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Expression of the extracellular domain of OB-cadherin as an Fc fusion protein using bicistronic retroviral expression vector

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Abstract

Osteoblast cadherin (OB-cadherin, also known as cadherin-11) is a Ca^{2+} -dependent homophilic cell adhesion molecule that is expressed mainly in osteoblasts. OB-cadherin is expressed in prostate cancer and may be involved in the homing of metastatic prostate cancer cells to bone. The extracellular domain of OB-cadherin may be used to inhibit the adhesion between prostate cancer cells and osteoblasts. In this report, we describe the expression of the extracellular domain of OB-cadherin as an Fc fusion protein (OB-CAD-Fc) in human embryonic kidney 293FT cells using a bicistronic retroviral vector. Coexpression of GFP and OB-CAD-Fc through the bicistronic vector permitted enrichment of OB-CAD-Fc-expressing cells by fluorescence-activated cell sorting. Recombinant OB-CAD-Fc proteins were secreted into cell medium, and about 0.85 mg of purified OB-CAD-Fc protein was purified from 1 liter of the conditioned medium using immobilized protein A-affinity chromatography. The purified OB-CAD-Fc was biologically active because it supported the adhesion of PC3 cells and L cells transduced with OB-cadherin. The availability of OB-CAD-Fc offers opportunities to test whether OB-CAD-Fc can be used to inhibit OB-cadherin-mediated prostate cancer bone metastasis *in vivo* or to generate antibodies for inhibiting the adhesion between prostate cancer cells and osteoblasts.

Keywords

Cell adhesion; Osteoblast; OB-cadherin; Retroviral expression vector

Introduction

Adhesion molecules have been postulated to play a role in the metastasis of cancer to distant organ sites. Thus, interfering adhesion molecule-mediated events for cancer treatments have

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actively been pursued [1]. One of the strategies is to use recombinant proteins containing the functional domains of adhesion molecules to block the adhesion. Expression of the functional domains of adhesion molecules for *in vivo* studies is a challenge. The *Escherichia coli* expression system, which lacks post-translational modifications, especially proper disulfide bond formation, has limited utility. Large-scale expression of proteins in mammalian expression systems is time consuming and costly. In this study, we attempted to improve the current method of protein expression in mammalian cells and express the functional domain of osteoblast cadherin (OB-cadherin) for therapeutic exploration.

The cadherins, a family of cell adhesion molecules that mediate Ca^{2+} -dependent homophilic adhesion, are important both for tissue morphogenesis during development and for maintaining stable cell–cell adhesion in adult tissues [2,3]. On the basis of protein domain composition, genomic structure, and phylogenetic analysis of the protein sequences, the cadherins form a superfamily with at least six subfamilies [4]. Among them, the type I cadherins include N-, E-, M-, and R-cadherins, and type II cadherins include cadherins 5 through 12 [4].

OB-cadherin, also known as cadherin-11, is a type II cadherin that is expressed preferentially in osteoblasts, with only weak signals detectable in brain, lung, and testicular tissue [5–7]. OB-cadherin, like other classical cadherins, is composed of an extracellular domain with five repeated subdomains (EC 1–5), a single transmembrane domain, and a cytoplasmic C-terminal tail [5]. The calcium binding sites are located in the extracellular domain and participate in the homodimerization of cadherin present on neighboring cells [8–11]. Expression of OB-cadherin is associated with osteoblast differentiation and has been proposed to function in cell sorting, migration, and alignment during the maturation of osteoblasts [12]. As a result, OB-cadherin has also been used as a marker for the selection of osteoblastic lineage cells from embryonic stem cells induced to differentiate into various lineages [13].

In prostate cancer, studies by Tomita et al [14] showed that OB-cadherin becomes expressed in poorly differentiated prostate cancer cells. Because prostate cancer has a propensity to metastasize to bone, this observation has led us to hypothesize that OB-cadherin, by mediating the adhesion of metastatic prostate cancer cells and osteoblasts, plays a role in the metastasis of prostate cancer cells to bone. Consistent with this hypothesis, we found that bone-derived prostate cancer cell lines express high levels of OB-cadherin and exhibit specific binding to OB-cadherin in a cell-to-substrate assay (Chu et al., manuscript submitted). Further, OB-cadherin-expressing prostate cancer cells exhibited a high incidence of colonization in bone when these cells were injected intracardially into mice, and knockdown of OB-cadherin in prostate cancer cells decreased their colonization in bone (Chu et al., manuscript submitted). These results suggest that OB-cadherin is one of the adhesion molecules involved in the homing of prostate cancer cells to bone. Because the current therapies used to control prostate cancer progression have only limited efficacy, strategies that block OB-cadherin-mediated adhesion may prevent the dissemination of prostate cancer cells to bone. One possible method is to use the extracellular domain of OB-cadherin to inhibit the binding between prostate cancer cells and osteoblasts via competition for binding with OB-cadherin. A large amount of the biologically active form of OB-cadherin will be needed for functional studies *in vivo*.

Because OB-cadherin is a membrane protein that has extensive posttranslational modification, including signal sequence cleavage, disulfide bond formation, and glycosylation, it may need to be expressed in mammalian cells to retain the proper conformation and function. To avoid the transfection and selection of permanent cell lines, which is time consuming, for the expression of the extracellular domain of OB-cadherin, we have chosen to use a bicistronic vector that expresses two proteins from the same promoter through an internal ribosome entry site (IRES).² An IRES is a noncoding RNA fragment that has the ability to induce protein synthesis in mammalian cells and cell-free extracts in a cap-independent way. Therefore, the

presence of an IRES sequence allows for initiation of translation in the middle of a messenger RNA. When an IRES sequence is located between two genes in an mRNA molecule, both proteins are produced in the cell [15–17]. Thus, the expression of genes of interest can be reflected by the fluorescence reporter whose expression is directed by the IRES placed between the two protein coding sequences. Coexpression of a fluorescence reporter with the gene of interest allows the selection of cells through fluorescence-activated cell sorting (FACS) analysis, which bypasses the long period associated with selecting drug-resistant cell lines.

Retroviruses are highly efficient gene delivery vehicles for mammalian cells in that they can infect nearly all dividing mammalian cells. After its entry into the cells, the retroviral DNA frequently integrates into the host genome, making this technique suitable for creating stable cell lines [18]. Combining the high transduction rate of retroviruses, the detection of the expression of the gene of interest by coexpression of a fluorescence reporter through IRES, and the enrichment of cells that express the recombinant protein by FACS will greatly shorten the time needed to generate cell lines that express proteins of interest.

In this study, we demonstrate the expression of the extracellular domain of OB-cadherin as an Fc fusion protein in the human embryonic kidney 293FT cell line using a bicistronic retroviral vector. Because cadherins have been shown to be functional on dimerization [9–11], expression of the protein as Fc fusion will allow the protein to form dimers. We show that stable cell lines expressing OB-CAD-Fc protein can be obtained within a short time. Recombinant OB-CAD-Fc proteins were produced and secreted into cell medium and could be purified from the conditioned medium in one step using immobilized protein A-affinity chromatography.

Materials and methods

Retroviral vector construction

The pBMN-I-GFP, a bicistronic retroviral vector that allows for the expression of genes of interest with the green fluorescent protein (GFP) through an IRES, was kindly provided by Dr. Gary Nolan's laboratory (Stanford University). The extracellular domain of OB-cadherin was linked to glutamic acid residue 211 of the human immunoglobulin G₁ (IgG1) Fc region (Fig. 1A) as follows. The human IgG1 Fc region, starting at glutamic acid residue 211, was amplified by polymerase chain reaction (PCR) using *Bam*HI-Fc-F and *Not*I-Fc-R primers (GGATCCGAGCCCAAATCTTGTGACAAAAC and GCGGCCGCGTCGACTTATCATTTACC) with GoTaq polymerase (Promega, Madison, WI). The PCR conditions were as follows: 94 °C 5 min (1 cycle); 94 °C 30 sec, 53 °C 30 sec, 72 °C 30 sec (30 cycles); and 72 °C 10 min (1 cycle). The PCR product was subcloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), and positive clones were sequenced. The positive clones were digested with *Bam*HI and *Not*I enzymes and subcloned into pBMN-I-GFP through *Bam*HI and *Not*I sites to give pBMN-Fc-I-GFP. The cDNA encoding the human extracellular domain of OB-cadherin, truncated after tyrosine 587 of the mature OB-cadherin protein, was generated by PCR using oligonucleotide primers (*Bgl*III-OBCECD-F and *Bam*HI-OBCECD-R: AGATCTCCATGAAGGAGAACTACTGT and GGATCCGTAGCCTCTGCGTTGCAGGA, respectively) with PrimeSTAR HS DNA polymerase (Takara Bio, Madison, WI). The PCR conditions were as follows: 94 °C 5 min (1 cycle); 94 °C 30 sec, 53 °C 30 sec, 72 °C 30 sec (30 cycles); and 72 °C 10 min (1 cycle). The PCR product was subcloned using a StrataClone Blunt PCR cloning kit (Stratagene, La Jolla, CA). Positive clones were sequenced to confirm the integrity of the sequence. The positive clones were then cut with *Bgl*III and *Bam*HI and subcloned into *Bam*HI-digested pBMN-Fc-I-

²Abbreviations used: OB-cadherin, osteoblast cadherin; IRES, internal ribosome entry site; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; IgG, immunoglobulin; PCR, polymerase chain reaction; BSA, bovine serum albumin;

GFP. The resulting plasmid, pBMN-(OB-CAD-Fc)-I-GFP, contains the extracellular domain of OB-cadherin linked to glutamic acid residue 211 of the human IgG1 Fc region [19] through an artificial *Bam*HI adapter, which creates a junction sequence, “GSEPKSCD,” with the amino acids GS (glycine and serine) encoded from the restriction-site sequence of *Bam*HI.

Retrovirus production and transduction

The pBMN-(OB-CAD-Fc)-I-GFP plasmid was transfected into Phoenix cells, a 293-derived retroviral packaging cell line (American Type Culture Collection, Manassas, VA), using Eugene 6 (Roche Diagnostics, Indianapolis, IN). The virus-containing supernatant was collected 48 h posttransfection, and used to infect 293FT cells (Invitrogen).

Generation of 293FT cell lines expressing OB-CAD-Fc

The virus-containing supernatant from the previous step was mixed with Polybrene (hexadimethrine bromide H9268; Sigma-Aldrich, St. Louis, MO) to a final concentration of 8 µg/µl and added to the 293FT cells. Transduction of the 293FT cells with retrovirus was performed at 32 °C for 72 hours in a 10-cm plate. The cells were then expanded in 10-cm tissue culture plates to increase the total cell numbers before performing FACS analysis.

FACS analysis

Cells from a subconfluent 10-cm plate were released from the plate by trypsin digestion. The cells were resuspended in DMEM/10% FBS and sorted on a FACScan analyzer (Becton Dickinson, Mountain View, CA). Cells that were positive with green fluorescence were collected, washed with DMEM/10% FBS, and plated on 10-cm tissue culture plates.

Large-scale expression of human OB-CAD-Fc in 293FT cells

293FT cells expressing OB-CAD-Fc were grown on 15-cm plates in DMEM/10% FBS until they were 80–90% confluent. Cells from 30 plates were washed once with PBS and allowed to grow on CD293 medium (chemically defined medium, protein-free; Invitrogen). The medium was collected every 2 days for 6 days and was replenished with fresh medium each time. The medium from days 2 and 4 was collected, centrifuged for 20 min at 8000 rpm, filtered through a 0.22-µm filter (Corning Inc., Corning, NY), and concentrated 10-fold by low-speed centrifugation in a Centricon Plus-70 (Millipore, Billerica, MA).

SDS-PAGE and Western blot analyses

The conditioned medium and fractions from the purification steps were heated at 95 °C for 5 min in sample buffer (0.075 M Tris-HCl [pH 6.8], 1.5% SDS, 15% glycerol) in both the presence and absence of 1.5% 2-mercaptoethanol and analyzed on 4–12% gradient NuPage gels (Novex, San Diego, CA). Gels were stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL) or electrotransferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) in Tris-glycine-methanol transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol [v/v]). The membranes were stained with 1% Ponceau S (Sigma, St Louis, MO) to check the protein loading. Blots were blocked with 4% blotting grade blocker nonfat dry milk (Bio-Rad) in TBS-T (50 mM Tris-HCl [pH 8.0], 138 mM NaCl, 27 mM KCl, 0.1% Triton X-100) at room temperature for 30 min and probed with a 1:1000 dilution of goat anti-human OB-cadherin polyclonal antibody (R&D Systems, Minneapolis, MN) in TBS-T containing 4% of blotting grade blocker nonfat dry milk for 18 h at 4 °C. The blots were then washed five times with TBS-T for 5 min each at room temperature and probed with a 1:5000 dilution of a horseradish peroxidase labeled donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS-T containing 4% of blotting grade blocker nonfat dry milk for 1 h at room temperature. Bands were detected with SuperSignal West Pico (Pierce) according to the manufacturer's instructions.

Protein A-agarose affinity chromatography

The concentrated medium was filtered through a 0.22- μ m filter, and protease inhibitors (Complete Mini EDTA-free, Roche) were added following instructions from the manufacturer. To each concentrated sample (from days 2 and 4), 0.5 ml (50% v/v in PBS) of protein A-agarose beads (Pierce) were added, and the mixtures were incubated overnight at 4 °C with end-over-end rotation. The medium with protein A-agarose beads was loaded onto a chromatography column, and the resin was allowed to settle with gravity flow. The beads were washed six times with washing buffer (150 mM NaCl, 20 mM Tris [pH 8.0], 2 mM CaCl₂, and protease inhibitors). The protein was then eluted with 100 mM glycine-HCl, pH 2.5. The fraction (1 ml eluate) was immediately neutralized with 60 μ l of 1 M Tris-HCl, pH 9.5, previously added to the collection tubes. Six elution fractions were collected, and the protein A-agarose beads were regenerated by washing them with washing buffer.

Preparation of PC3 and L cells expressing OB-CAD for assay

PC3 cell, a prostate cancer cell line that expresses OB-cadherin, was kindly provided by Dr. I. J. Fidler (M. D. Anderson Cancer Center). PC3 cells were released from the culture plate with PBS containing 2 mM EDTA (ethylene-diamine-tetra-acetic acid) and then resuspended to a final concentration of 1×10^6 cells/ml in binding buffer. L cells (CCL1.3) were obtained from American Type Culture Collection. The plasmid containing the human OB-cadherin cDNA, pCMV-Sport6-hOBC, was obtained from Open Biosystems. The human OB-cadherin cDNA was subcloned on *NotI/XhoI* site of pBMN-I-GFP to generate pBMN-OB-CAD-I-GFP. Retrovirus were produced as described above and used to infect L cells to generate L cells/ OB-CAD. Control L cells were prepared by infecting L cells with retrovirus generated from pBMN-I-GFP plasmid.

Cell-to-substrate adhesion assay

The wells of Linbro 96-well microtiter plates (ICN Flow Laboratories, Horsham, MA) were coated with 100 μ l of 10 ng/ml of goat anti-human IgG (Fc γ , cat. no. 109-005-098; Jackson ImmunoResearch, West Grove, PA) in Tris-buffered Saline (TBS) solution (50 mM Tris-HCl [pH 8.0], 138 mM NaCl, 27 mM KCl) containing 1 mM CaCl₂ for 18 h at 4 °C and then washed twice with binding buffer (10 mM HEPES [pH 7.4], 1 mM CaCl₂, 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 0.1% glucose). The wells were then blocked with 1% bovine serum albumin (BSA) (Calbiochem-Novabiochem Corp.) in binding buffer for 30 min at room temperature and washed twice with binding buffer. Various concentrations of OB-CAD-Fc or human Fc (Jackson ImmunoResearch) were added to the wells and allowed to bind with goat anti-human IgG for 1 h at 37 °C.

To perform cell-to-substrate adhesion assay, cells were labeled by incubation with 1 mM Calcein-AM (Invitrogen) for 10 min at room temperature. Cells were washed twice and resuspended in binding buffer to a final concentration of 5×10^5 cells/ml, and 100 μ l of cells were added to the wells of the microtiter plates prepared as described above. The fluorescence (excitation at 485 nm, emission at 528 nm) in each well was immediately measured and recorded. The cells were then allowed to bind for 2 h at 37 °C. Unbound cells were removed, and the wells were washed 3 times with 1 mM CaCl₂ in binding buffer. The fluorescence emitted by the cells bound to OB-CAD-Fc was measured, and the percentage of bound cells was determined as the output-input ratio.

Results

Generation of 293FT cell lines expressing OB-CAD-Fc

To express OB-CAD-Fc for functional studies, we constructed a retroviral expression vector containing cDNAs encoding for the entire extracellular domain (EC 1 through 5) of OB-cadherin fused with Fc (OB-CAD-Fc) and GFP in a bicistronic unit (Fig. 1A and B). The recombinant retroviral vector was transfected into Phoenix cells to generate recombinant OB-CAD-Fc retrovirus. The conditioned medium that contained the retroviral particles from the Phoenix cells was used to infect 293FT cells, a cell line derived from human embryonic kidney. FACS analysis indicated that about 42.5% of 293FT cells expressed GFP protein (Fig. 1C), and these GFP-positive cells were collected from FACS for protein production. As shown in Fig. 1D, about 88% of the sorted cells expressed GFP, according to FACS analysis.

OB-CAD-Fc in 293FT conditioned medium

Western blotting was used to detect the expression of OB-CAD-Fc protein in the cells and the conditioned medium (Fig. 2). The extracellular domains of the OB-cadherin and the Fc regions in the expression vector contain 587 (excluding the signal sequence) and 234 amino acids, respectively, and the two respective regions have two and one potential N-linked glycosylation sites. If we estimate that each site after N-linked glycosylation is around 3 kDa, the apparent molecular masses for the monomeric and dimeric forms of OB-CAD-Fc would be expected to be 100 kDa and 200 kDa, respectively.

There are also 11 cysteines in the OB-CAD-Fc. Western blot analysis of proteins indicated that OB-CAD-Fc was expressed and secreted into the culture medium, and the apparent molecular mass of OB-CAD-Fc was around 250 kDa under nonreducing conditions (Fig. 2A). The discrepancy between the apparent and calculated molecular masses of the dimeric form of OB-CAD-Fc was likely due to the presence of disulfide bonds. The parental 293FT cells did not express OB-CAD-Fc, and the faint band at around 180 kDa detected on Western blotting was due to immunoglobulin present in the culture medium because it was also detected by using secondary antibody alone (data not shown). Under reducing conditions, the apparent molecular mass of OB-CAD-Fc was reduced to around 100 kDa (Fig. 2B), as detected by both a polyclonal goat anti-OB-cadherin antibody and an anti-OB-cadherin monoclonal antibody. These observations suggested that OB-CAD-Fc in the conditioned medium was present as a dimer.

Time course of OB-CAD-Fc expression

To enable large-scale production of OB-CAD-Fc recombinant protein, we first examined the time course of OB-CAD-Fc secretion in the 293FT cells. To avoid the presence of immunoglobulin, which would interfere with the purification of OB-CAD-Fc through a protein A-agarose affinity gel matrix, the cell culture medium was changed to a protein-free chemically defined medium, CD293. The conditioned medium was collected every day for 6 days with medium changes every other day. Western blot analysis of the conditioned medium showed that a high level of OB-CAD-Fc was expressed for up to 4 days. However, on days 5 and 6, OB-CAD-Fc secretion significantly decreased, likely because of the overconfluent state of the cell culture (Fig. 3A). The OB-CAD-Fc was maintained as a dimer in serum-free conditioned medium but could be reduced to a monomer by treating it with β -mercaptoethanol (Fig. 3A).

Protein A-agarose purification of OB-CAD-Fc

The conditioned medium from days 2 and 4 were concentrated and incubated with protein A-agarose affinity matrix, and then OB-CAD-Fc was eluted from the matrix by using glycine buffer (pH 2.5). The eluted OB-CAD-Fc was immediately neutralized with Tris buffer. The elution profile from protein A-affinity chromatography is shown in Fig. 3B. Most of the protein

that bound to protein A-agarose was eluted in the first fraction (Fig. 3B); this first-eluted fraction contained a major protein with an apparent molecular mass of about 250 kDa on SDS-PAGE under nonreducing conditions (Fig. 3C). On Western blot analysis (Fig. 3C), the 250 kDa protein reacted with anti-OB-cadherin antibody, suggesting that this purified protein was likely OB-CAD-Fc.

To estimate the yield of the chromatographic purification and the degree of purification, quantitative Western blot analysis was done. We found that OB-CAD-Fc was enriched about fourfold after purification, and about 89% of the protein could be recovered in the eluted fractions. From 1 liter of conditioned medium, approximately 0.85 mg of OB-CAD-Fc could be obtained (Table 1).

OB-CAD-Fc mediates adhesion

To examine whether the purified OB-CAD-Fc could mediate adhesion, we used a cell-to-substrate adhesion assay to assess the ability of L cells stably expressing OB-cadherin or control cells, to bind to the various concentrations of purified OB-CAD-Fc coated onto 96-wells plates. A plate coated with various concentrations of purified Fc was used as a control. L cells expressing OB-cadherin bound to OB-CAD-Fc in a dose-dependent manner, but not to Fc. On the other hand, L cells infected with the control virus were not able to bind to any of the coated plates (Fig. 4), suggesting that the binding is specific to OB-cadherin. To further examine the ability of OB-CAD-Fc to mediate adhesion, PC3 cells, which express OB-cadherin, were tested in the same assay. PC3 cells bound to OB-CAD-Fc, but not to Fc, in a dose-dependent manner. Maximal binding of PC3 to OB-CAD-Fc was obtained at a concentration of about 200 ng/well (Fig. 4). PC3 cells did not bind significantly to Fc (Fig. 4). These observations thus demonstrated that OB-CAD-Fc can mediate adhesion.

Discussion

The findings from this study enabled us to describe the generation of the extracellular domain of OB-cadherin as an Fc fusion protein, OB-CAD-Fc, in 293FT cells. OB-CAD-Fc was secreted into the conditioned medium and could be purified in one step by protein A-agarose affinity chromatography. Generation of an OB-CAD-Fc-producing cell line was achieved in a relatively short time by using a bicistronic retroviral vector containing GFP, and those cells could be enriched by FACS. From 1 liter of conditioned medium, about 0.85 mg of OB-CAD-Fc was obtained. In addition, we found that the purified OB-CAD-Fc is biologically active because it could support the adhesion of PC3 cells and L cells transduced with OB-cadherin. The availability of OB-CAD-Fc offers opportunities to test whether it can be used to inhibit OB-cadherin-mediated bone metastasis of prostate cancer *in vivo* or to generate antibodies for inhibiting prostate cancer bone metastasis. Although we did not examine this in the current study, it is possible that the efficiency of protein expression could be improved by adapting OB-CAD-Fc-expressing 293FT cells to grow in suspension culture or by expressing OB-CAD-Fc in a 293 cell line that has already been adapted to suspension growth. However, this needs to be tested empirically.

A bicistronic expression cassette that allows coexpression of the protein of interest and a fluorescence reporter from the same promoter can greatly simplify the process of generating a recombinant protein-producing cell line. To produce proteins in mammalian cells, it is common to transfect the plasmids of interest into the cells. This transfection usually has low efficiency, and the level of protein expressed in mammalian cells is, in general, very low. Therefore, it is necessary to transfect large numbers of mammalian cells if a transient protein-expression method has to be used. This could become expensive and time consuming because multiple transfection events would be needed. Thus, it is common to generate permanent cell lines with the genes of interest integrated into the genome. This is generally achieved by

incorporating a drug-resistant gene into the mammalian expression vector, therefore allowing the selection of cell lines with specific drugs, e.g., G418. However, the generation of permanent cell lines is also time consuming, since not only does it take weeks or months to select for drug-resistant cells but also the selected cells frequently exhibit drug resistance without expressing the protein of interest. Thus, it is necessary to characterize the selected clones individually, and the cell lines are then further expanded for protein production. Furthermore, because membrane proteins are usually expressed in a relatively low level in the cells and secreted proteins are diluted in the conditioned medium, screening for cell lines that express these types of proteins in the initial stage of selection is difficult. The process of cell-line selection would be substantially improved by using a bicistronic expression vector with GFP coexpressed with the protein of interest. The use of retroviral particles, which are highly efficient in infection and integration into mammalian cells, further improves the method of protein expression.

Replication-deficient adenovirus can also be used as a method for producing a high level of protein expression. However, the expression of protein from adenoviral infection is transient, with protein expression lasting only between 24 and 72 h. In addition, this transient nature of protein expression from an adenoviral expression system requires the production of a high titer of adenoviral particles, which is time consuming. Furthermore, the adenovirus is highly infectious to humans, making it unsuitable for infecting a large quantity of cultured cells for protein production.

Whether OB-cadherin-mediated adhesion depends on the interactions of partner EC1 domains or on multiple EC domains is not clear. A report by Patel et al. [8] described the expression of the first domain of mouse OB-cadherin protein, i.e., amino acids 1–98, in *E. coli*, for crystal-structure determination. They showed that the protein assumed a dimeric conformation similar to E-cadherin and proposed that the first domain of OB-cadherin is the adhesion domain. We also have this protein from *E. coli*, but this domain neither conferred nor was able to compete with OB-cadherin-mediated cell adhesion activity in the cell-to-substrate assay (data not shown). Studies on the structural basis of adhesion on other cadherins have shown that multiple EC domains may be involved in cadherin binding [20–23]. Attaining the expression of OB-CAD-Fc will permit the generation of recombinant OB-CAD-Fc proteins containing various lengths of the extracellular domain—e.g., EC (1–4), EC (1–3), EC (1–2), and EC1—for the identification of the domain that mediates the homophilic adhesion. Delineating the minimal amino acid sequences required for OB-cadherin binding will allow the design of small molecules to inhibit the OB-cadherin-mediated adhesion. We expect that this improved expression method will also facilitate the expression of other membrane or secretory proteins for functional study.

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Fig. 1. Generation of 293FT cells expressing OB-CAD-Fc

(A) Schematic representation of OB-CAD-Fc protein, which consists of the five extracellular subdomains of OB-cadherin (EC 1 through 5) fused to human Fc(hFc). (B) Map of the pBMN-(OB-CAD-Fc)-I-GFP vector used to express OB-CAD-Fc and green fluorescent protein (GFP) in a bicistronic unit. IRES, internal ribosome entry site. (C) Distribution of forward-scatter signal (FSC-A) versus GFP expression by 293FT cells after infection with the virus that contained the pBMN-(OB-CAD-Fc)-I-GFP vector. The population of cells expressing GFP (P2), that represents 42.5% of the total cells, was sorted from the nonexpressing cells (P1) by fluorescence-activated cell-sorting (FACS) analysis. (D) Expression of GFP by 293FT/OB-

CAD-Fc cells after the FACS was checked by fluorescence microscopy (top) and FACS (bottom). After sorting, about 88% of the cells expressed GFP.

Fig. 2. Expression of OB-CAD-Fc from 293FT cells

(A) The presence of OB-CAD-Fc in the conditioned medium (Med) and in the cell lysate (Cell) of 293FT control or OB-CAD-Fc-expressing cells was determined by Western blotting (right). The arrow indicates the OB-CAD-Fc dimer band, and the arrowhead indicates the immunoglobulin G band. The protein loading of the Western blot was shown by Ponceau S staining of the membrane (left). (B) Western blots of OB-CAD-Fc under reducing (in the presence of β -mercaptoethanol, + β -ME) and nonreducing ($-\beta$ -ME) conditions, using an anti-OB-cadherin goat polyclonal antibody (left) and a mouse monoclonal antibody that recognizes the extracellular domain of OB-cadherin (right).

Fig. 3. Purification of OB-CAD-Fc

(A) The time course of OB-CAD-Fc expression in 293FT cells. The cell culture medium was refreshed on days 2 and 4 (arrows), and an aliquot of the conditioned medium was collected every day to examine the expression of OB-CAD-Fc in it. The conditioned medium from days 0, 2, 4, and 6 was also examined for the expression of OB-CAD-Fc under reducing conditions (labeled with + β -ME). (B) Elution profile from protein A-affinity chromatography. (C) Coomassie blue stained SDS-PAGE and corresponding western blot of the fractions eluted from protein A-affinity chromatography. FT indicates the flow through fraction of the affinity chromatography.

Fig. 4. OB-CAD-Fc-mediated adhesion activity

The wells of a 96-well plate were coated with various amounts of purified OB-CAD-Fc or Fc protein as indicated. L cells transduced with OB-cadherin (L-cell/OB-CAD) adhered to OB-CAD-Fc in a dose-dependent manner (●) but did not adhere to Fc (○). Control L cells, which do not express any cadherin, did not adhere to either OB-CAD-Fc (■) or to Fc (□). PC3 cells, which express high levels of OB-cadherin, bound to OB-CAD-Fc (●), but not to Fc (○), in a dose-dependent manner.

Table 1
Purification of OB-CAD-Fc in 293FT cells

Protein fraction	Total volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Specific activity (arbitrary units/ mg protein)*	Degree of purification (fold)	Yield (%)
Conditioned medium	95	0.04	3.80	6,670	1.0	100
Eluted fraction	5	0.17	0.85	26,700	4.0	89

* Determined by Western blot analysis.