による抗腫瘍効果（2）

7）OBP-702と放射線照射によるアポトーシス誘導効果の検討

OBP-702と放射線照射による相乗効果の検討

H358ヒト非小細胞肺癌細胞およびT.Tnヒト食道扁平上皮癌細胞において、OBP-301、OBP-702いずれも容量依存性の放射線との増強効果が認められ

た。しかし、CIの算出では、OBP-301はH358細胞においてのみ、またOBP-702はH358、T.Tn細胞いずれにおいても相乗効果が確認された。

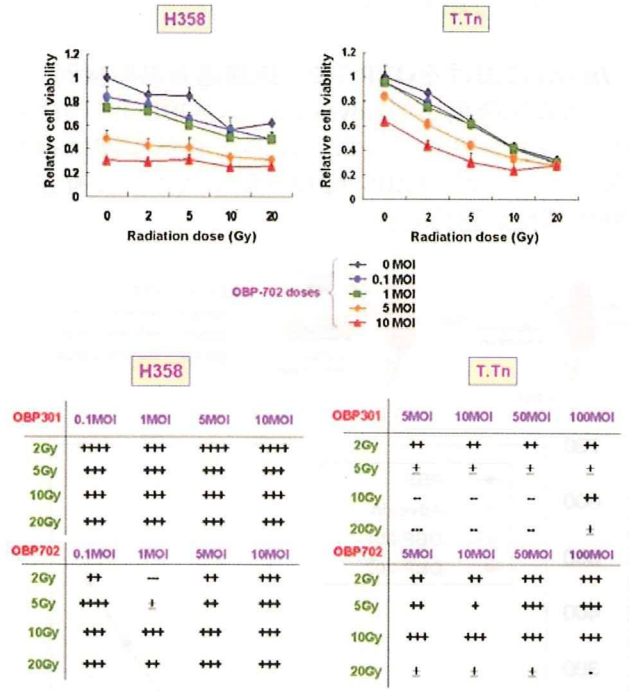


図9　OBP-702に放射線感受性増強効果
（上段 容量反応曲線、下段 Combination Index）

さらに、OBP-702と放射線によるアポトーシス誘導能を活性化caspase-3の発現で比較したところ、OBP-301やAdvexinに比べてOBP-702と放射線の併用でもっとも高率にアポトーシス細胞の増強が確認された。

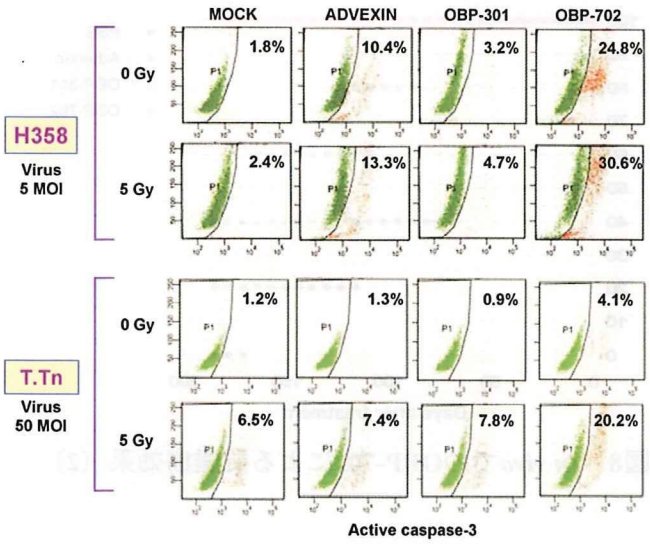


図10　OBP-702による放射線感受性増強効果

8）OBP-702製剤の大量製造

Wave 20バイオリアクターにてHeLa細胞を大量培養し、OBP-702の種ウイルスを感染させ、細胞およびウイルスが十分増殖した時点でTween 20にて細胞を融解した。安全キャビネット内でフィルトレーションを繰り返してウイルスを濃縮し、最後にAKTA100カラムクロマトグラフィーでウイルス分画を回収して高品質ウイルスストックを作成した。最終的には、2.2×10^{12} virus particle (vp)/ml濃度のウイルス液が54 ml採取できており、1.2×10^{14} vpという高濃度のウイルスストックが調達できた。

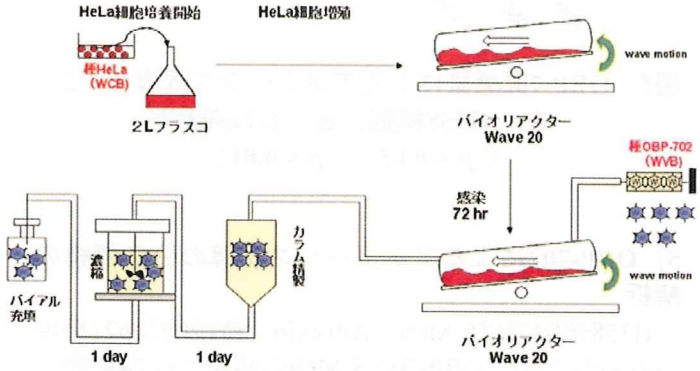


図11　Wave 20 Bioreactorによる製造工程

D．考察

本研究で開発したOBP-702は、hTERT遺伝子のプロモーターでアデノウイルス*E1*遺伝子を駆動し、放射線感受性プロモーターEgr-1でヒト正常型p53遺伝子を発現する腫瘍融解ウイルスである。

平成19年度には、肺癌、大腸癌、肝臓癌、食道癌、頭頸部癌、乳癌、悪性中皮腫などの癌細胞で選択的に増殖することによりOBP-702は標的細胞死を引き起こすと同時に、*p53*遺伝子を発現して強力なアポトーシス誘導を介した抗腫瘍効果を発揮することが明らかになった。Egr-1プロモーターの放射線制御性の根拠は得られなかったが、サイトメガロウイルス・プロモーターなどに比べて活性が低いため、十分なウイルス増殖が可能となり、その相乗効果が顕著にみられたと考察できる。

平成20～21年度には、p53遺伝子を持たないOBP-301およびp53遺伝子発現非増殖型アデノウイルスAdvexinとの比較を行い、OBP-702はAdvexinの約10倍のアポトーシス誘導効果を有することが明らかとなった。その分子機構として、Advexinではp21が持続的に誘導されるため細胞周期停止が優位となるが、OBP-702感染ではp21発現が抑制されるため、アポトーシスが強く誘導されると推測される。

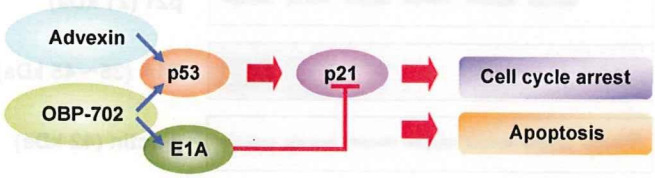


図12　OBP-702とAdvexinの作用機序の違い

ヌードマウスの背部に移植したH358ヒト肺癌腫瘍にOBP-702を腫瘍内投与したところ、同容量のAdvexinやOBP-301に比べて有意に強力な*in vivo*抗腫瘍効果がみられ、さらに生存期間の延長が認められた。

さらに、OBP-702の放射線感受性増強効果を検討したところ、OBP-301に感受性のあるH358ヒト肺癌細胞、OBP-301に抵抗性のT.Tnヒト食道癌細胞のいずれでもアポトーシス誘導に伴う明らかな放射線増感作用が観察されており、OBP-702は放射線増感作用を有する生物製剤と言える。

今後は、前臨床研究としての毒性試験や体内動態の分析を進めることで、OBP-702によるトランスレーショナルリサーチの実現を目指す。

E．結論

p53遺伝子を搭載したテロメラーゼ特異的増殖アデノウイルスOBP-702製剤は、基盤となったp53遺伝子を持たないOBP-301製剤やp53遺伝子を発現する非増殖型アデノウイルスより強い抗腫瘍活性を示し、その有用性が示された。また、放射線感受性増強効果も明らかであり、今後のトランスレーショナルリサーチが期待される。

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研究成果の刊行に関する一覧表（1）

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研究成果の刊行に関する一覧表（2）

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ouchi, M., Kawamura, H., Urata, Y., Fujiwara, T.	Antiviral activity of cidofovir against telomerase-specific replication-selective oncolytic adenovirus, OBP-301 (Telomelysin).	**Investigational New Drugs**	27	241-245	2009
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Review Article

Understanding and exploiting *hTERT* promoter regulation for diagnosis and treatment of human cancers

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Telomerase activation is a critical step for human carcinogenesis through the maintenance of telomeres, but the activation mechanism during carcinogenesis remains unclear. Transcriptional regulation of the human telomerase reverse transcriptase (*hTERT*) gene is the major mechanism for cancer-specific activation of telomerase, and a number of factors have been identified to directly or indirectly regulate the *hTERT* promoter, including cellular transcriptional activators (c-Myc, Sp1, HIF-1, AP2, ER, Ets, etc.) as well as the repressors, most of which comprise tumor suppressor gene products, such as p53, WT1, and Menin. Nevertheless, none of them can clearly account for the cancer specificity of hTERT expression. The chromatin structure via the DNA methylation or modulation of nucleosome histones has recently been suggested to be important for regulation of the *hTERT* promoter. DNA unmethylation or histone methylation around the transcription start site of the *hTERT* promoter triggers the recruitment of histone acetyltransferase (HAT) activity, allowing *hTERT* transcription. These facts prompted us to apply these regulatory mechanisms to cancer diagnostics and therapeutics. Telomerase-specific replicative adenovirus (Telomelysin, OBP-301), in which *E1A* and *E1B* genes are driven by the *hTERT* promoter, has been developed as an oncolytic virus that replicates specifically in cancer cells and causes cell death via viral toxicity. Direct administration of Telomelysin was proved to effectively eradicate solid tumors *in vivo*, without apparent adverse effects. Clinical trials using Telomelysin for cancer patients with progressive stages are currently ongoing. Furthermore, we incorporated green fluorescent protein gene (*GFP*) into Telomelysin (TelomeScan, OBP-401). Administration of TelomeScan into the primary tumor enabled the visualization of cancer cells under the cooled charged-coupled device (CCD) camera, not only in primary tumors but also the metastatic foci. This technology can be applied to intraoperative imaging of metastatic lymphnodes. Thus, we found novel tools for cancer diagnostics and therapeutics by utilizing the *hTERT* promoter. (*Cancer Sci* 2008; 99: 1528–1538)

In the past decade, research in the field of telomerases has progressed tremendously, especially in relation to cellular immortality and carcinogenesis. Telomerase activation is observed in approximately 90% of human cancers, irrespective of tumor type, while most normal tissues contain inactivated telomerase.[1] The role and timing of telomerase activation in carcinogenesis has been revealed by telomerase-knockout mouse studies.[2,3] Significant telomere erosions and age- and generation-dependent increases in cytogenic abnormalities are exhibited in telomerase-knockout mice, providing evidence that telomere dysfunction with critically short telomeres causes genomic instability.[2] This concept is further supported by studies using telomerase–/– p53–/– double-knockout mice.[3] These mouse cells demonstrate high levels of genomic instability, exemplified by increases in both formation of dicentric chromosomes and susceptibility to oncogenic transformation. These mice exhibit significantly decreased tumor latency and overall survival. Thus, in the absence of genome checkpoint functions, telomere dysfunction accelerates genomic instability, facilitating cancer initiation.[4] According to this concept, the genomic instability caused by telomere dysfunction occurs in the early stages of carcinogenesis, before telomerase activation. Subsequently, telomeres in these initiated cells undergo further progressive shortening, generating rampant chromosomal instability and threatening cell survival. Telomerase activation necessarily occurs at this stage to stabilize the genome and confer unlimited proliferative capacity upon the emerging and evolving cancer cell. In other words, cells that have acquired telomerase activity can obtain the capacity for cancer progression. Eventually, most cancer cells exhibit telomerase activity.

This cancer-specific telomerase activity provides an opportunity for us to utilize it for cancer diagnosis and treatment. Continuous effort has been made to uncover the molecular mechanisms of telomerase activation during carcinogenesis. The discovery of the telomerase subunit human telomerase reverse transcriptase (hTERT),[5,6] a catalytic subunit bearing the enzymatic activity of telomerase,[7,8] was the starting point for uncovering the cancer-specific activation of telomerase. Numerous studies have demonstrated that hTERT expression is highly specific to cancer cells and tightly associated with telomerase activity, while the other subunits are constitutively expressed both in normal and cancer cells.[9–12] Therefore, there is no doubt that hTERT expression plays a key role in cancer-specific telomerase activation. In this review article, we discuss the cancer-specific regulation of *hTERT* and its application for cancer diagnosis and treatment.

Cloning of the *hTERT* promoter and identification of the core promoter region containing *cis*- and *trans*-elements for cancer-specific transcription

In 1999 we and other groups successfully cloned the 5′-promoter region of the *hTERT* gene.[13–15] Transient expression assays using the 3.0 kb of the flanking sequences of the *hTERT* gene revealed that the transcriptional activity was up-regulated

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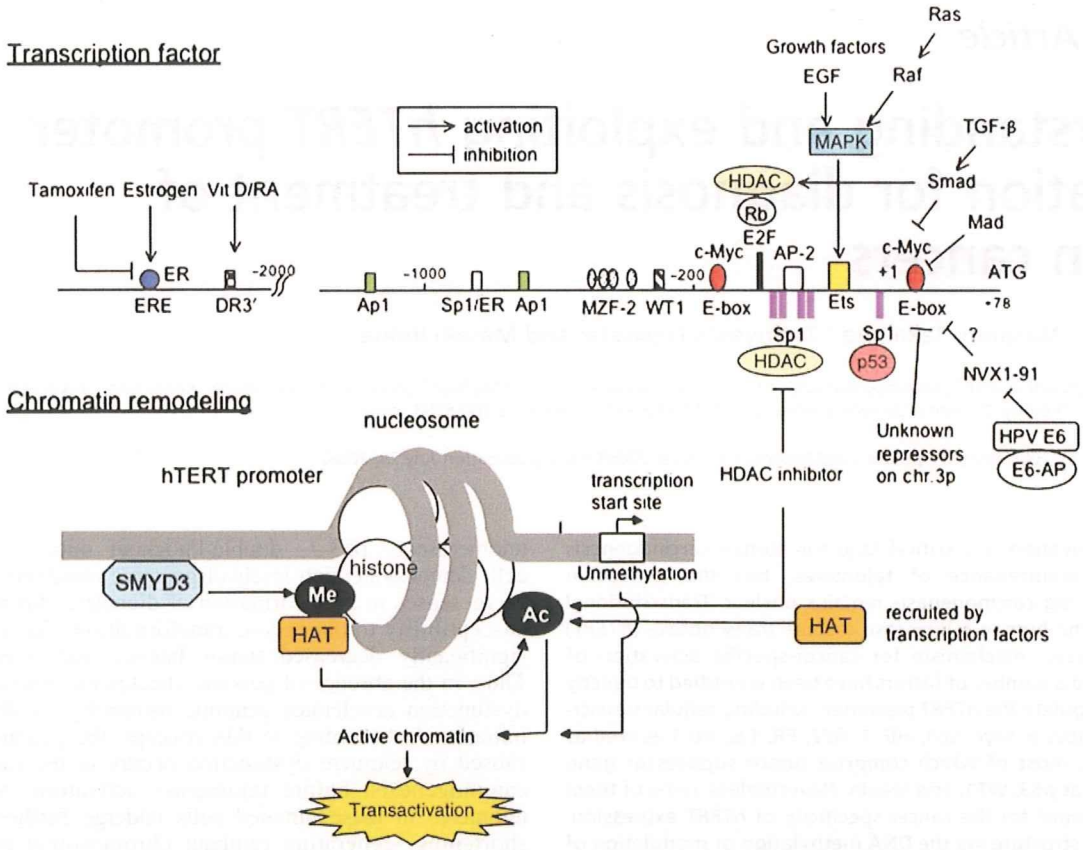

Fig. 1. Complex molecular mechanisms of transcriptional regulation of human telomerase reverse transcriptase (*hTERT*). Representative transcription factors and their upstream factors essential for *hTERT* regulation are shown in the upper panel. The sites on the promoter are not precisely in scale. +1 indicates the start site of transcription.[13] The proposed model of chromatin remodeling for the regulation of *hTERT* promoter is shown in the lower panel. Me, methylation of histone; Ac, acetylation of histone.

specifically in cancer cells, while it was silent in most normal cells.[13] Deletion analysis of the promoter identified the proximal 260 bp region functioning as the core promoter essential for cancer-specific transcriptional activation. Within the core promoter, several distinct transcription-binding sites are present; E-boxes (CACGTG) located at −165 and +44 (numbering based on the transcription start site determined by CapSite Hunting method[11]) are potential binding sites of basic helix-loop-helix zipper (bHLHZ) transcription factors encoded by the Myc family oncogenes. The existence of E-boxes on the *hTERT* promoter stirred telomerase researchers since c-Myc has been known to activate telomerase.[16] In fact, several groups confirmed that c-Myc binds to E-boxes on the *hTERT* promoter and activates the transcription[15–19] which established the scenario that c-Myc is a key regulator of *hTERT* transcription during carcinogenesis. However, several studies found that Myc and hTERT expression levels are not necessarily tightly correlated in some cancer cells.[20,21] Furthermore, it should be noted that most of these studies used overexpressed c-Myc for the luciferase reporter assay as well as recombinant c-Myc for the electrophoretic mobility shift assay (EMSA) to demonstrate binding to the E-boxes. Therefore, it remains unclear whether endogenous binding of c-Myc on the *hTERT* promoter plays a critical role in *hETRT* transcription *in vivo*, especially during carcinogenesis. Xu *et al.* reported the important finding that endogenous c-Myc binding to the E-boxes on the *hTERT* promoter was well correlated with the induction of *hTERT* in proliferating leukemic cells.[22] Nevertheless, it remains unclear whether up-regulation of *in vivo* binding of c-Myc to the *hTERT* promoter occurs during carcinogenesis and how critical it is for continuous hTERT expression in cancer.

Other characteristic sequences that exist on the *hTERT* promoter are the GC-boxes (GGGCGG), which are binding sites for zinc finger transcription factor Sp1. There are at least five GC-boxes within the core promoter of *hTERT*, proven by EMSA to bind Sp1.[22] Introduction of mutations in these GC-boxes significantly decreased the transcriptional activity of the promoter, while overexpression of Sp1 in cells that contain relatively low levels of endogenous Sp1 enhanced the promoter activity.[17] In particular, the *hTERT* core promoter activity was almost completely diminished by introducing mutations in all five GC-boxes, while mutation in one site moderately decreased it. Therefore, the GC-boxes function synergistically to maintain the promoter activity of *hTERT*. However, Sp1 is ubiquitously expressed in a wide range of normal cells, and is not therefore a strong candidate to cause cancer-specific hTERT expression.

Overall, while the *hTERT* core promoter is highly specific to cancer cells, the key transcription factors identified are far from accounting for cancer-specific *hTERT* expression.

Critical factors that regulate *hTERT* transcription

A number of factors that regulate *hTERT* transcription have been identified to regulate the *hTERT* promoter. The representative regulators of *hTERT* promoter with regard to the clinical aspects are shown in Fig. 1.

Cellular transcription factors. Several transcription factors, as well as c-Myc and Sp1, have been identified to regulate the *hTERT* promoter. Activating Enhancer-binding Protein-2 (AP-2) was recently identified as a transcriptional activator of the *hTERT* promoter[23] and, of particular interest, it exhibited tumor-specific

binding to the core promoter region. Although this study examined only one tumor type (lung cancer), this may partly explain tumor-specific *hTERT* transcription.

Hypoxia-inducible factor-1 (HIF-1), a key regulator of O_2 homeostasis, regulates the expression of several genes linked to angiogenesis and energy metabolism. The presence of putative HIF-1 binding sites on the *hTERT* promoter prompted us to examine the involvement of HIF-1 in regulation of *hTERT* in tumor hypoxia: we found that hypoxia activated hTERT mRNA in cancer cells *in vitro*.[24,25] Luciferase reporter assays revealed that *hTERT* transcription was significantly activated in hypoxia and by HIF-1α overexpression, and that the two putative HIF-1 binding sites within the core promoter are responsible for this activation. The chromatin immunoprecipitation assay identified specific binding of HIF-1α to these sites, which was enhanced in hypoxia. siRNA inhibition of HIF1-α abrogated hypoxia-induced hTERT mRNA expression. Thus, hypoxia activates telomerase mainly via transcriptional activation of *hTERT*, and HIF-1 plays a critical role as a transcription factor. In contrast to these findings, Koshiji *et al.* observed that HIF-1 inhibited *hTERT* expression in colon cancer cells.[26] In this study, they demonstrated that HIF-1 induces cell-cycle arrest even in the absence of hypoxia by functionally counteracting Myc. Eventually, HIF-1 down-regulates Myc-activated genes including *hTERT*. The reasons for this discrepancy remain unclear, but experimental conditions, such as the concentration of oxygen and constitutive levels of HIF-1 in cell types used, may significantly affect the results. A recent study underscored the importance of HIF-2 in regulating *hTERT* promoter.[27] While HIF2-α enhances *hTERT* expression in renal-cell carcinoma, it represses *hTERT* transcription in glioma cells, adding a further layer of complexity to the relationship between hypoxia and telomerase activity.

We also found the transcription activator protein AP-1 to function as a transcriptional repressor.[28] There are two AP-1 sites (at −1655 and −718) within the 2.0 kb promoter of *hTERT*. EMSA revealed that JunD is the major factor binding to them, which was further supported by chromatin immunoprecipitation (ChIP) assay *in vivo*. Overexpression of Jun family members with c-fos significantly reduced the promoter activity while mutation of AP-1 sites increased it. Of particular interest is the observation that AP-1 had no effect on the mouse *TERT* (*mTERT*) promoter although it has similar binding sites for AP-1. Since *mTERT* is constitutively expressed both in tumor and normal cells, this species-specific function of AP-1 in *TERT* expression may in part help explain the difference in telomerase activity between normal human and mouse cells.

Hormones. Hormonal regulation of *hTERT* and the molecular mechanisms involved have been analyzed most extensively in relation to estrogen. We and other groups found that estrogen activates *hTERT* transcription via binding of ligand-activated estrogen receptor-α (ERα) to the estrogen-responsible element (ERE) in the *hTERT* promoter.[29,30] ER-Sp1 half-sites located downstream of the ERE similarly function as *cis*-acting elements in response to estrogen stimulation. Estrogen also activates *hTERT* expression via post-transcriptional mechanisms with the stimulation of nuclear accumulation of hTERT via its phosphorylation, which is mediated by Akt signaling.[31] Tamoxifen, a selective estrogen receptor modulator, also regulates *hTERT* expression in a cell-type-specific manner;[32] tamoxifen inhibits the growth of breast cancer cells, as well as hTERT mRNA expression in the presence of estrogen (E2), antagonizing the E2 effects, in which the ERE on the promoter is involved. In contrast, tamoxifen stimulated the growth of endometrial cancer cells and activated hTERT mRNA expression in the absence or presence of E2, exhibiting estrogen-agonistic action, in which MAP kinase signaling pathways are involved. Androgen was also shown to activate hTERT mRNA in androgen-sensitive prostate cancer cells but this regulation was not due to *hTERT* promoter activation.[33] Progesterone exerts diverse effects on hTERT mRNA expression in a time-dependent manner in progesterone-receptor-positive breast cancer cells;[34] in the short term, it activates *hTERT* transcription, but prolonged exposure to progesterone antagonizes estrogen and inihibits *hTERT* transcription. Interestingly, both short- and long-term regulation is mediated via the MAP kinase signaling pathway.

Cytokines. Telomerase activation is known to be tightly associated with cell proliferation, which suggests that growth signaling might directly regulate hTERT expression.[35–37] We established an *in vitro* model in which telomerase activity can easily be induced upon stimulation of EGF in EGF-receptor-positive cancer cells.[38] Luciferase reporter assays revealed that EGF activates the *hTERT* promoter: an Ets motif located in the core promoter of *hTERT* is responsible. Notably, MAP kinase signaling pathways mediate this regulation. A number of growth signals have been known to be mediated through MAP kinase pathway, with Ets factors playing critical roles as final mediators regulating the target-gene expression. Therefore, EGF-mediated Ets-based *hTERT* transcription may be one representative pathway through which various growth signals are transduced to the *hTERT* promoter. This scenario can partly account for telomerase activation associated with cell proliferation.

TGF-β is a representative cytokine that represses *hTERT* transcription.[39] The mechanisms through which TGF-β down-regulates *hTERT* transcription are controversial: while some studies demonstrated that TGF-β repressed *hTERT* transcription via indirect down-regulation of c-Myc expression,[40,41] others reported direct interaction of Smad3 and c-Myc disturbing c-Myc activity.[42] Another study identified several negative regulatory factors for *hTERT* by means of gene screening using enhanced retroviral mutagenesis (ERM) and found that Smad interacting protein-1 (SIP1) is a repressor for *hTERT*, possibly mediating TGF-β signals.[43] A more recent study using siRNA inhibition of the Smad family confirmed that TGF-β-mediated repression of *hTERT* transcription is largely mediated through Smad3, not Smad1 or Smad2.[44] However, this study found no role for E-boxes in this repression, but found four E2F-binding sites within the proximal promoter of *hTERT* to be responsible, based on the data that mutation of these four sites reversed TGF-β-mediated repression of *hTERT* transcription. The transcriptional activity of E2F family members is regulated by interactions with pocket proteins (Rb, p107, p130) that recruit histone deacetylase (HDAC) proteins to repress target genes. Interestingly, overexpression of the dominant negative E2F gene lacking the ability to bind pocket protein (Rb, p107, p130) and to recruit HDAC significantly abrogated TGF-β-mediated repression of *hTERT* transcription. Furthermore, trichostatine A (TSA), a HDAC inhibitor, completely reversed the inhibitory effect of TGF-β. These findings highlight E2F and HDAC as central mediators of TGF-β-mediated repression of *hTERT* transcription. The involvement of HDAC in *hTERT* transcription is also discussed below.

Oncogenes. High-risk human papillomaviruses (HPV) are representative oncoviruses whose E7 protein can bind to Rb and alleviate repression of E2F-dependent target genes, thereby allowing rapid progression into S phase[45] while E6 protein facilitates the degradation of p53 through the actions of E6-associated protein (E6-AP), which results in the abrogation of the G_1/S and G_2/M checkpoints.[46–48] The initial study found that telomerase is activated in keratinocytes stably expressing HPV16 E6.[49] Since E6 had been known to activate c-Myc expression[50] it seemed likely that E6 activates *hTERT* transcription via up-regulating c-Myc. However, subsequent studies confirmed that high-risk HPV E6 activates *hTERT* transcription but is not associated with up-regulation of c-Myc.[51–53] Several studies found that *hTERT* transactivation by HPV16 E6 correlates with its ability to bind E6-AP.[54] A correlation between E6-AP binding and *hTERT* induction prompted the search for possible targets of

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the E6/E6-AP complex by a yeast two-hybrid screen, which identified a transcriptional repressor known as NFX1 that binds to 48-bp sequences surrounding the proximal E-box on the *hTERT* promoter.[54] It is supposed that the E6/E6-AP complex induces hTERT expression by destabilizing NFX-1. In support of this, decreased expression of NFX1 using siRNAs was sufficient to induce *hTERT* expression and telomerase activity in primary human epithelial cells.

Some human oncoproteins specifically activate *hTERT* promoter. In hTERT-negative normal cells, HER2/Neu signals (by overexpressing oncogenic HER2/Neu mutant) alone failed to activate the endogeneous *hTERT* expression.[55] However, coexpression of HER2/Neu with one ETS family member (ER81) successfully activated *hTERT* expression in these cells. There are five putative binding core GGAA/T sites for ETS family in exon1 to intron1 of the *hTERT* gene, and ER81 specifically binds to two of them and activates *hTERT* promoter in cooperation with HER2/Neu signals. Notably, this activation was mediated via the ERK-MAP kinase pathway, in which upstream Ras and Raf-1 play critical roles. Thus, three prominent oncoproteins, HER2/Neu, Ras, and Raf, facilitate *hTERT* expression via an Ets family member in hTERT-negative normal cells.

Epigenetic regulation of *hTERT* transcription

The *hTERT* promoter contains a cluster of CpG sites, and many researchers therefore supposed its regulation to involve DNA methylation. Several groups examined the methylation status of these CpG sites on this promoter. It was initially expected that methylation of the *hTERT* promoter was associated with gene silencing; indeed, some groups showed such association.[56–58] However, other reports indicated no significant correlation between *hTERT* expression and methylation status either overall or at a specific site.[59,60] Furthermore, contradictory results have been reported: increased DNA methylation in the *hTERT* promoter was observed in hTERT-positive cancer cells while lack of methylation was found in normal hTERT-negative cells.[61] These unusual correlations between DNA methylation and hTERT expression in normal and cancer cells generated confusion among telomerase researchers. Recently, Zinn *et al.* aimed to clarify the discrepancies:[62] using bisulfite sequencing, they first identified that all telomerase-positive cancer cell lines examined retained alleles with little or no methylation around the transcription start site despite being densely methylated in more upstream regions. ChIP assay revealed that both active (acethyl-H3K9 and dimethyl-H3K4) and inactive (trimethyl-H3K9 and trimethyl-H3K27) chromatin marks are present across the *hTERT* promoter. Subsequent Chip-MSP (methylation-specific polymerase chain reaction [PCR]) assay identified that active chromatin mark DNA around the transcription start site was tightly associated with unmethlated DNA. These data suggest that the absence of methylation and the association with active chromatin marks around the transcription start site allow for the expression of *hTERT* (Fig. 1), indicating that the DNA methylation pattern of the *hTERT* promoter is consistent with the usual dynamics of gene expression.

Modification of nucleosome histones, including acetylation/deacetylation as well as methylation, is known to regulate chromatin structure and thereby affect gene transcription.[63] Roles for histone-modification-mediated chromatin remodeling in the regulation of *hTERT* transcription have been revealed (Fig. 1). We and other groups found that treatment with TSA induced significant elevation of hTERT mRNA expression and telomerase activity in normal cells, but not in cancer cells.[64,65] Transient expression assays revealed that TSA activates the *hTERT* promoter, for which the proximal core promoter was responsible. Overexpression of Sp1 enhanced responsiveness to TSA, and mutation of Sp1 sites but not c-Myc sites of the core promoter of *hTERT* abrogated this activation. Introduction of the dominant-negative form of the Sp family inhibited TSA activation. These results indicate that HDAC inhibitor activates the *hTERT* promoter in normal cells in an Sp1-dependent manner (Fig. 1). It is possible that endogeneous Sp1 interacts with HDAC and recruits it to the *hTERT* promoter[66] resulting in the deacetylation of nucleosome histones, leading to the repression of transcription. While Sp1 contributes to the transactivation of *hTERT* as a potent transcriptional activator[22] it might be involved in gene silencing of *hTERT* in normal cells, possibly by recruiting HDACs. Compelling evidence suggests that Sp1 interacts with a p300 coactivator possessing intrinsic histone acetyltransferase (HAT) activity.[67] Therefore, it is possible that Sp1 interacts with various factors that have HAT or HDAC activity, and that this switching explains the different actions of Sp1 on the *hTERT* promoter in normal and cancerous cells. The E-box binding activator c-Myc and repressor Mad1[21,22,68] which compete with each other for the common binding partner Max are also involved in histone-modification-mediated chromatin remodeling of the *hTERT* promoter. The endogenous c-Myc/Max complex to the *hTERT* promoter in proliferating leukemia cells was found to be associated with the acetylated histones, resulting in enhanced hTERT expression.[22] In contrast, the complex was replaced by the endogeneous Mad1/Max complex that was associated with deacetylated histones and decreased *hTERT* expression in differentiated status.

Recently, a role for histone methylation in *hTERT* regulation has also been demonstrated. Atkinson *et al.* observed that highly trimethylated H3-K4 was associated with the actively transcribed *hTERT* gene in telomerase-proficient tumor cells.[69] More recently, we reported the interesting finding that SET- and MYND-domain-containing protein-3 (SMYD3), a histone H3-K4-specific dimethyltransferase and trimethyltransferase, respectively, play critical roles in H3-K4 methylation of the *hTERT* promoter.[70] Of the various SET-domain-containing proteins, SMYD3 is unique because not only does it have methyltransferase activity but it also binds to a specific DNA sequence (CCCTCCC) in its target promoters, as do transcription factors. In fact, SMYD3 was confirmed to bind some of the CCCTCCC motifs within the core promoter of *hTERT* and activate *hTERT* transcription. Overexpression of *SYND3* induced hTERT mRNA expression in hTERT-negative normal and cancer cells. Disruption of SMYD3 binding motifs in the *hTERT* promoter led to significant reduction of transcription. Expectedly, siRNA-knockdown of *SMYD3* resulted in abolishment of H3-K4 trimethylation of the *hTERT* promoter in cancer cells; interestingly, this knockdown also led to defects in binding c-Myc and Sp1. Furthermore, histone H3 acetylation within the core promoter of *hTERT* was diminished by the *SMYD3*-knockdown. These data suggest a model in which SMYD3 binding to the *hTERT* promoter leads to increased H3 trimethylation, a critical event that recruits HAT and promotes Sp1 and c-Myc access to the *hTERT* promoter (Fig. 1). Thus, SMYD3-mediated trimethylation of H3-K4 may function as a licensing element for subsequent transcription-factor binding to the *hTERT* promoter, which may trigger further recruitment of HAT activity.

Identification of *hTERT* repressors

Recently, Lin *et al.*[43] identified several negative regulatory factors for *hTERT* by means of gene screening that used enhanced retroviral mutagenesis (ERM). They identified menin, SIP1, Mad1, hSIR2, and BRIT1 as candidates for the *hTERT* repressor, generating the idea that multiple tumor suppressors might involve telomerase repression, especially in normal cells. p53 was also shown to repress *hTERT* transcription in a Sp1-dependent manner.[71,72] It was proved that p53 can form a complex with Sp1, which disturbs the transcriptional activity of Sp1 and leads to transcriptional repression.[72] Several transcriptional repressors,

including Wilms' tumor 1 tumor suppressor (WT1) and myeloid-specific zinc finger protein-2 (MZF-2) are also known to repress *hTERT* transcription via binding to their specific sites on the promoter, although the mechanisms of repression remain unclear.[73,74] We also found that on combinatorial treatment with Vitamin D3 and 9-*cis*-retinoic acid, the heterodimer complex, vitamin D[3] receptor/retinoid X receptor (RXR), binds to the distal sites on the *hTERT* promoter and represses transcription.[75]

There has been an extensive search for telomerase repressors, one of which was based on microcell-mediated chromosome transfer.[76] Several normal human chromosomes, including chromosomes 3, 4, 6, 7, 10, and 17, have been shown to repress telomerase activity in some but not all cancer cells.[77–85] Horikawa *et al.* established a nice system to investigate an endogenous mechanism for telomerase repression using a telomerase-positive renal carcinoma cell line (RCC23) and telomerase-negative counterpart (RCC23 + 3) generated by transferring a normal chromosome 3 into RCC23 cells.[86] By comparing the molecular characteristics of these cells, they identified the E-box downstream of the transcription initiation site that was responsible for telomerase repressive mechanisms restored by normal chromosome 3 targets. They also found that the factors binding to the E-box, other than c-Myc/Mad or USF families, were involved in the transcriptional repression of *hTERT* although they remained to be cloned. This E-box-mediated repression functions in various types of normal human cells, while it is inactive in some, but not all, hTERT-positive cancer cells, providing evidence for an endogenous mechanism for *hTERT* transcriptional repression that becomes inactivated during carcinogenesis.

hTERT promoter for cancer therapeutics

***hTERT* promoter for cancer-specific transgene expression.** In the field of cancer gene therapy, the researchers have a great interest in efficiently expressing target genes in the tumor tissue while decreasing adverse effects in normal tissue. Control of gene expression via tissue- or cell-specific promoters has been tested extensively as a means of targeting transgene expression. Several promoters have been identified that are more active in particular tumor types than in the tissues from which they arise, and these promoters have been exploited to target transgene expression in tumors. These promoters include the tyrosinase gene promoter in melanomas,[87] the carcinoembryonic antigen promoter in colorectal and lung cancer,[88] the MUC1 promoter in breast cancer,[89] and the E2F promoter in cancers that carry a defective retinoblastoma gene.[90] However, while reports on these promoters suggest that achieving relatively tumor-specific transgene expression is possible, several limitations have also been revealed. First, most of these promoters are limited to specific tumor histologies and cannot be used universally in tumors of various origins. Second, most of these promoters are much weaker than commonly used viral promoters such as the CMV early promoter, the Rous sarcoma virus long-terminal repeat (RSV-LTR), and the SV40 early promoter. Consequently, their use is hampered by the problem of low expression.

The *hTERT* promoter is ideal to overcome the shortcoming of these promoters. Gu *et al.* first established the binary adenoviral system, which uses two adenoviral vectors to induce *Bax* gene expression.[91] One of these vectors contains a human *Bax* cDNA under the control of a minimal synthetic promoter comprising five Gal-4-binding sites and a TATA box, which is silent in 293 packaging cells, thus avoiding the toxic effects of the *Bax* gene on the 293 cells and allowing vector (Ad/GT-Bax) production. Expression of the *Bax* gene can be induced by coinfecting the Ad/GT-Bax virus with the second adenoviral vector in the binary system (Ad/PGK-GV16), which consists of a fusion protein comprising a Gal-4 DNA-binding domain and a VP 16 activation domain under the control of a constitutively active PGK promoter. Ad/PGK-GV16 is expected to produce VP16 with Gal-4 DNA binding domain preferentially in tumor cells and thereby induce *Bax* gene expression via interaction with Gal-4-binding sites. This binary infection system was reported to suppress tumor growth *in vitro* and *in vivo*. More simple vector systems to achieve cancer-specific transgene expression have been tried, in which several apoptosis-inducible genes such as *FADD*,[92,93] *caspace*[94,95] or suicide gene (human herpes simplex virus thymidine kinase (*HSVtk*) gene),[96] tumor-necrosis-factor-related apoptosis-inducing ligand gene (*TRAIL*),[97] or chemoattractant protein gene (*MCP-1*)[98] have been driven by the *hTERT* promoter in various tumor types. Most of these studies successfully demonstrated tumor-specific transgene expression *in vivo*, achieving long-term survival benefit and minimizing its expression in normal tissues following direct injection of the vectors and even with systemic injection. Systemic toxicity is one concern in this treatment modality because telomerase activity has been reported to exist in some normal cells, such as hematopoietic crypt and endometrial cells, most of which have high regenerative potentials. Gu *et al.* tested *hTERT*-promoter-driven transgene expression in human CD34(+) bone marrow progenitor cells and found very low *hTERT* promoter activity in these cells as well as no detectable change in blood-cell profiles under long-term observation.[99] Basically, the *hTERT* promoter activity in these normal cells with telomerase activity is much lower than that in cancer cells, and toxicity is expected to be minimized.

***hTERT* promoter for cancer-specific replication-competent adenovirus.** Despite these efforts, levels of transgene expression were insufficient to eradicate tumors, especially when vectors were systemically administrated. This is mainly due to the characteristics of adenoviral vectors used, in which the *E1* gene was deleted to inhibit replicative capacity. These nonreplicative vectors had limited distribution within the tumor mass even after direct intratumoral administration. To confer specificity of infection and increase viral spread to neighboring tumor cells, the use of replication-competent adenoviruses has become a reality. The use of modified adenoviruses that replicate and complete their lytic cycle preferentially in cancer cells is a promising strategy for the treatment of cancer. Many efforts have been made to realize cancer-specific adenoviral replication using a variety of gene promoters, including the prostate-specific antigen,[100] MUC1,[101] osteocalcin,[102] L-plastin,[103] midkine,[104] and *E2F-1* genes.[105] Unfortunately, these promoters have tissue-type specificity and exhibit transcriptional activity only in cells that express such tumor markers. Furthermore, the transcriptional activity is relatively low. We were prompted by these studies to use the *hTERT* promoter, hypothesizing that an adenovirus containing the *hTERT* promoter-driven *E1* genes could target a variety of tumors and kill them with high replicative capacity.

We developed a novel telomerase-dependent replicative adenovirus type 5 vector (Telomelysin, OBP-301) in which *E1A* and *E1B* genes, required for adenoviral replication, were transcribed under the *hTERT* promoter.[106] In most vectors that replicate under the transcriptional control of the *E1A* gene, *E1B* is driven by the endogeneous adenovirus *E1B* promoter. However, the insertion of internal ribosome entry site (IRES) between *E1A* and *E1B* improved the promoter specificity of *E1B* transcription. We selected the 455 bp-proximal promoter region of the *hTERT* gene to drive *E1A* and *E1B* genes because our previous experiments showed that this region exhibits the highest transcriptional activity, comparable to the proximal core promoter.[13] The construction of Telomelysin is shown in Fig. 2. Similar replicative adenoviruses controlled by the *hTERT* promoter have also been developed by other groups.[107–109]

In vitro replication assays revealed that Telomelysin induced selective expression of *E1A* and *E1B* in cancer cells, resulting in viral replication at 5–6 orders of magnitude by 3 days after infection, while it was attenuated by up to 2 orders of magnitude

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