ganization as well as induction of the antiapoptotic mediator Bcl-2. A Rac1-dependent pathway of *Bcl-2* induction could be a critical process during positive selection by preventing TCR-mediated apoptosis, and thus, a detailed mechanism of *Bcl-2* induction needs to be elucidated in future studies.

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Oral Therapeutics and Pharmacology

Functional Significance of Intracellular Form of Osteopontin in the Migration and Fusion of Osteoclasts

Keiko Suzuki* and Shoji Yamada*

There is growing evidence that osteopontin (OPN) plays an important role in the cell migration, which is one of the key events in the immune response, and tumor metastasis and invasion. Although OPN is recognized generally as a secreted protein, we have shown the presence of an intracellular form of OPN (iOPN) in migrating fibroblasts, activated macrophages, motile osteoclasts and metastatic cells, which indicates the importance of iOPN in the cell migration. In this study, we examined the effects of exogenous OPN on the impaired cell spreading and migration observed in the OPN-null osteoclasts, to determine whether iOPN has functional significance in the osteoclast motility. The results show that 1) in osteoclasts, generated from OPN-null bone marrow cells in the presence of M-CSF and RANKL, cell spreading and protrusion of pseudopodia were reduced and cell fusion was impaired, which resulted in smaller osteoclasts with fewer nuclei, 2) osteoclast migration towards M-CSF was significantly compromised in OPN-null mice, and 3) exogenous OPN, which was pre-coated onto the Transwell™ membrane, failed to rescue the impaired osteoclast spreading and migration observed in the OPN-null mice. These findings have identified an intracellular form of OPN that appears to function in the osteoclast migration and fusion.

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INTRODUCTION

That osteopontin (OPN) has a prominent role in bone resorption was first indicated by the demonstration that it co-localizes with the $\alpha v\beta 3$ vitronectin receptor and mediates the attachment of osteoclasts to the bone surface.[1] In addition to mediating attachment, OPN is required to stimulate resorptive activity of osteoclasts,[2,3] reflecting its cytokine properties. TRACP-negative cells involved in the bone resorption, together with their immediate precursors, differentiate into TRACP-positive cells that express OPN,[4] which is expressed during the early stages of osteoclast differentiation and its attachment function required for the osteoclastogenesis.[5] Consistent with these observations, we have previously shown that OPN is required for the migration and fusion of osteoclast precursors.[2]

Although OPN is recognized as a secreted protein, we have identified an intracellular form of OPN (iOPN) that is associated with the CD44 in migrating fibroblastic cells, activated macrophages, metastatic tumor cells[6] and motile osteoclasts.[2] In this study, to determine the functional significance of iOPN in the osteoclast migration, we have investigated the effects of exogenously added OPN on the cell spreading and migration towards M-CSF in OPN-null osteoclasts.

MATERIALS AND METHODS

1. Preparation of osteoclasts

Bone marrow cells obtained from femurs and tibias of 5- to 7-week-old male C57BL/6 (provided by Drs. S. Rittling and D.T. Denhardt[7]) were plated at a density of 5×10^5 cells/cm^2 and then cultured in α-MEM supplemented with 10% FCS, 10 ng/ml of M-CSF and 50 ng/ml of RANKL for up to six days. All the experiments were performed in accordance with Showa University Animal Care and Use Committee guidelines (#13029).

2. Cell migration assays

TRACP-positive prefusion osteoclasts were trypsinized and re-plated onto the Transwell™ membrane (polycarbonate, pore size: 8 μm, Corning Costar Japan, Tokyo, Japan) and allowed to attach for two hours. M-CSF (10 ng/ml) was added to the lower compartment as a chemoattractant and the cells allowed to migrate for up to three hours. In some experiments,

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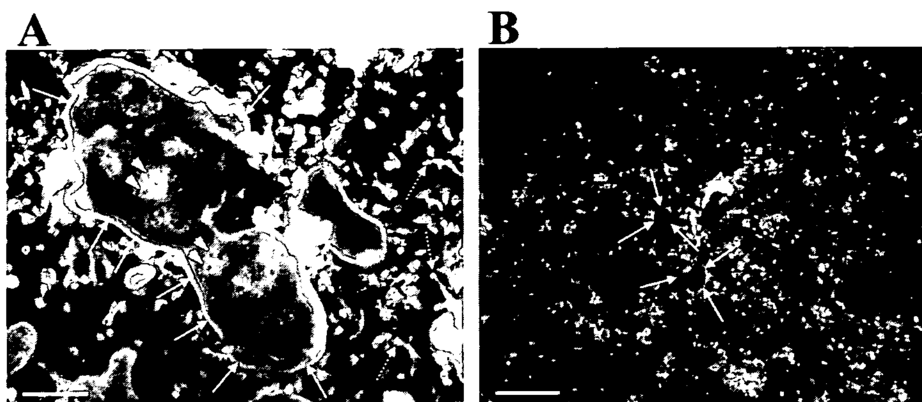

Fig. 1 Morphological characteristics of osteoclasts generated from WT (A) and OPN-null (B) cells and stained for F-actin. Solid and dotted arrows indicate the actin-ring structure and cell processes, respectively. Arrowheads show cell nuclei. Bar = 100μm.

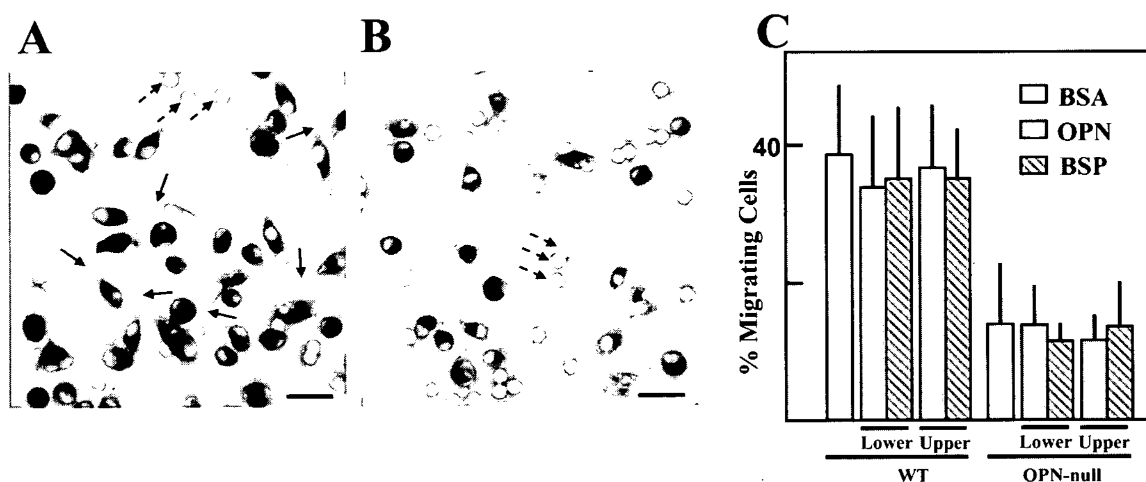

Fig. 2 Cell migration assay of osteoclasts from WT (A) and OPN-null mice (B), and the effects of exogenous OPN or BSP on migration of these osteoclasts (C). cell processes and membrane pores, Bar = 20μm.

Transwell™ membranes were pre-coated on both sides with BSA (100μg/membrane), or on either the upper or lower surface with rat OPN (100 ng membrane, provided by Dr. J. Sodek), or porcine BSP (100 ng/membrane, provided by Dr. J. Sodek). Membranes were fixed and stained for TRACP activity. Images were acquired using an air-cooled charge-coupled device camera mounted on an inverted fluorescent microscope, and the cell number determined.

3. Statistical analysis

Quantitative data obtained for cell migration assay was averaged from a minimum of 12 values in replicate experiments from which mean values ± SD were calculated. Leven's test and two sample *t*-tests were performed to determine homogeneity of variance and the difference between two means, respectively, using SPSS for Windows™ 11.0.1J (SPSS Japan Inc., Tokyo, Japan).

RESULTS

1. Impaired cell fusion in pre-osteoclasts derived from OPN-null mice

To determine whether the cell fusion is impaired in the OPN-null mice, mouse bone marrow mononuclear cells were cultured in the presence of M-CSF and RANKL for six days. Morphologically, the cells derived from the OPN-null mice (Fig. 1B) were smaller and did not appear to spread as well as the wild-type (WT) cells (Fig. 1A), and extended fewer cell processes, as shown by staining for cytoskeletal actin. The reduced pseudopod formation is suggestive of an impaired

ability of pre-osteoclasts to fuse, which is an important step in osteoclastic differentiation, as we have previously observed both in the monolayer osteoclast culture and calvaria.

OPN-null osteoclasts stained for actin filaments, cortical actin ring structures (arrows in Fig. 1B), typically seen in multinucleated osteoclasts, were much smaller than those seen in the WT cells, which extended long pseudopodia towards adjacent cells (dotted arrows in Fig. 1A). The small size of the TRACP-positive cells from OPN-null mice appeared to result from reduced cell fusion since the number of nuclei (arrowheads in Fig. 1A) in the osteoclasts from OPN-null mice was significantly lower ($p<0.001$) than in WT osteoclasts (data not shown).

2. Osteoclast migration towards M-CSF

Because reduced fusion in the OPN-null cells could, in part, result from impaired migration, the ability of mouse osteoclasts to migrate in response to a chemotactic gradient of M-CSF was determined using a modified Boyden chamber system. Osteoclasts generated from bone marrow cells were plated on a Transwell™ polycarbonate membrane of an insert placed in a culture well containing the same medium and serum, but with 10 ng/m*l* M-CSF added to the lower compartment.

Significantly fewer TRAP-stained cells obtained from the OPN-null (mean ± SD: 13.9 ± 8.9%; $p>0.001$) mice migrated through the 8-μm pores compared to the WT cells (mean ± SD: 38.6 ± 10.3%) after two hours, despite their smaller size. Furthermore, migrated cells in WT have morphological features of motile cells, which protrude cell processes as shown by TRACP staining (arrows in Fig. 2A), whereas OPN-null cells have rounded, less motile features (Fig. 2B).

3. Effects of exogenous OPN on the migration of OPN-null osteoclasts

Since OPN has been reported to stimulate cell migration of osteoclasts through activation of the $\alpha v\beta 3$ integrin, we coated the upper or lower surfaces of the polycarbonate membrane with either rat bone OPN or porcine BSP. However, neither the OPN nor BSP had a significant effect on the migration of either the WT or OPN-null cells (Fig. 2C). Since the pore size restricted the migration of the large multinucleated cells, the migrant cells were relatively enriched in TRACP-positive prefusion osteoclasts and smaller multinucleated osteoclasts. These results indicate that OPN which secreted as an extracellular matrix protein may be not fully functional in osteoclast spreading and migration.

DISCUSSION

Osteopontin, which interacts with the $\alpha v\beta 3$ integrin through an RGD sequence[8] and mediates the attachment of osteoclasts to the bone surface[1] has a prominent role in bone resorption. It is obvious, from accumulated evidence in a variety of cells, that the interaction between OPN and several types of integrins, through an RGD motif, is necessary for cells to migrate to the controlled direction.

Although OPN is recognized as a secreted protein, we have identified an iOPN that is associated with the CD44 in migrating fibroblastic cells, activated macrophages and metastatic tumor cells.[6] Further, in osteoclasts treated with cycloheximide, perinuclear staining for OPN, which is characteristic to the secreted form, was lost, but iOPN staining was retained within cell processes for up to three hours.[2] The extracellular OPN can regulate the migratory processes by activating intracellular signaling pathways through its receptors. However, the present study strongly suggested that the iOPN was required for the osteoclasts to migrate. Also, the existence of functional iOPN was supported by pulse-chase studies in which a thrombin-sensitive, phosphorylated protein immunoprecipitated with OPN antibodies was retained inside macrophage.[9]

In conclusion, our present results indicate that 1) in osteoclasts generated from OPN-null mice, cell spreading and protrusion of pseudopodia were reduced and cell fusion was impaired, which resulted in smaller osteoclasts with fewer nuclei, 2) osteoclast motility towards M-CSF was significantly compromised in OPN-null mice, and 3) exogenous OPN failed to rescue the impaired osteoclast spreading and migration which was observed in the OPN-null mice. These findings have identified an intracellular form of OPN which seems to function in the osteoclast migration and fusion.

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Expert Opinion

Atelocollagen-mediated drug discovery technology

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1. Introduction
2. Present applications for cancer treatments
3. Small interfering RNA as a nucleic acid drug
4. The delivery method is critical for successful RNAi treatment of cancers
5. Atelocollagen drug delivery system-mediated small interfering RNA delivery
6. Identification of a drug target using cell transfection arrays
7. Local delivery of nucleic acid drugs
8. Systemic delivery of small interfering RNA with atelocollagen complexes
9. Principle of the atelocollagen drug delivery system
10. Expert opinion

RNA interference (RNAi) was first reported in nematodes in 1998. Since that time, RNAi has been discovered in fish, insects and mammals. Novel treatments and drug discovery in preclinical studies based on RNAi are targeting a wide range of diseases at present, including viral infections and cancers. In addition to the local administration of synthetic small interfering RNA (siRNA) targeting local lesions, specific methods for the systemic administration of these molecules to treat infectious diseases or metastatic cancers have also been reported. *In vivo* delivery technology is a key hurdle that must be addressed for the successful clinical application of synthetic siRNA. In this review, the authors evaluate the recent findings on atelocollagen–siRNA complexes for the treatment for metastatic cancers and outline the cancer therapies and drug discovery studies that are based on RNAi technology.

Keywords: atelocollagen, cancer, delivery, gene therapy, RNAi, siRNA

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1. Introduction

RNA interference (RNAi) is a method of post-transcriptional gene silencing in which double-stranded RNA (dsRNA) corresponding to a transcribed sequence is introduced into an organism, resulting in the degradation of the corresponding mRNA [1-7]. In RNAi, dsRNA blocks gene expression in a sequence-specific manner. When introduced into cells, dsRNA is processed by the RNase III family nuclease dicer into small interfering RNA (siRNA), a 21-base pair dsRNA with two overhanging bases at each 3′-terminus. The double-stranded siRNA is passed to the RNA-induced silencing complex (RISC), a RNA–nuclease complex, which is activated as it unwinds the duplex and incorporates one of the antisense strands; the RISC then selectively degrades RNA containing the sequence complementary to the incorporated antisense strand. Antisense oligonucleotide drugs [8] were used prior to the discovery of RNAi and several antisense molecules are at present in late-stage preclinical or clinical development [9]. Although researchers continue to explore and develop antisense reagents for therapeutic use by morpholino oligomers, a fourth class of oligonucleotide-based compounds consisting of siRNAs has recently become widely used for gene knockdown *in vitro* and *in vivo*. Another group of catalytically active RNA molecules (ribozymes) have also been considered for therapeutic use. However, only a few ribozymes have turned out to be efficient compounds in clinical trials [10]. RNAi is effective because siRNA is highly specific for the target gene and the single-strand RNA molecule incorporated into the RISC is used to recognise multiple copies of the target RNA; therefore, an extremely small amount of siRNA can generate reliable gene suppression, making toxicity less of a concern. Furthermore, effective antisense oligonucleotide sequences are determined empirically, resulting in uncertain efficacies. In contrast, siRNA can be designed to silence the expression of a target gene through a simple search using computer software. Due to the ease with which siRNA can be designed, the higher levels of gene suppression and the reproducibility of the results obtained with these

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molecules, more researchers are using siRNA. However, RNAi is a relatively new field of science that requires additional research to uncover its underlying mechanisms. For example, the process of gene silencing by siRNA has not yet been fully elucidated and that several off-target effects of certain siRNA molecules have been reported; therefore, care must be taken in the clinical application of siRNA to avoid unforeseen adverse side effects.

2. Present applications for cancer treatments

The effectiveness of siRNA molecules in cancer therapy has already been demonstrated [11]. Changes in cancer-related genes (oncogenes, tumour-suppressor genes and so on) are thought to induce cell abnormalities, resulting in tumorigenesis. RNAi-based therapies use siRNA to specifically block the expression of a mutated gene, which, in turn, inhibits cancer cell proliferation. Injecting siRNA that targets the vasculature (which supplies nourishment to cancer cells or induces apoptosis in cancer cells) is actively being investigated. In addition to methods that seek to directly reduce the expression of causal genes in cancer formation, some studies are considering using RNAi in conjunction with other chemotherapies, such as anticancer drugs. For example, silencing multi-drug-resistance genes with siRNA successfully restored the drug sensitivity of cancer cells [12,13]. Furthermore, siRNA libraries have been developed by a number of companies and screening via these libraries has led to the identification of novel target genes for the treatment of cancers [14].

3. Small interfering RNA as a nucleic acid drug

When studying the clinical uses of RNAi, the first step is to design siRNA for the target genes and to prepare an *in vitro* assay system. The outcome of the treatment depends on the cell line selected as an assay system and the most effective siRNA sequence for that cell line. It is preferable to match the method by which the siRNA is introduced to the culture cells with the method that is used to introduce siRNA to the corresponding animal. The next step includes the construction of *in vivo* delivery and assay systems. Maintaining the stability of siRNA within blood and tissue, as well as the aspect of cellular uptake, is the primary issue when introducing siRNA to the body. Among the innovations addressing this matter, chemically modified siRNAs with enhanced resistance to nucleases have been shown to be effective [15] and several companies have begun manufacturing these types of products. Resistance to nucleases, which is considered to be a key index of the stability of siRNA in blood, is also conferred to these molecules following liposome binding or complexation with a delivery carrier such as atelocollagen. Also important is selection of appropriate assay systems to test the biological activities of the selected siRNA, including the level of *in vivo* delivery, the suppression of target gene expression, or the degree of tumour contraction. Of the variety of methods that have been reported [16], the authors use an assay system based on luminescence imaging [17-19]. In this method, the cancer cells to be transplanted to the animal are engineered to express luciferase and siRNA that inhibits luciferase expression is used to quantitate the efficiency of siRNA delivery. By measuring the luciferase activity (photon number), the level of siRNA that reaches the target cells can be determined. Furthermore, targeting siRNA to a gene associated with the proliferation of cancer cells enables the quantification of the inhibition of cancer cell growth using luciferase as an indicator. However, the most important issue is how to deliver siRNA effectively without causing non-specific biological reactions as a result of interferons or inflammatory cytokines.

4. The delivery method is critical for successful RNAi treatment of cancers

Synthetic siRNA delivery has been tested in a variety of animals. For example, it was reported that siRNA was successfully introduced into murine liver cells using hydrodynamics; a large amount of synthetic siRNA in phosphate buffer solution (PBS) solution (as much as 10% of the body weight) was injected into mice by the tail vein [20-22]. To evaluate the efficacy of this delivery method, mice were co-injected with plasmids expressing the luciferase gene as the reporter gene along with synthetically prepared siRNAs targeted to the luciferase mRNA. Suppression of luciferase expression was observed in such varied tissues as the liver, spleen, lungs, kidneys and pancreas, observations that influenced other groups to investigate the potential of siRNAs for the treatment of liver failure. The *in vivo* silencing effect of siRNA directed against the gene encoding the Fas receptor was tested in murine liver for its potential to protect mice from liver failure and fibrosis in models of autoimmune hepatitis [23]. There is a unique report from another group that hydrodynamic injection of naked siRNA into a distal vein of a limb transiently isolated by tourniquet or blood pressure cuff was able to show an efficient and repeatable delivery of nucleic acids to muscle cells (myofibres) throughout the limb muscle of rats [24]. However, although the high-pressure or hydrodynamic intravascular injection technique used to deliver siRNA is effective in mice and non-human primates, it is unlikely to be applicable to human therapy.

Furthermore, viral vectors need to be used to achieve long-term gene silencing. Several studies have reported successful siRNA delivery using siRNA incorporated into viral vectors such as adenovirus, lentivirus, retrovirus and adeno-associated virus. Zhang *et al.* showed that the intratumoural injection of an adenovirus encoding the HIF-1-α targeted siRNA had a significant effect on tumour growth when combined with ionising radiation [25]. Sumimoto *et al.* reported that intratumoural administration of an adenovirus siRNA vector for Skp-2, which is involved in the degradation of cell-cycle regulators including p27Kip1, p21 and c-myc,

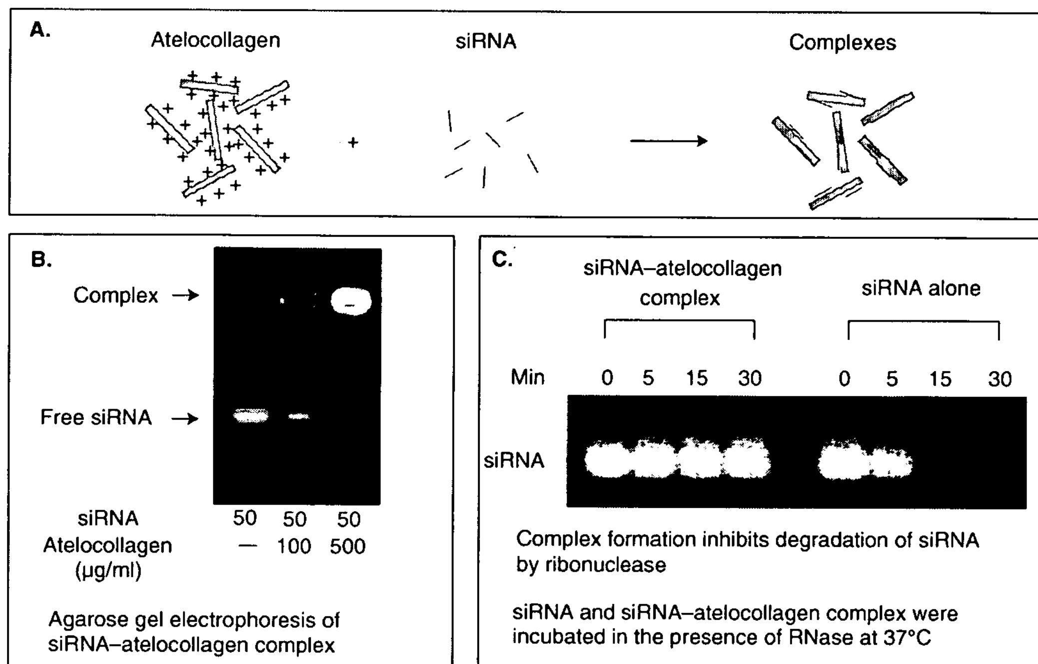

Figure 1. Characteristics of a siRNA–atelocollagen complex. A. Schematic image of complex formation between atelocollagen and siRNA. Atelocollagen is an enzymatically digested and highly purified type I collagen. A total of three polypeptides (containing both acidic and basic amino acids) form a linear triple helix. Atelocollagen has a slightly positive charge and forms an electrostatic complex with siRNA, charged negatively from its internucleotide phosphate. **B.** Agarose gel electrophoresis of the siRNA–atelocollagen complex. Complex formation could be confirmed by gel electrophoresis. The complex formation depends on the atelocollagen concentration and the complex remains in the slots of the gel when electrophoresed. **C.** Complex formation inhibits the degradation of siRNA by ribonuclease. The siRNA–atelocollagen complex and siRNA alone were treated with ribonuclease. The results show that atelocollagen protects siRNA from enzymatic degradation.
siRNA: Short interfering RNA.

efficiently inhibited the growth of an established subcutaneous tumour of human small-cell lung carcinoma cells on non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice [26]. Tiscorina *et al.* developed Cre recombinase-inducible RNAi mediated by a lentiviral vector system [27,28]. However, a clinically applicable *in vivo* siRNA delivery method has yet to be established.

Cationic liposomes have been successfully used as an alternative approach to viral systems to deliver nucleic acids. Although past experience with antisense oligonucleotides and ribozymes suggests that most cationic lipid (liposome) delivery systems are too toxic when used *in vivo*, some companies (e.g., Nippon Shinyaku Co. Ltd, Kyoto, Japan) have developed novel cationic liposomes that can be safety administered in this way. Yano *et al.* have used such a type of liposome to demonstrate that anti-bcl-2 siRNA complexed with liposome has a strong antitumour activity when administered intravenously in the mouse model of liver metastasis [29]. In addition, Nogawa *et al.* also reported that the intravesical injection of polo-like kinase (PLK)-1 siRNA/liposomes successfully prevented the growth of bladder cancer in an orthotopic mouse model [30]. A more attractive method is through the delivery of siRNA using a cancer cell-specific antibody. Song *et al.* showed that an antibody against ErbB2 fused to a protamine fragment specifically and effectively only delivers siRNAs to ErbB2-expressing breast cancer cells [31]. Chien *et al.* developed a novel cationic liposome based on a synthetic cationic cardiolipin analogue (CCLA) and found that the treatment of a c-raf siRNA–CCLA complex in SCID mice bearing human breast xenograft tumours resulted in significant tumour growth suppression [32]. However, issues regarding transfer efficiency and the duration of the silencing effect restrict the spectrum of the applications of synthetic siRNAs *in vivo*. These limitations of siRNAs have been resolved by the cellular expression of short hairpin RNAs (shRNAs) from DNA vectors [33,34]. Thus, stable target gene suppression by RNAi *in vivo* using shRNA delivery is now possible.

In the case of systemic injection, tissue-targeting technologies are required. One interesting study reported that liver-targeted delivery of siRNA may be enhanced using chemical modification of the oligonucleotide with, for example, cholesterol conjugates. These conjugates are more resistant to nuclease

Table 1. High-throughput loss-of-function methods in mammalian cells.

System	Nucleic acids	Platform	Spotting materials	Transfection reagents	Cells	Refs.
Microarray-based cell transfections	shRNA siRNA	Glass slide	Gelatine	Lipofectamine plus	C2C12 and HeLa	[51]
RNAi microarray	siRNA	Glass slide	Matrigel	Lipofectine	HeLa	[52]
Cell transfection array	siRNA	96-Well plate	Atelocollagen	Atelocollagen	293T, NEC8 and PC3	[44,45,70, 74]
Surfection	shRNA	Mini wells	Gelatine	PEI	293T	[53]
RNAi microarrays	shRNA siRNA	Glass slide	Gelatine	Effectine	293T	[54]
Cell microarrays	siRNA	Glass slide	Gelatine	Effectine	293T	[55]
siRNA cell microarrays	siRNA	Chambered cover glass	Gelatine and fibronectin	Effectine	HeLa, MCF7 and COS7L	[56]
Transfection microarrays	siRNA	Glass slide	Fibronectin	JetPEI	hMSCs	[57]
Transfection microarrays	siRNA	Glass slide	Oleyl polyethylene glycol) ether	Lipofectamine 2000	K562	[58]
Small-molecule cell microarray	siRNA	Glass slide	PLGA	Effectine	A549 and HeLa	[59]

PEI: Polyethylenimine; PLGA: Copolylactic acid/glycolic acid; RNAi: RNA interference; shRNA: Short hairpin RNA; siRNA: Short interfering RNA.

degradation, the cholesterol attachment stabilising the siRNA molecules in the blood by increasing binding to human serum albumin and increased uptake of siRNA molecules by the liver [35]. Recently, the same group reported success with this approach in primates. This report provided encouraging evidence for the therapeutic use of RNAi in the near future [36]. Pirollo *et al.* has shown that a nanoscale non-viral liposome-based complex that includes an anti-transferrin receptor single-chain antibody fragment can efficiently and specifically deliver siRNA to both primary and metastatic tumours after systemic delivery in mice [37]. One promising approach is an *in vivo* siRNA delivery through complexation of siRNAs with polyethylenimine (PEI) [38]. Urban-Klein *et al.* showed that the non-covalent complexation of synthetic siRNAs with PEI efficiently stabilises siRNAs by protecting them against nucleolytic degradation and delivers siRNAs targeting the c-erbB2/neu (HER-2) receptor into a subcutaneous mouse tumour model by intraperitoneal administration, resulting in a marked reduction of tumour growth through siRNA-mediated HER-2 downregulation [39].

Recently, McNammara *et al.* have succeeded in developing aptamer–siRNA chimaeras that target specific cell types through binding to prostate-specific membrane antigen (PSMA), a cell surface receptor overexpressed in prostate cancer cells and tumour vascular endothelium [40]. They also suggested that the aptamer–siRNA chimaeras specifically inhibit tumour growth and mediate tumour regression in a xenograft model of prostate cancer. These studies implicate an approach for targeted delivery of siRNAs with numerous potential applications for cancer therapeutics.

5. Atelocollagen drug delivery system-mediated small interfering RNA delivery

Atelocollagen is prepared from type I collagen extracted from the bovine dermis. Type I collagen, which consists of three polypeptide chains that form a spiral structure, is a rod-like molecule 300 nm in length and 1.5 nm in diameter. The collagen molecule has a sequence of amino acids called a 'telopeptide' on both its N and C termini, which accounts for most of the antigenicity of collagen. As the telopeptides are removed by pepsin treatment during the purification of atelocollagen, this protein rarely elicits immunological responses or causes toxicity even if it is administered *in vivo*. Furthermore, atelocollagen has a variety of medical uses, such as the restoration of depressed areas in soft tissues, and the safety of this biomaterial in humans has been verified [41]. Atelocollagen is a liquid (gel) at low temperatures (2 – 10°C); therefore, it can be mixed with solutions containing nucleic acids. As shown in **Figure 1**, atelocollagen forms complexes with negatively charged nucleic acid molecules because it is positively charged [42] and the complex becomes resistant against nuclease digestion. It is thought that atelocollagen interacts with antisense oligonucleotides and siRNA to form nano-size particles that can be easily taken up into cells [43].

6. Identification of a drug target using cell transfection arrays

Because the nano-size particles of these complexes can be immobilised on a plate, they are amenable to high-throughput

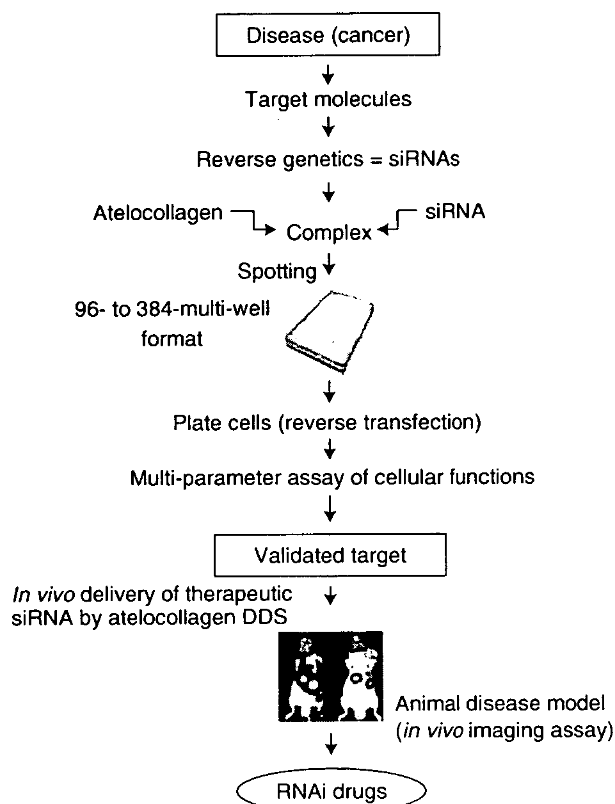

Figure 2. Atelocollagen DDS-mediated RNAi drug discovery. Based on information about gene groups (several hundred in each pool) that have changed (increased) their expression levels in tumours, the corresponding siRNAs are synthesised and introduced into target culture cells (cancer cell lines) using a cell transfection array. The plates are assayed for apoptosis, cell proliferation and other desired effects to select the candidate siRNAs. The selected siRNAs are introduced into the animal disease models (tumour-bearing animals) using the atelocollagen DDS. The tumour-suppressing effect for each siRNA was examined and the candidate siRNAs that have potential clinical uses were identified.
DDS: Drug delivery system; siRNA: Short interfering RNA.

screening (HTS) of nucleic acid drugs by reverse transfection [44-47]. The term 'reverse transfection' was first described in the *Biotechniques* journal by Amarzguioui in 2004 [48] and was later adapted for high-throughput applications as described in the *RNA* journal article '*High-throughput RNAi screening* in vitro*: from cell lines to primary cells*' [49]. RNAi-based reverse genetics using these complex particles will become a powerful tool to understand gene functions in cells and will allow the identification of drug targets. The authors' group [44] and Sabatini (MIT) *et al.* [50] concurrently published the first reports on the principle of reverse transfection in 2001 and many other laboratories have since then reported similar results. In particular, this method is indispensable for the analysis of gene functions using siRNAs, shRNAs and siRNA libraries that cover entire genomes [51-59]. In **Table 1**, the authors have summarised cell-based reverse transfection array technologies for the analysis of RNAi function. The basic strategy for the discovery of RNAi drugs for cancer therapies is illustrated in **Figure 2**. Based on information about gene groups (several hundred in a pool) that possess altered expression levels (increased) in cancer cells as detected by microarray analyses of clinical samples, the authors synthesise siRNAs against genes. These siRNA molecules are screened using a cell transfection array on target culture cells (a cancer cell line) and then the siRNAs that induce the desired activities, such as apoptosis, cell proliferation or infiltration, are identified. Next, the authors used the atelocollagen drug delivery system (DDS) to deliver these candidate siRNAs into animals that serve as models of diseases (tumour-bearing animals). Finally, tumour-suppressing effects are examined to identify functions that can be clinically applied to humans. In addition, siRNA and shRNA libraries have been developed by several groups [60-65] to allow for the systematic analysis of genes required for disease processes such as cancer using high-throughput RNAi cell transfection arrays. Therefore, these RNAi-based technologies are extremely important for functional analysis and target validation.

7. Local delivery of nucleic acid drugs

In 1999, the authors' group reported that atelocollagen is a biomaterial that is suitable for the controlled release of nucleic acids [42]. Since that time, atelocollagen has been used as a carrier material for the delivery of various nucleic acid drugs as summarised in **Table 2**. It is clear that precise drug delivery is critical for the treatment of many types of cancer. Human testicular tumour cells that are dependent on the fibroblast growth factor human stomach tumour (HST)-1/FGF-4 were transplanted into the testes of nude mice. A complex of antisense oligonucleotides targeting HST-1/FGF-4 and atelocollagen was directly administered into the testicular tumour, which resulted in the suppression of tumour cell proliferation as well as metastases to other organs [66]. Takei *et al.* reported that repeated administration of a complex of antisense oligonucleotides targeting midkine and atelocollagen in the mice transplanted with murine rectal carcinoma cells resulted in the enhanced suppression of cancer cell proliferation compared with the antisense oligonucleotides alone [67,68]. With regard to drug delivery for diseases other than cancer, Ando *et al.* used oligonucleotide–atelocollagen complexes to convert specific bases of the transthyretin gene, a causative gene of familial amyloid neuropathy, and reported that the conversion efficiencies were 11 and 8.7% for culture cells and mouse liver cells, respectively [69]. In contrast, oligonucleotides alone were not capable of inducing these conversions, demonstrating that the atelocollagen delivery system improved the efficiency of the oligonucleotide-mediated base conversion and its usefulness for the treatment of genetic diseases.

Table 2. *In vivo* delivery of nucleic acid medicines by atelocollagen.

Nucleic acids	Route	Therapeutic model (cell lines)	Infected site (target organs)	Target genes	Refs.
Synthetic siRNA	Intratumoural	Prostate cancer (PC-3)	Subcutaneous	VEGF	[71]
Synthetic siRNA	Intratumoural	Germ cell tumour (NEC8)	Testis	FGF-4	[70]
Synthetic siRNA	Intravenous	Prostate cancer (PC-3M-Luc)	Bone metastasis	EZH2 and p110α	[74]
Synthetic siRNA	Intravenous	Cervical cancer	Subcutaneous	HPV (E6 and E7)	[72]
Antisense oligonucleotides	Intratumoural	Rectal carcinoma, mouse (MT-93)	Subcutaneous	Midkine	[67,68]
Antisense oligonucleotides	Intratumoural	Germ cell tumour (NEC8)	Testis	FGF-4	[66]
Antisense oligonucleotides	Intravenous	Allergic dermatitis by 2,4-dinitrofluorobenzene	Ear	ICAM-1	[73]

HPV: Human papillomavirus; ICAM: Intercellular adhesion molecule; siRNA: Short interfering RNA.

To determine the effectiveness of the complexation with atelocollagen, Minakuchi *et al.* administered a siRNA–atelocollagen complex and siRNA alone to nude mice carrying tumour cells that had been transplanted subcutaneously and into the testes. The results revealed that the level and duration of the suppression of tumour cell proliferation increased in the mice treated with the complex [70]. Furthermore, Takei *et al.* reported that the administration of a complex of siRNA targeting the VEGF and atelocollagen substantially inhibited the proliferation of human prostate cancer cells that had been subcutaneously transplanted into nude mice. The authors reported that the complexation enhanced the incorporation of siRNA into the cancer cells in addition to extending the half-life of the siRNA [71]. Fujii *et al.* successfully established a treatment model of human cervical cancer using siRNA targeting the E6 and E7 oncogenes of human papilloma virus 18 [72].

8. Systemic delivery of small interfering RNA with atelocollagen complexes

Inflammation was experimentally elicited in the periphery of the mice and was treated with an intravenous administration of a complex of antisense oligonucleotides targeting intercellular adhesion molecule-1 and atelocollagen via the tail vein. The suppression of the inflammation was confirmed and the effect was observed even when the complex was injected 3 days before the initiation of inflammation [73]. This suggests that atelocollagen DDS is an excellent carrier for the systemic delivery of nucleic acid drugs. Human prostate cancer cells were transplanted to the left ventricle of nude mice as a bone metastasis model [74]. A siRNA–atelocollagen complex was administered via the tail vein to examine the delivery of the nucleic acids to the systemic metastatic foci. As a result, siRNA alone reduced the expression level of the target gene product by ~ 40%, whereas the siRNA–atelocollagen complex exhibited an inhibition level of ≥ 90%, indicating that atelocollagen is suitable for drug delivery to bone metastatic foci [74]. Furthermore, atelocollagen-mediated delivery of siRNA targeting two genes, *EZH2* and *p110α*, which are associated with the malignancy of prostate cancers, significantly inhibited the cell proliferation of bone metastatic tumours. These two genes are also reported to be associated with the malignancy of human breast cancers. However, in these models, siRNA was delivered to various normal organs in addition to the bone metastatic tumour lesions. When delivering drugs for cancer treatment, it is important to achieve effective drug delivery to the cancer lesions as well as minimise the delivery of the drug to normal organs. Therefore, this issue must be overcome in atelocollagen DDS-mediated systemic drug delivery.

Atelocollagen suitable for siRNA for local or systemic delivery is supplied by Koken Bioscience Co. Ltd (Tokyo, Japan) [101].

9. Principle of the atelocollagen drug delivery system

The development of drugs that target cancer cells has been conducted based on the principle of active targeting, in which anticancer drugs are targeted to cancer cells using antibodies against antigens specifically expressed on the cancer cells. Although this method was somewhat effective in culture cells, few successful results have been reported for animal experiments. Antigens similar to the cancer antigens are found in blood and on the surface of other cells, making it difficult to maintain an effective concentration of the drug at the target sites. On administration of the atelocollagen DDS to normal animals, siRNA–atelocollagen complexes are distributed to a number of organs. However, it is worth noting that the delivery rate of siRNA to tumours in these animals was ~ 1.7- to 2.2-times faster than the rate of delivery to normal tissues. The difference in the delivery rates may be partially explained by the enhanced permeation and retention (EPR) effect [75] discovered by Maeda *et al.* in 1986. In fact, the discovery of the EPR effect has significantly changed

studies about targeting solid cancers. Nascent blood vessels in cancer tissues possess higher substrate permeability than those in normal tissues; therefore, a larger amount of relatively large polymer compounds is transferred to cancer tissues. Furthermore, these polymer compounds are likely to be retained in cancer tissues because cancer tissues do not effectively remove polymer compounds via lymph ducts; therefore, some cancer therapies (such as the atelocollagen DDS-mediated siRNA delivery) are now based on a passive targeting theory, which states that the drugs in blood are taken up by cancer cells due to the EPR effect.

10. Expert opinion

In this review, the authors describe the usefulness of atelocollagen DDS in identifying the target genes associated with diseases or abnormal cell functions using an *in vitro* transfection array, and in the local and systemic delivery of synthetic siRNA for cancer treatment. In addition to siRNA, micro-RNA (miRNA) is likely to play a key role in future cancer therapies. miRNA is a 20- to 24-nucleotide single-stranded RNA; it is thought to control protein expression at the translational level and > 400 molecular species have been identified in humans. Depending on the progression of future cancer studies, miRNA may become an effective tool to elucidate disease mechanisms and novel treatments; therefore, the application of an atelocollagen DDS-based delivery to miRNA may identify diseases that can be controlled by miRNA and establish effective therapies. However, to improve the safety of this technology, our next challenge is to modify the complex of the nucleic acid molecule and atelocollagen to generate tumour-targeting specificity as well as reduce the effects on normal organs. The clinical development of nucleic acid drugs, particularly siRNA, is likely to be further accelerated by a novel technology, including cell-based transfection array technologies, as well as *in vivo* delivery systems.

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Type I collagen gene suppresses tumor growth and invasion of malignant human glioma cells

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Abstract

Background: Invasion is a hallmark of a malignant tumor, such as a glioma, and the progression is followed by the interaction of tumor cells with an extracellular matrix (ECM). This study examined the role of type I collagen in the invasion of the malignant human glioma cell line T98G by the introduction of the human collagen type I α1 (HCOL1A1) gene.

Results: The cells overexpressing HCOL1A1 were in a cluster, whereas the control cells were scattered. Overexpression of HCOL1A1 significantly suppressed the motility and invasion of the tumor cells. The glioma cell growth was markedly inhibited *in vitro* and *in vivo* by the overexpression of HCOL1A1; in particular, tumorigenicity completely regressed in nude mice. Furthermore, the HCOL1A1 gene induced apoptosis in glioma cells.

Conclusion: These results indicate that HCOL1A1 have a suppressive biological function in glioma progression and that the introduction of HCOL1A1 provides the basis of a novel therapeutic approach for the treatment of malignant human glioma.

Background

The processes of tumor cell invasion into the stromal tissue are closely related to the interactions between tumor cells and extracellular matrix (ECM). Furthermore, the alteration of the expression and modification of ECM proteins in tumor cells is relevant to their invasiveness into surrounding normal tissue. For example, loss of fibronectin, which is an ECM glycoprotein from the cell surface, has been indicated to be closely associated with malignant transformation of cells [1]. It was then shown that the overexpression of fibronectin in human fibrosarcoma cells was able to suppress the motility and growth potential of tumor cells [1]. Collagens are the most important components of ECM and play an important role in cell adhesion, movement, differentiation, proliferation, and metastasis of tumor cells. Recently, fragments of type IV, type XV, and type XVIII collagen, which are components of various basement membranes, have been extensively studied for their potential in the reduction of angiogenesis and tumorigenesis [2,3]. Endostatin, a non-collagenous C-terminal domain (NC1) fragment of type XVIII collagen, was the first endogenous fragment characterized with anti-angiogenic properties [4]. The NC1 fragments of type XV collagen and type IV collagen were also identified as an endogenous angiogenesis inhibitor [5-11].

Type I collagen is a fibrillar collagen that consists of two polypeptide chains, the α1(I) chain and the α2(I) chain.

Most of type I collagen molecules are distributed as heterotrimers $[\alpha 1(I)]_2\alpha 2(I)$, and a small number of molecules exist as homotrimers $[\alpha 1(I)]_3$. Although type I collagen is the most abundant collagen in skin, bone, and other tissues and organs, no type I collagen molecule and/or fragments have been demonstrated to have suppressive efficacy on malignant phenotypes of a tumor. However, variations in the production of type I collagen by tumors have been reported in the relationship with tumor progression, and transformed cells generally synthesize less collagen than their counterparts [12-15]. The differential expression between hepatocellular carcinomas and normal liver was revealed, and type I collagen was down-regulated in hepatocellular carcinomas [15]. In a neuroblastoma, type I collagen biosynthesis is a helpful marker for studying specific patterns of transdifferentiation associated with the loss of malignant potential [16]. In addition, type I collagen was able to inhibit growth and malignant transformation in human glioma cells [17,18]. In the present study, a type I collagen gene was introduced to glioma, which is characterized by marked tumor cell proliferation and extensive local invasion, in order to examine the inhibitory effects of the overexpressed HCOL1A1 on invasion and tumorigenesis. The results showed that overexpression of HCOL1A1 in malignant glioma cells suppressed cell proliferation, inhibited cell motility and invasiveness, and arrested tumor formation *in vivo*.

Results

Overexpression of HCOL1A1 in T98G cells

Human type I collagen cDNA (HCOL1A1) was transfected into the human malignant glioma cell line T98G. Twenty-nine colonies of G418-resistant cells were cloned, and G418-resistant clones were screened for expression of HCOL1A1 by immuno-detection with a polyclonal anti-$\alpha 1(I)$ collagen antibody. Two independent clones stably over-expressing HCOL1A1, designated HOCL1A1-I and HCOL1A1-II, were isolated. By immunocytochemical staining HCOL1A1-I and HCOL1A1-II cells showed strong immunoreactivity, while HCOL1A1 peptides were not detectable in wild cells and Mock cells (Fig. 1a). Expression of HCOL1A1 peptides were also confirmed in the whole cell lysates and the conditioned medium from HCOL1A1-transfected cells by immunoblot analysis. Although immunoreactive protein bands, which are absent in parental wild cells and Mock-transfected cells, were clearly identified, HCOL1A1 peptides were digested into fragments and did not fold into triple helices characteristic of type I collagen (data not shown).

The cell densities of the HCOL1A1-transfected cells (4 days) were significantly different from that of wild cells and Mock cells (Fig. 1b): this was clearly shown that the growth inhibition of HCOL1A1-transfected cells, as well as they were tightly cell-cell contacted in clusters, while control cells were dispersed and spindle-shaped in scatter-

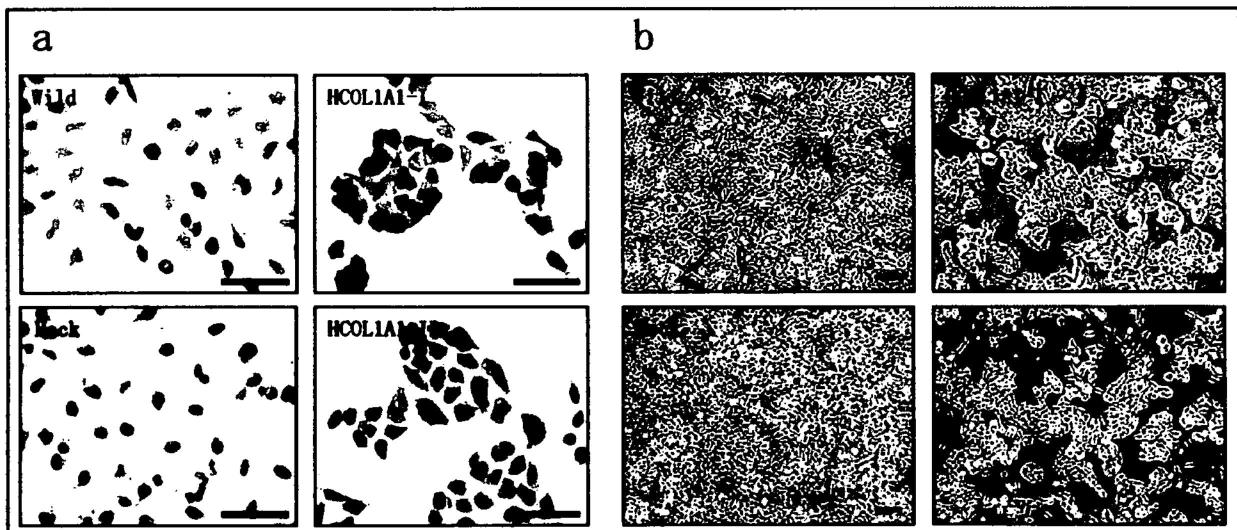

Figure 1
Morphological changes of the HCOL1A1-overexpressing T98G cells. Wild cells (Wild), control transfected cells (Mock), and HCOL1A1-transfected cells (HCOL1A1-I and HCOL1-II) were seeded at 1 × 10^5 cells on 6-cm culture dishes and grown for 2 to 4 days. (a) Immunocytochemical staining of T98G cells (day 2). HCOL1A1 peptides were immunostained with polyclonal anti-$\alpha 1(I)$ collagen antibody. Scale bar: 100 μm. (b) Phase-contrast photomicrographs of T98G cells (day 4). Scale bar: 200 μm.

ing as usual (Fig. 1a). These results suggest that forced expression of HCOL1A1 resulted in apparent cell density changes in T98G cells.

Inhibition of cell growth by HCOL1A1

The effect of HCOL1A1 expression on the growth of T98G cells was further evaluated kinetically (Fig. 2). Cell growth was significantly suppressed in HCOL1A1-I and HCOL1A1-II cells, $P < 0.001$ and $P < 0.0001$ versus Mock cells, respectively. These results showed that overexpression of HCOL1A1 was able to inhibit the growth of T98G cells *in vitro*.

Suppression of tumor cell motility and invasion by HCOL1A1

Whether the HCOL1A1 peptide inhibits tumor cell motility was examined. (Fig. 3a, 3b). The motility of HCOL1A1-transfected cells was dramatically lower than that of Mock cells. The migration of HCOL1A1-I and HCOL1A1-II cells was inhibited more than 80% as compared to Mock cells.

Furthermore, the effect of HCOL1A1 overexpression on the invasiveness of T98G cells was assessed. Figure 3c and 3d shows the ability of these cells to invade through the reconstituted Matrigel ECM. HCOL1A1-transfected cells displayed a significant reduction in invasion of more than 55%.

In the *in vitro* invasion model using reconstituted basement membrane wafers, HCOL1A1-I cells hardly invaded the Matrigel wafer on day 7 (Fig. 3e). In contrast, a number of Mock cells invaded the Matrigel wafer. Thus, it

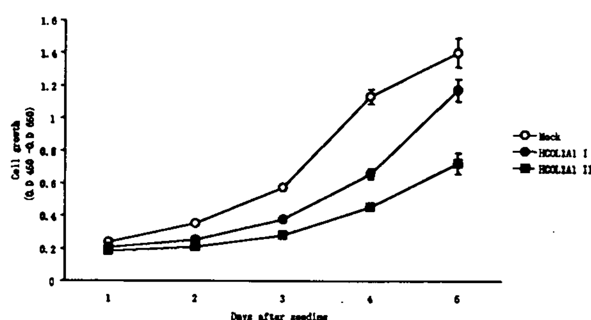

Figure 2
Inhibition of T98G cell growth *in vitro* by HCOL1A1 expression. The graph shows the growth curves of Mock cells (Mock, ○) and HCOL1A1-transfected cells (HCOL1A1-I, ●; HCOL1A1-II, ■). The same number of cells were seeded and cultured for 5 days. At each time point, the cells were assayed for proliferation. Each value represents mean ± SD (n = 8).

was clearly demonstrated that the HCOL1A1 peptide suppressed the cell motility and invasion of T98G cells.

Inhibition of tumor formation in vivo by HCOL1A1

To assess the effects of forced HCOL1A1 expression on T98G glioma cell growth *in vivo*, HCOL1A1-I cells and Mock cells were injected into nude mice s.c., and the tumor volumes were determined 87 days later (Fig. 4). Figure 4b shows that HCOL1A1-I cells had grown more poorly and formed smaller nodules than the Mock cells. At 87 days, the animals were sacrificed, and complete tumor regression was observed in the mice transplanted with HCOL1A1-I cells. In contrast, the control cell-transplanted animals had developed large tumors of around 1,000 mm^3. These results suggest that the introduction of HCOL1A1 was able to function as a growth suppressor in human glioma cells *in vivo*.

Induction of apoptotic cells in vitro by HCOL1A1

The effect of overexpressed HCOL1A1 on the apoptosis of glioma was studied by the TUNEL assay (Fig. 5). Figure 5a shows that numerous apoptotic cells were detected in HCOL1A1-transfected cells, whereas apoptotic cells were scarcely seen in the Mock cells. The number of apoptotic cells was counted in the microscopic fields, and the percentage of apoptotic cells is revealed in Figure 5b. The apoptosis rates of HCOL1A1-I and HCOL1A1-II were 17% and 29%, respectively. In contrast, Mock cells showed less than 1% of apoptotic cells. Therefore, the over-expression of HCOL1A1 induced the apoptosis of T98G glioma cells and this may partially contribute to the growth suppression of HCOL1A1 tumors in mice.

Discussion

The present study shows that the overexpression of HCOL1A1 in T98G malignant glioma cells markedly changed the cell morphology and significantly suppressed the motility and invasion of cells. In addition, the inhibition of glioma cell growth was indicated *in vitro* and *in vivo*; in particular, tumorigenicity had regressed completely in nude mice. Moreover, HCOL1A1 induced the apoptosis of glioma cells, and this finding suggested that apoptosis participates in a mechanism for the suppression of malignancy of T98G glioma cells by forced expression of HCOL1A1. Interestingly, overexpression of HCOL1A1 in 293 cells, which are transformed human embryo kidney cell line and do not express HCOL1A1, showed no influence on the cell morphology, growth and apoptosis induction (data not shown). This finding suggests that ectopic forced expression of HCOL1A1 protein in cells dose not cause of unfolded protein response which induces apoptosis, however, the effects of accumulation of unfolded HCOL1A1 protein on various cell types should be further considered.

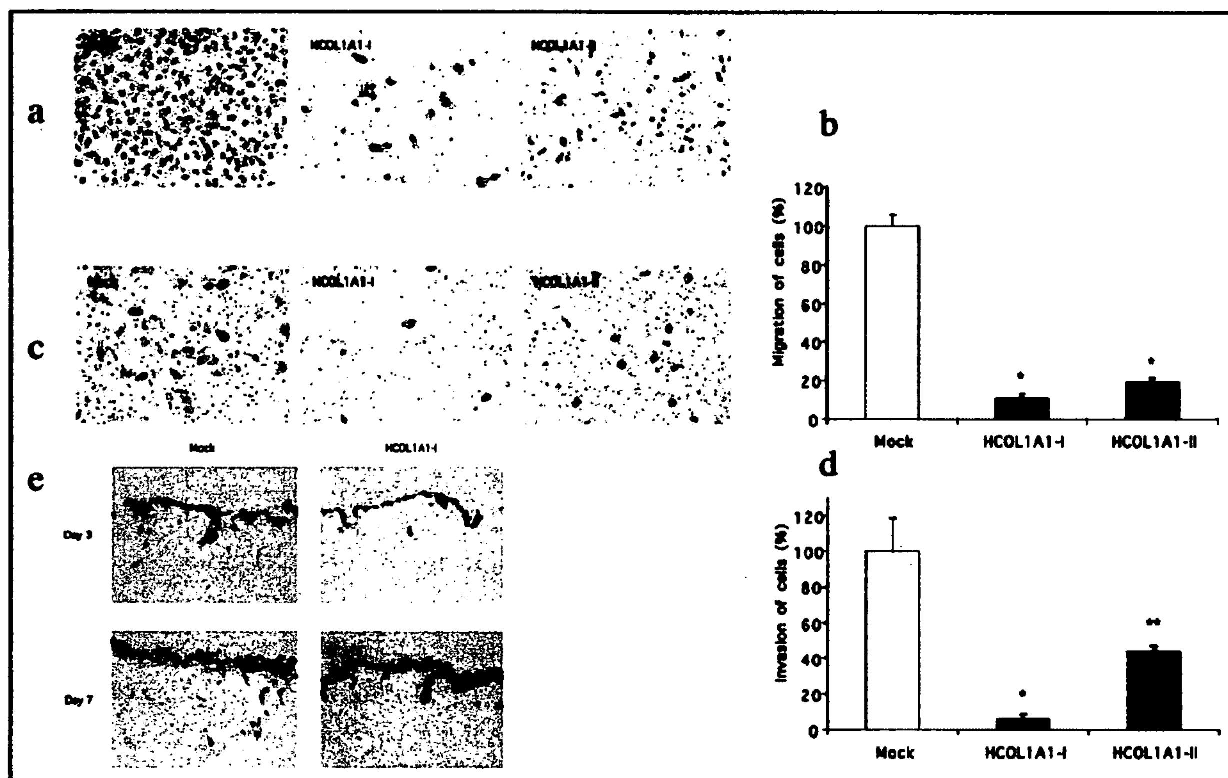

Figure 3
Suppression of T98G cell motility and invasion by overexpression of HCOL1A1. Mock cells (Mock) and HCOL1A1-transfected cells (HCOL1A1-I and HCOL1A1-II) were added to a transwell chamber and the cell motility was evaluated. (a) The migrated cells were stained and photographed under the microscope at × 100 magnification. (b) The number of migrated cells was counted, and the results represent a percentage of Mock cells. Each value is a mean ± SD (n = 4). *, $P < 0.001$, when tested against the Mock. Mock cells (Mock) and HCOL1A1-transfected cells (HCOL1A1-I and HCOL1A1-II) were added to a transwell chamber coated with Matrigel and the cell invasion was evaluated. (c) The invaded cells were stained and photographed under a microscope at × 100 magnification. (d) The number of invaded cells was counted, and the results are expressed as a percentage of Mock cells. Each value is a mean ± SD (n = 4). *, $P < 0.001$, **, $P < 0.01$ when tested against the Mock. The cell invasion into an *in vitro* invasion model was evaluated using reconstituted basement membrane wafers. (e) Mock cells (Mock) and HCOL1A1-transfected cells (HCOL1A1-I) were plated onto Matrigel wafers. On days 3 and 7 after plating, Matrigel wafers and adherent cells were fixed, and sections were stained with hematoxylin and eosin. Magnification, × 200.

It has been pointed out that loss of ECM control is a characteristic feature in malignant tumor progression toward invasion [19]. In glioma cells, the expression of ECM components, such as decorin, tenascin, vitronectin, laminin, fibronectin, type I collagen, type IV collagen, neuronal cell adhesion molecule (NCAM), N-cadherin, and beta-catenin, dramatically changes during tumor progression, and these ECM proteins have been reported to play a significant role in the migration and invasion of gliomas [18,20-29]. As an example of glioma therapy using ECM protein, decorin, which is poorly expressed by glioma cell lines, was ectopically expressed in glioma cells and successfully used to abrogate the growth of experimental gliomas [20,30,31]. Similarly, the overexpression of type I collagen should be useful for suppression of glioma progress.

It has become clear that ECM proteins have unknown physiological cell functions. Recently, the biological functions of various collagen fragments have been described; fragments of type IV, type XV, and type XVIII collagen have been noted for their potential [2,3]. Type IV, type XV, and type XVIII collagen are major components of various basement membranes. Although their functional role in basement membrane architecture is well known, the NC1 fragments of these collagens are involved in the regulatory functions of various cellular events, such as angiogenesis,

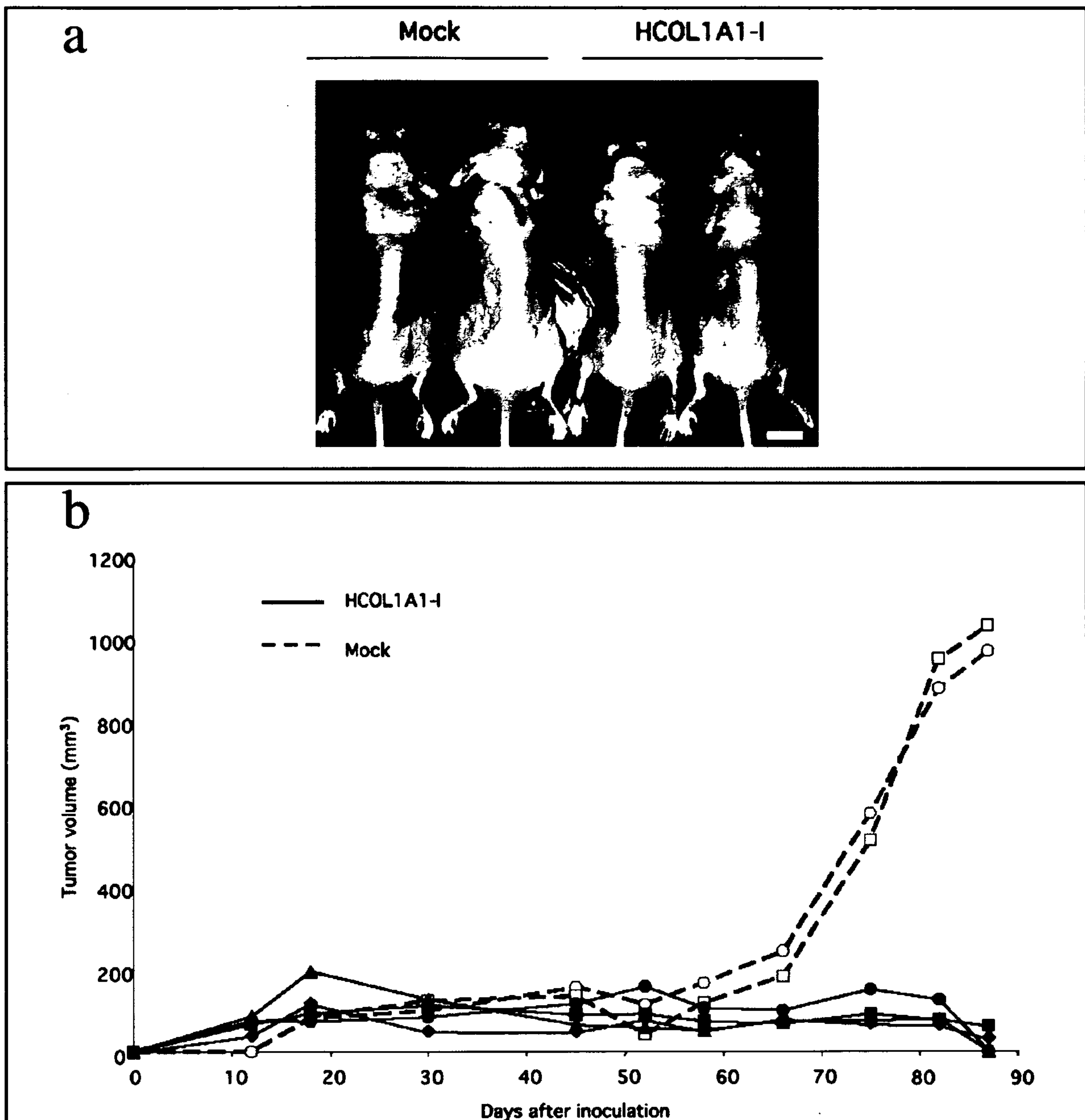

Figure 4
Inhibition of tumor formation of T98G cells *in vivo* by introduction of the HCOL1A1 gene. T98G cells were implanted s.c in the forelegs of nude mice, and the tumor volume was measured at each time point. (a) Tumor formation in nude mice at 87 days after inoculation with Mock cells (Mock) or HCOL1A1-transfected cells (HCOL1A1-I). Scale bar, 1 cm. (b) The *in vivo* tumor growth of HCOL1A1-transfected cells (HCOL1A1-I, n = 4) was compared with that of Mock cells (Mock, n = 2).