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LC/MS in glycomics

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Adenovirus Vector-Mediated Gene Transfer into Stem Cells

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Abstract: Stem cells, including embryonic stem (ES) cells, mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs), are defined by their capacity for self-renewal and multilineage differentiation. Efficient gene transfer into stem cells is essential for the basic research in developmental biology and for therapeutic applications in gene-modified regenerative medicine. Adenovirus (Ad) vectors, based on Ad type 5, can efficiently and transiently introduce the exogenous gene into many cell types via the primary receptor, coxsackievirus, and adenovirus receptor (CAR). However, some kinds of stem cells, such as MSCs and HSCs, cannot be efficiently transduced with conventional Ad vectors based on Ad serotype 5 (Ad5), because of the lack of CAR expression. To overcome this problem, fiber-modified Ad vectors and an Ad vector based on another serotype of Ad have been developed. Here, we review the advances in the development of Ad vectors suitable for stem cells and discuss their application in basic biology and clinical medicine.

Keywords: Adenovirus; stem cell; gene therapy; regenerative medicine; review

Introduction

Adenovirus (Ad) is a nonenveloped virus containing an icosahedral protein capsid with a diameter of approximately 80 nm. At least 51 serotypes of human Ad have been identified and classified into six different subgroups (A–F), many of which are associated with respiratory, gastrointestinal, or ocular diseases. Of them, Ad serotype 5 (Ad5) and Ad serotype 2, both belonging to subgroup C, have been the most extensively studied for use as vectors in gene therapy applications. Ad capsids consist of three major protein components: the hexon, the penton base, and the fiber. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices (Figure 1A). The two components of the penton capsomer, the fiber and penton base, interact with distinct cell surface receptors during the entry of Ad into susceptible cells. Fiber proteins consist of three distinct domains: the tail, the shaft, and the knob. Each domain has distinct functions in host cell infection. The amino-terminal tail anchors the fiber to the Ad capsid through association with the penton base.[1] The shaft extends away from the virion surface and, in Ad5, is composed of 22 pseudorepeats of 15 amino acids in a triple-β-spiral conformation.[2] By extending the knob away from the virion, the shaft facilitates its interaction with the host

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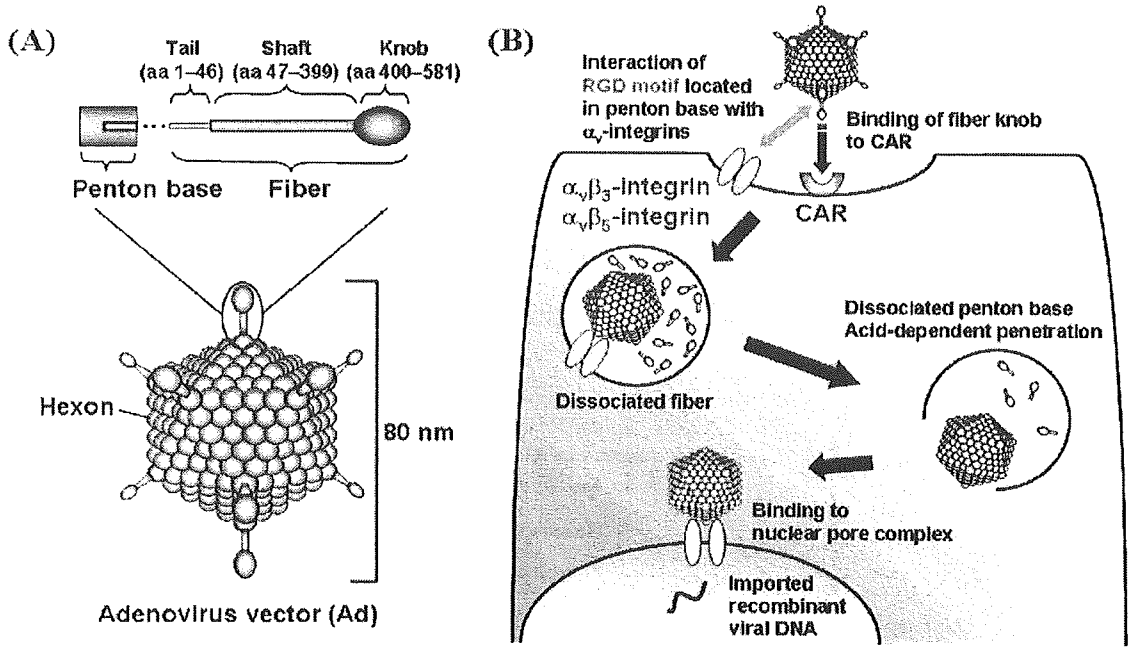

Figure 1. Structure and gene transduction pathway of the Ad vector. (A) The double-stranded virus genome is packaged within an icosahedral protein capsid. Hexon proteins comprise each geometrical face of the capsid, while penton bases associate with fiber proteins to form penton capsomer complexes at each of the 12 vertices. The fiber is composed of the tail, shaft, and knob domain. (B) The Ad vector binds to CAR following internalization in the cells and releases the viral DNA into the nuclei.

receptor.[1] The trimeric subunits of the carboxyl C-terminal knob domain are responsible for binding to the host's primary cellular receptor.[3,4]

Human Ad5 contains a linear, approximately 36 kb, double-stranded DNA genome encoding more than 70 gene products. The viral genome contains five early transcription units (E1A, E1B, E2, E3, and E4), two early delayed (intermediate) transcription units (pIX and IVa2), and five late units (L1–L5), which mostly encode structural proteins for the capsid and internal core. Inverted terminal repeats (ITRs) at the end of the viral genome function as replication origins. The E1A gene is the first transcription unit to be activated shortly after infection and is essential to the activation of other promoters and the replication of the viral genome. In the first-generation Ad vectors, the E1 (E1A and E1B) gene is deleted and the virus propagated in E1-transcomplementing cell lines, such as 293,[5] 911,[6] or PER.C6 cells.[7] The E3 region-encoded proteins modulate the host defense but are not required for viral replication in vitro; thus, the E3 region is often deleted to enlarge the packagable size limit for foreign genes. Since up to 3.2 and 3.1 kb of the E1 and E3 regions, respectively, can be deleted[8] and approximately 105% of the wild-type genome can be packaged into the virus without affecting the viral growth rate and titer,[9] E1/E3-deleted Ad vectors allow the packaging of approximately 8.1–8.2 kb of foreign genes.[8]

The coxsackievirus and adenovirus receptor (CAR), which is a broadly distributed type I membrane protein, has been identified as the primary receptor for Ad of subgroups A and C–F.[10–12] The entry of Ad5 into cells is initiated by the

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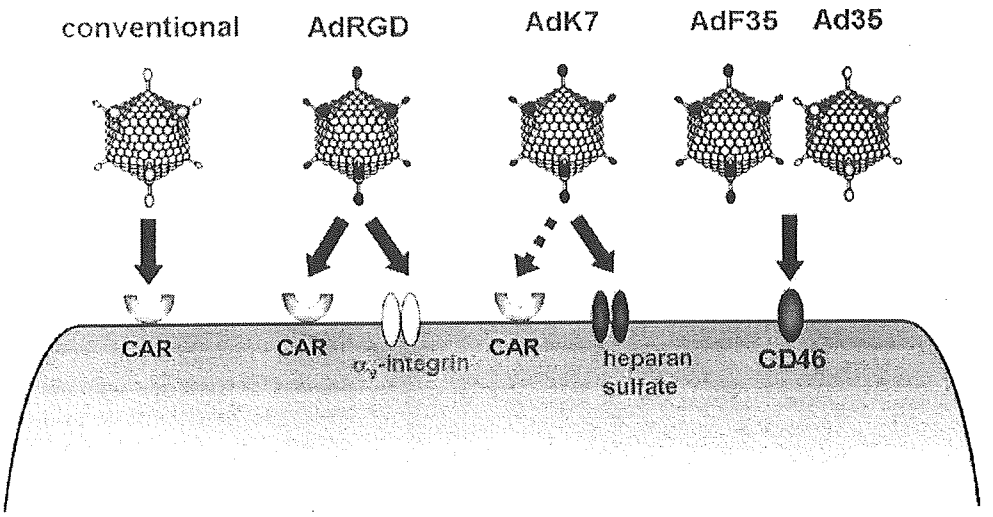

Figure 2. Characteristics of gene delivery by various types of Ad vectors. The conventional Ad vector infects via CAR. The AdRGD vector contains a RGD peptide motif in the HI loop of the fiber knob and infects via αv integrin as well as CAR. The AdK7 vector contains a polylysine peptide in the C-terminus of the fiber knob and infects via heparan sulfate as well as CAR. It is uncertain whether the AdK7 vector infects via CAR. The Ad35 and AdF35 vectors, which contain a fiber protein derived from the Ad5 fiber tail and the Ad35 fiber knob and shaft, infect via CD46.

attachment of fiber on the surface of the capsid to the CAR on the cell surface (Figure 2). The affinity of the RGD (Arg-Gly-Asp) peptide at the penton base of the Ad5 capsid for the cell surface molecules of the integrin family, such as $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_1$, aids in the internalization of Ad5 into the cell.[13−15] Furthermore, heparan sulfate glycosaminoglycans have also been reported to serve as primary attachment sites for Ad2 and Ad5.[16] The abundant expression of these receptors in various cells determines the wide tropism of Ad vectors. Internalized Ad reaches the endosomal pathway and avoids lysosomal degradation (Figure 1B). Inside the endosome, a stepwise disassembly program takes place, allowing the Ad to release its genome into the nucleus. During this process, the pH of the endosome decreases, leading to the release of the fiber from the virion and the dissociation of the penton base.[17] The resulting endosome rupture allows viral DNA to escape from inside the degraded capsid and to enter the nucleus (Figure 1B). During this process, the terminal protein plays a crucial role in translocating the Ad genome into the nucleus. This uncoating process of the Ad starts immediately after internalization and ends 40 min after infection with the translocation of the Ad into the nucleus. As early as 60 min after infection, the Ad begins to transcribe its genome in the host cell.[18]

Although Ad vectors mediate extremely high transduction efficiency, gene transfer with Ad vectors is less efficient in some kinds of cells, such as mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), dendritic cells, T cells, smooth muscle cells, skeletal muscle cells, and others because of the scarcity of CAR on their cell surfaces. Modification of the Ad fiber proteins has been used to successfully overcome this obstacle.[19,20] One is constructed by the addition of foreign peptides to the HI loop or C-terminus of the fiber knob of an Ad vector.[21−25] Enhanced gene transfer has been

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reported, on the basis of the use of mutant fiber proteins containing either an RGD peptide (AdRGD vector)[21−26] or a stretch of lysine residues [K7 (KKKKKKK) peptide] (AdK7 vector),[21,25,26] which target αv integrins or heparin sulfates on the cell surface, respectively (Figure 2). Altered vector tropism was reported with the substitution of the Ad5 fiber protein with that of Ad belonging to subgroup B, such as Ad types 3, 11, and 35.[27−31] These fiber-modified Ad vectors infect cells via CD46, CD80, and CD86, which have recently been identified as the cellular receptors of Ad belonging to subgroup B (Figure 2).[32−36] Mercier et al. described the creation of a chimeric Ad vector encoding the reovirus attachment protein σ1, which targets cells expressing junctional adhesion molecule 1.[37]

Several groups have developed an Ad vector from the entire Ad type 35 (Ad35) or Ad type 11 (Ad11) and have demonstrated that the Ad35 and Ad11 vectors exhibit higher transduction efficiencies into hematopoietic progenitor and dendritic cells compared with the conventional Ad5 vector (Figure 2).[38−43] As other approaches to changing the vector tropism, modification of the Ad vector with the antibodies, the fusion protein composed of CAR and the cell binding domain, cationic lipid, or macromolecules has been reported.[19,20] Here, we highlight the genetic manipulations of stem cells by the Ad vector and fiber-modified Ad vector for basic research and therapeutic usage. Recent advances in Ad vector-mediated gene transfer into stem cells, such as embryonic stem (ES) cells, mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs), will be discussed.

Gene Transfer into Stem Cells

Stem cells are defined as cells which possess the abilities of self-renewal and multilineage differentiation. Stem cells have been isolated from a wide variety of tissues, and in general, their differentiation potential may reflect the local environment. They lack tissue-specific characteristics but under the influence of appropriate signals can differentiate into specialized cells with a phenotype distinct from that of their precursor. Gene therapy applications that target stem

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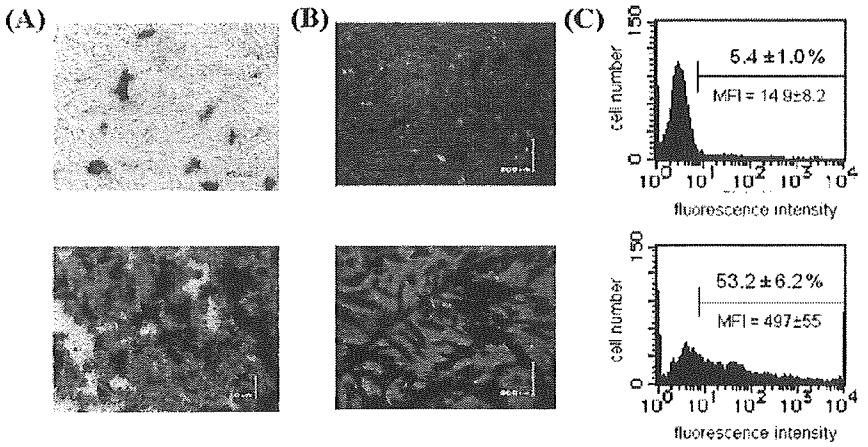

Figure 3. Improved transduction efficiency in the stem cells by the optimized Ad vectors. (A) mES cells were transduced with the LacZ-expressing conventional Ad5 vector containing the CMV promoter (top) or EF-1α promoter (bottom). (B) hMSCs were transduced with the LacZ-expressing Ad5 vector (top) or AdK7 vector (bottom). Both vectors have the CA promoter. (C) Human CD34+ cells were transduced with the GFP-expressing Ad5 vector (top) or Ad35 vector (bottom). Both vectors have the CMV promoter. MFI is the mean fluorescence intensity.

choice of a promoter is important for the efficient expression of exogenous genes in mES cells (Figure 3A). In the transient expression system using a cationic liposome−plasmid complex, the EF-1α (elongation factor 1α) and CA promoter (β-actin promoter/CMV enhancer) were shown to be highly active in mES cells while the CMV promoter was inactive.[62] More recently, we reported that the Ad vector containing the EF-1α or CA promoter has mediated the efficient expression of the reporter gene in mES cells, whereas the Ad vector containing the Rous sarcoma virus (RSV) or the CMV promoter has exhibited little expression.[63] Because CAR was highly expressed in mES cells but not in feeder cells,[63] the Ad vector could be a powerful tool for the genetic manipulation of mES cells when an appropriate promoter is used. To date, although we have no idea about the expression of CAR in hES cells, the Ad vector was reported to mediate the reporter gene expression in both mES cells and hES cells,[64] suggesting that hES cells may also express CAR on their cell surfaces.

As a result of the comparative analysis of mES cells transduced with various types of fiber-modified Ad vectors, the conventional Ad vector exhibited highly efficient and specific transduction, whereas the AdRGD and AdK7 vectors transduced mES cells and feeder cells (embryonic fibroblasts) to the same degree.[63] Therefore, the conventional Ad vector containing the EF-1α or CA promoter should be appropriate when only ES cells are transduced. In turn, the AdRGD or AdK7 vector is adequate when both ES cells and feeder cells are transduced.

The conventional Ad vector containing the EF-1α promoter was applied for the transduction of functional genes. It is well-known that the activation of signal transducer and activator of transcription 3 (STAT3) is essential for leukemia inhibitory factor (LIF)-mediated mES cell self-renewal, and the inhibition of LIF/STAT3 signaling leads to either apoptosis or differentiation.[65] It is also known that transcription factor Nanog maintains the pluripotency of mES cells in a manner that is independent of LIF/STAT3 signaling.[66,67] Ad vector-mediated STAT3F (STAT3 dominant-negative mutant) transduction strongly promoted mES cells to cell differentiation into three germ layers without any nonspecific toxicity.[63] The co-infection of the STAT3F-expressing Ad vector and the Nanog-expressing Ad vector showed that the differentiation suppressing ability of Nanog negated the differentiation promoting function of STAT3F and that mES cells maintained their undifferentiated state.[63] Thus, the differentiation of ES cells could be controlled by the transduction of differentiation-key regulator genes with the Ad vector. ES cells might differentiate into hematopoietic progenitor, pancreatic β cells, or neurons by the Ad vector-mediated introduction of HoxB4,[68,69] Pax4,[70] or nuclear receptor-related I,[71] respectively.

Gene Transfer into Mesenchymal Stem Cells. MSCs, which reside within the stromal compartment of bone

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cells offer great potential for the treatment of many kinds of diseases. Despite this promise, clinical success has been limited by poor rates of gene transfer and poor levels of gene expression. Therefore, an efficient gene delivery system needs to be developed for stem cell gene therapy.

Gene Transfer into Embryonic Stem Cells. ES cells are pluripotent cell lines derived from the inner cell mass of the developing blastocyst.[44−46] With the establishment of human ES (hES) cells, they have been used as a renewable source of transplantable tiss e-specific stem cells.[47−49] ES cells differentiate spontaneously in vitro in a random manner into a mixture of differentiated cells. The protocols for the differentiation of ES cells enriched for a specific lineage have been developed in both the mouse ES (mES)[50,51] cell and hES cell systems,[52,53] although the differentiated cells are still relatively heterogeneous. Therefore, further research is needed to allow controlled directed differentiation of ES cells into pure cultures of committed cells. One of the most powerful techniques for controlled differentiation is genetic manipulation. Electroporation methods,[54] retroviral vectors,[55,56] lentiviral vectors,[57−59] and a supertransfection method based on a replication system using the polyoma replication origin and large T antigen[60] have been used for exogenous gene expression in ES cells, although lentiviral vectors have been shown to be ineffective at expressing exogenous genes in mES cells, but not in hES cells.[57,59] In plasmid-based systems such as eletroporation and supertransfection methods, stable cell lines are generated by selection using a drug resistance gene. All these methods mediate long-term constitutive gene expression, although a long-term gene expression system such as that as described above may be problematic for use in therapeutic applications, because the gene is continuously expressed even after cell differentiation. There is thus a need for efficient vector systems for transient expression.

The Ad vector has been thought to be inappropriate for gene transfer into ES cells.[61] It has been reported that the retrovirus vector preferentially transduced ES cells, while the Ad vector containing the cytomegalovirus (CMV) promoter preferentially transduced embryonic fibroblasts as feeders in the ES culture.[61] However, it was found that the

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marrow, were first identified as bone-forming progenitor cells from rat marrow.[72] MSCs represent a very small fraction, 0.001−0.01% of the total population of nucleated cells in marrow.[73] They have the capacity to differentiate into cells of connective tissue lineages, including bone, fat, cartilage, and muscle. Recently, it has been reported that MSCs can differentiate into other lineages, such as neurons,[74] hepatocytes,[75] and insulin-producing cells.[76] Therefore, MSCs have attracted a great deal of interest because of their potential use in regenerative medicine and tissue engineering. To date, MSCs could be differentiated in vitro into proper lineages via a change in the culture conditions.[77] Another method for the in vitro differentiation is to genetically modify MSCs.[78,79] Although exogenous gene transfer into human MSCs (hMSCs) has been reported by using a conventional Ad vector, its transduction efficiency is quite low due to the scarcity of CAR.[80,81] Therefore, hMSCs have been transduced with high titers (more than 1000 infectious units/cell) of Ad vectors.[80,81] Fiber-modified Ad vectors have been applied for hMSCs to improve the transduction efficiency.[79,82,83] hMSCs infected with the AdRGD vector containing the BMP2 gene produced larger amounts of BMP2 than cells infected with the conventional Ad vector and efficiently differentiated into the osteogenic lineage.[82,83] Highly efficient transduction of hMSCs was achieved with tropism-modified Ad5 vectors carrying fiber shaft domains and knobs of different serotypes of Ad, such as Ad16, Ad35, or Ad50.[84] In a systematic comparison with various types of fiber-modified Ad vectors, the AdK7 vector is the most efficient for hMSCs and exhibited a 460-fold higher transduction efficiency than the conventional Ad vector.[79] The AdRGD vector or the Ad vector containing the Ad35 fiber (AdF35) exhibits a 16 or 130 times higher transduction efficiency, respectively, than the conventional Ad vector.[79] hMSCs are found to express CD46, which is the primary receptor for Ad35, but not CAR.[79] In conclusion, the AdK7 or AdF35 vector is the most appropriate for the transduction of hMSCs (Figure 3B).

Gene Transfer into Hematopoietic Stem Cells. Hematopoietic stem cells (HSCs) are capable of self-renewal and multilineage differentiation into all mature blood cells.[85] HSCs comprise only 0.01% of the whole bone marrow, the tissue in which they primarily reside.[86] Efficient transduction into HSCs would afford the opportunity to treat a number of hematopoietic disorders and would be a powerful tool for

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the study of the proliferation, differentiation, and trafficking of HSCs. Although the retroviral and lentiviral transduction of HSCs to achieve stable gene expression has been established,[87,88] stable expression is not always desirable. For example, stable expression of MDR1 gene results in HSC expansion but can cause leukemia upon transplantation to recipient mice.[89] As the Ad vector mediates the exogenous gene expression transiently, this vehicle can be safe for gene therapy. However, the application of conventional Ad vectors for the transduction into human CD34+ cells, which contain a population of HSCs, has been limited because CAR is not expressed at sufficient levels in human CD34+ cells.[90,91] It has been shown that Ad serotype 35 (Ad35), which belongs to subgroup B, is efficient at binding to human CD34+ cells and hematopoietic cell lines.[90,92] We showed that the Ad35 vector, which is composed from the whole Ad35, achieved higher levels of transduction efficiency in human bone marrow CD34+ cells than both conventional Ad5 vectors and AdF35 vectors.[39,93] The expression level of reporter genes in the CD34+ cells transduced with the Ad35 vector was 12−76 and 1.4−3 times higher than that in the cells transduced with the Ad5 and AdF35 vectors, respectively.[39] The transduction efficiency of the Ad35 vector was slightly higher than that of the AdF35 vector, although the reason remains unknown. CD46 is ubiquitously expressed in almost all human cells, including human cord blood CD34+ cells.[94] Therefore, human CD34+ cells would be considered to be a suitable target for the Ad35 vector (Figure 3C). As a result of the systematic comparison of promoters with Ad35 vectors, significantly higher transduction efficiencies were achieved with the EF-1α, CA, and CMV promoter/enhancer with the largest intron of CMV (intron A) (CMVi) promoters. In particular, the CA promoter was found to allow for the highest transduction efficiencies in both the whole human CD34+ cells and the immature subsets.[93] In mice, a population of mouse bone marrow highly enriched for HSC, called side population (SP) cells, has been reported to be transduced with the conventional Ad5 vector.[95] This suggests that pure mouse HSCs might express CAR on the cell surface. Further studies are needed to clarify this. The Ad vector-mediated transduction of hematopoietic regulator genes, such as HoxB4,[68,69] Bmi-1,[96] or SCL/Tal-1,[97] into HSCs may be effective for therapeutic use such as HSC expansion, although the Ad vector expressing HoxB4 was unsuccessful because of unexpected HSC differentiation due to its high transduction efficiency.[98]

Conclusions

We have reviewed recent advances in the development of improved Ad vectors for stem cells. Ad vectors have advantages over other viral vectors: the high transduction efficiency, the ease of vector preparation, and the transient expression ability. By the Ad vector-mediated introduction of a differentiation master regulator gene, we could control the differentiation of stem cells. These technical advances should greatly facilitate the analysis of gene function in the stem cells as well as the therapeutic applications of gene-modified stem cells.

Abbreviations Used

ES, embryonic stem; mES, mouse ES; MSCs, mesenchymal stem cells; HSCs, hematopoietic stem cells; Ad, adenovirus; CAR, coxsackievirus and adenovirus receptor; Ad5, Ad serotype 5; ITR, inverted terminal repeats; Ad35, Ad serotype 35; AdRGD vector, Ad vector containing the RGD

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peptide; Ad K7 vector, Ad vector containing a polylysine stretch; hES, human ES; STAT3, signal transducer and activator of transcription 3; LIF, leukemia inhibitory factor; STAT3F, dominant-negative mutant of STAT3; hMSCs, human MSCs; BMP2, bone morphogenetic protein 2; AdF35, Ad vector containing the Ad35 fiber.

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RESEARCH ARTICLE

Optimization of adenovirus serotype 35 vectors for efficient transduction in human hematopoietic progenitors: comparison of promoter activities

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Adenoviral gene transfer to hematopoietic stem cells (HSCs)/progenitors would provide a new approach to the treatment of hematopoietic diseases and study of the hematopoietic system. We have previously reported that an adenovirus (Ad) vector composed of whole Ad serotype 35 (Ad35), which belongs to subgroup B, shows efficient gene transfer into human bone marrow CD34+ cells. However, Ad35 vector-mediated transduction into human HSCs/progenitors has not yet been fully optimized. In the present study, we have systematically examined promoter activity in the context of Ad35 vectors in human bone marrow CD34+ cells and primitive CD34+ subsets to optimize the transduction efficiency in human hematopoietic stem/progenitor cells. In the first of the transduction experiments, the improved in vitro ligation method was applied to Ad35 vector construction to allow for simple and efficient production of an E1/E3-deleted Ad35 vector. Using this method, we constructed a series of Ad35 vectors encoding the enhanced green fluorescence protein (GFP) under the control of a variety of strong viral and cellular promoters. Of the six types of promoters tested, significantly higher transduction efficiencies were achieved with the human elongation factor 1α promoter (EF1α promoter), the human cytomegalovirus (CMV) immediate-early 1 gene enhancer/β-actin promoter with β-actin intron (CA promoter), and the CMV promoter/enhancer with the largest intron of CMV (intron A) (CMVi promoter) in the human CD34+ cells and the immature subsets (CD34+CD38low/− and CD34+AC133+ subsets). In particular, the CA promoter was found to allow for the highest transduction efficiencies in both the whole human CD34+ cells and the immature hematopoietic subsets. Furthermore, the CA promoter-mediated GFP-expressing cells differentiated into progenitor cells of all lineages. These results indicate the construction of an optimized Ad35 vector backbone for efficient transduction into HSCs/progenitors.
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Keywords: *adenovirus serotype 35 vector; CD34+ cells; CA promoter; hematopoietic stem cells/progenitors*

Introduction

Hematopoietic stem cells (HSCs) have the potential for self-renewal and multilineage differentiation into all mature blood cells. Hence, efficient transduction into HSCs would afford the opportunity to treat a number of diseases that result from abnormal blood cell function, and would be a powerful tool for study of the regulation of proliferation, differentiation, and trafficking of HSCs. For gene transfer into HSCs, moloney-derived retrovirus vectors and lentivirus vectors are often used, although the transduction efficiencies of the retrovirus vectors are disappointingly low in immature HSC/progenitors, probably due to the quiescent state of HSCs and the lack of suitable receptors for vector binding.[1,2] Lentivirus vectors have recently shown promise,[3,4] but their safety remains to be established.

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Among the various types of vectors, adenovirus (Ad) vectors have been widely used for delivery of foreign genes in not only experimental studies but also clinical trials. Advantages which make Ad vectors an attractive vehicle for gene transfer include the ability to easily prepare high-titer stocks of purified vectors, efficient escape from the endosome, and the ability to transport their DNA genome into the nucleus, allowing for efficient transduction in quiescent cells. However, the utility of commonly used Ad vectors, which are based on Ad serotype 2 (Ad2) or Ad serotype 5 (Ad5) belonging to subgroup C, for transduction into human CD34+ cells has been limited[5,6] because a primary receptor for Ad2 and Ad5, coxsackievirus and adenovirus receptor (CAR), and second receptors, $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins, are not expressed at sufficient levels in human CD34+ cells.[5,7,8] In contrast, it has been shown that Ad serotype 35 (Ad35), which belongs to subgroup B, is efficient at binding to human CD34+ cells and hematopoietic cell lines.[5,9] We have therefore developed a novel Ad vector, Ad35 vector, which is composed of whole Ad35, and have demonstrated that Ad35 vectors achieve higher levels of transduction efficiency without significant

toxicity in human bone marrow CD34+ cells than both conventional Ad5 vectors and chimeric Ad5F35 vectors, which are fiber-substituted Ad5 vectors containing Ad35 fiber proteins.[10] Ad35 recognizes CD46 (membrane cofactor protein) as a cellular receptor,[11,12] and CD46 is ubiquitously expressed in almost all human cells except for erythrocytes,[13,14] including human cord blood CD34+ cells.[15] Therefore, human CD34+ cells would be considered to be a suitable target for Ad35 vectors.

In addition to receptor expression in target cells, the choice of promoters that drive expression of introduced genes is another crucial determinant for transduction efficiency. Optimization of the promoter leads not only to increased transgene expression but also to decreased vector dose and side effects. A variety of promoters have been used for transduction into human CD34+ cells, including the phosphoglycerate kinase 1 promoter (PGK promoter),[16] the human cytomegalovirus immediate-early region promoter/enhancer (CMV promoter),[17,18] and the CMV immediate-early enhancer/the chicken β-actin promoter with the β-actin intron sequence (CA promoter).[8] However, few studies have simultaneously compared the relative strength of various types of promoters in human CD34+ cells,[19] and information regarding promoter activities in human CD34+ cells is controversial. In addition, the promoter activities have not been fully evaluated in immature CD34+ subpopulations. It is well known that human CD34+ cells are morphologically and functionally heterogeneous and that HSCs/progenitors constitute only a fraction of all CD34+ cells. It is of great importance to evaluate the transduction efficiencies in immature hematopoietic subpopulations.

In the present study, we first applied the improved *in vitro* ligation method developed by Mizuguchi and Kay[20,21] to Ad35 vector construction to facilitate the generation of Ad35 vectors. Second, promoter activities in the context of Ad35 vectors were systematically evaluated in whole human bone marrow CD34+ cells and immature CD34+ subpopulations (CD34+CD38low/− and CD34+AC133+ subsets) to optimize Ad35 vector-mediated transduction into human hematopoietic stem/progenitors. Finally, the proliferative and differentiation potential of the Ad35 vector-mediated transduced CD34+ cells was examined by a colony-forming assay.

Results

Construction of E1/E3-deleted Ad35 vectors by the improved in vitro ligation method

To construct E1/E3-deleted Ad35 vectors simply and efficiently, the improved *in vitro* ligation method was applied to the construction of Ad35 vectors. This method, which was developed by Mizuguchi and Kay,[20,21] is a simple and efficient method by which conventional Ad5 vectors can be constructed. First, all of the E1a region and most of the E1b region were deleted to make the Ad35 vectors replication-incompetent. Then, the three unique restriction sites (I-*Ceu*I, *Swa*I, and PI-*Sce*I) were introduced into the E1 deletion site to efficiently insert foreign genes into the E1 deletion site of the vector plasmid by a single *in vitro* ligation. The I-*Ceu*I and PI-*Sce*I sites were used for insertion of foreign genes, while the *Swa*I site was used to reduce the generation of parental, nonrecombinant plasmid. Next, to increase the packaging capacity of the Ad35 vector genome, most of the E3a and E3b regions were deleted. The resulting vector plasmids, pAdMS2, pAdMS3, and pAdMS4, contain the complete Ad35 genome minus the E1 (pAdMS2) or E3 region (pAdMS3) or E1/E3 region (pAdMS4) (Figure 1). Approximately 3 and 1.9 kb of the E1 and E3 regions, respectively, were deleted in pAdMS4.

The Ad35 vector plasmids containing a green fluorescence protein (GFP) gene under the control of a variety of promoters were produced by *in vitro* ligation of I-*Ceu*I/PI-*Sce*I-digested pAdMS4 and the shuttle plasmids containing a GFP expression cassette. SbfI-linearized Ad35 vector plasmids were transfected into VK10-9 cells (293 cells expressing Ad5 E4 proteins as well as E1 proteins),[22] and the cells were cultured for 10–14 days to produce recombinant Ad35 vectors, followed by the routine method for Ad5 vector preparation. VK10-9 cells can support the replication of Ad35 vectors; however, Ad35 vectors can not grow on 293 cells, as described previously.[10] The final yields of the Ad35 vectors were equivalent to those described previously.[10,23]

Relationship between CD46 expression and transduction efficiency in human bone marrow CD34+ cells

Recently, the complement regulatory protein CD46 has been identified as a cellular receptor for Ad subgroup B.[11,12] To study the role of CD46 in Ad35 vector-mediated transduction into human bone marrow CD34+ cells, we evaluated the relationship between CD46 expression levels and the transduction efficiencies of Ad35 vector containing the CMV promoter. The CMV promoter is generally regarded as one of the most powerful

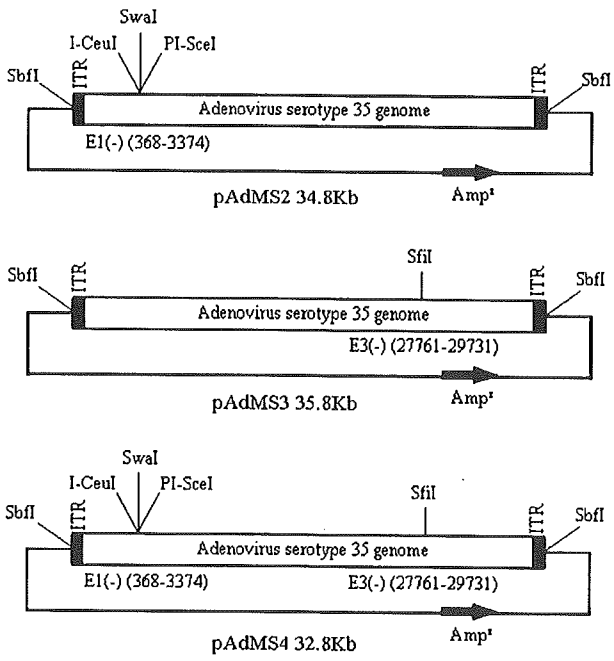

Figure 1 Structure of the vector plasmids pAdMS2, -3, and -4 for construction of Ad35 vectors by the improved in vitro ligation method.

promoters[24] and is widely used in transduction experiments. As shown in Figure 2a, almost all the bone marrow CD34$^+$ cells expressed high levels of CD46, similar to the cord blood CD34$^+$ cells.[15] Significant amounts of CD46 were detected in all the CD34$^+$ cells from three different donors (data not shown). In the transduction experiments using Ad35 vector containing the CMV promoter, the percentage of GFP-positive cells was proportional to the vector concentration at the lower vector doses of 1000–6000 VP/cell (Figure 2b). However, despite the high levels of CD46 expression, the transduction efficiencies of the Ad35 vector did not correlate with the vector concentrations at the higher vector dose reaching a plateau at more than 12 000 VP/cell. There was no correlation between the levels of CD46 expression and the GFP expression levels (Figure 2c). These data indicate that factors other than the CD46 expression levels also determine the transduction efficiencies of Ad35 vectors in human bone marrow CD34$^+$ cells.

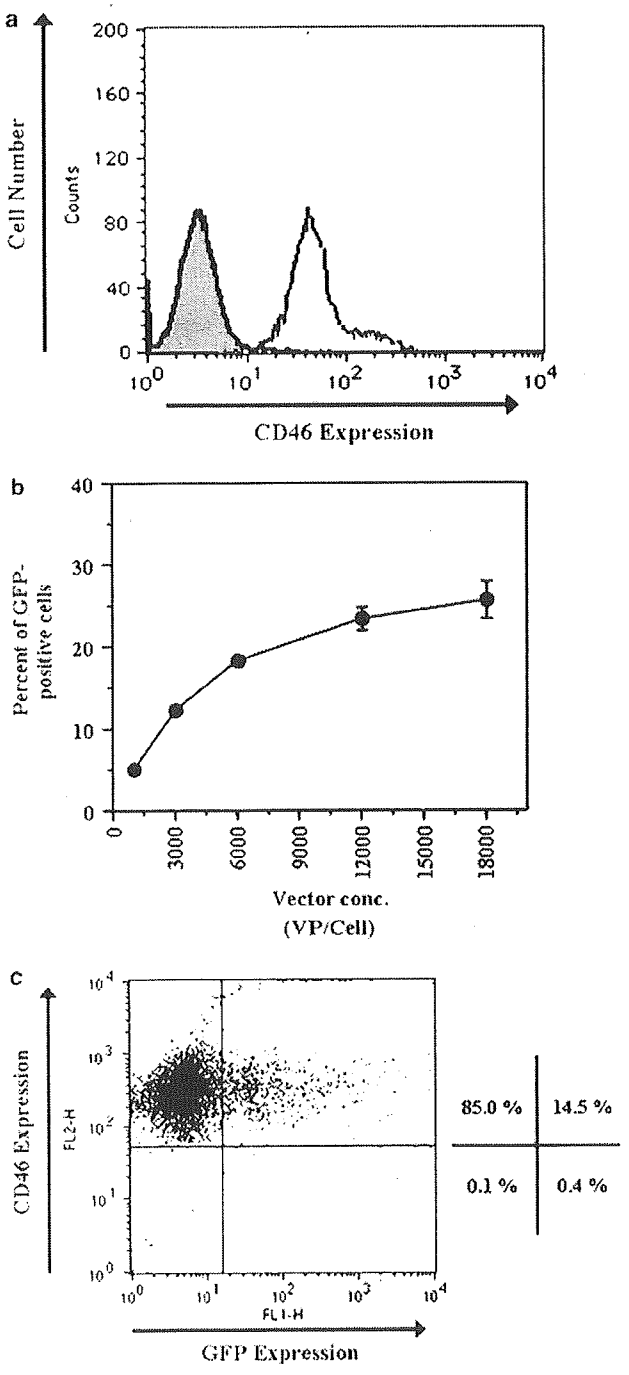

Figure 2 (a) CD46 expression in human bone marrow CD34$^+$ cells. The cells were incubated with FITC-conjugated anti-CD46 antibody. As a negative control, the cells were incubated with an irrelevant antibody (shaded histogram). Similar levels of CD46 were found in the cells from three different donors. (b) Dose–response of the percentage of GFP-positive cells following transduction with Ad35 vector containing a CMV promoter-driven GFP expression cassette. Human bone marrow CD34$^+$ cells were transduced with the Ad35 vector at the indicated vector concentrations for 6 h, washed, and resuspended in medium. After 48 h later, GFP expression was measured by flow cytometry. All data represent the means ± s.d. of three experiments. (c) Relationship between the CD46 expression level on human bone marrow CD34$^+$ cells and GFP expression levels following Ad35 vector transduction. The cells were transduced with the Ad35 vector containing the CMV promoter at 6000 VP/cell for 6 h, washed and resuspended in the medium. After 48 h of incubation and washing, the transduced cells were incubated with an anti-CD46 antibody. The cells were then washed, resuspended, and incubated with PE-conjugated second antibody. The percentage of stained cells found in each quadrant is indicated. Data shown are from one representative experiment of the three performed.

Transduction with Ad35 vectors containing various types of promoters in human bone marrow CD34$^+$ cells

The refractoriness of the human CD34$^+$ cells to Ad35 vector-mediated transduction might be due to promoters that drive expression of a foreign gene. To examine the promoter activities in the CD34$^+$ cells, we investigated the transduction efficiencies of the Ad35 vectors containing various types of promoters in the CD34$^+$ cells at 6000 VP/cell. The following promoters were tested: the CMV promoter, the EF1α promoter, the CA promoter, the mouse PGK promoter, the murine stem cell virus (MSCV) long terminal repeat (LTR) promoter (MSCV promoter), and the CMV promoter/enhancer containing the largest intron of CMV (intron A) (CMVi promoter). Of the six types of promoters, the CA, EF1α, and CMVi promoters were found to allow higher levels of GFP expression than the CMV, PGK, and MSCV promoters (Figure 3). In particular, the highest percentage of GFP-positive cells was obtained with the CA promoter (53.8%). The relative promoter strength in terms of percentage of GFP-positive cells was the CA (53.8%) > CMVi (44.0%) > EF1α (40.9%) > PGK (23.7%) > CMV (20.3%) > MSCV (10.5%). The mean fluorescence intensity (MFI) was also more than 3.5-fold higher with the CA and CMVi promoters than with the other types of promoters. Continuous incubation of the CD34$^+$ cells with Ad35 vector containing the CA promoter at 6000 VP/cell for 48 h led to an increase in GFP-positive cells up to 67% (data not shown). These data indicate that the transduction efficiencies in human bone marrow CD34$^+$ cells are largely dependent on the promoter and that the CA promoter is the most active in CD34$^+$ cells among the six types of promoters examined in the present study.

Next, to determine whether the Ad35 vectors had infected the CD34$^+$ cells that did not express GFP, the

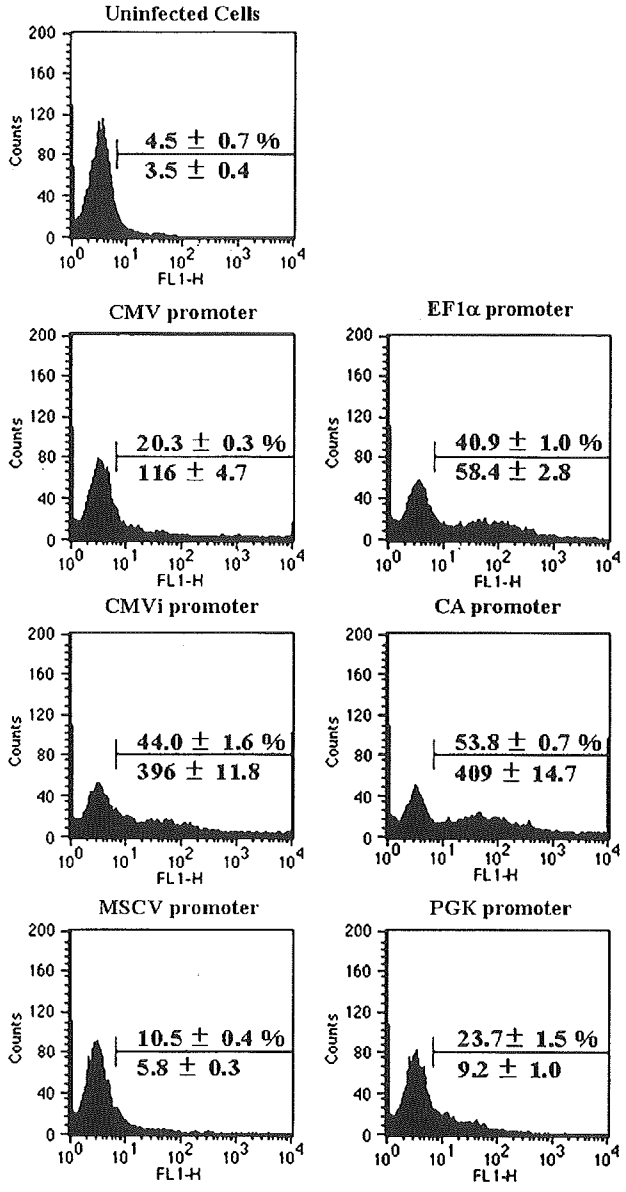

Figure 3 Comparison of promoter activities in human bone marrow CD34⁺ cells transduced with Ad35 vectors. The results are shown as a percentage of GFP-positive cells (upper) and the MFI (lower) in the panel. The CD34⁺ cells were transduced with Ad35 vectors at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP expression was measured by flow cytometry. All data represent the means ± s.d. of three experiments.

amounts of intracellular vector genomes in the GFP-positive and -negative fractions were measured by real-time PCR. As CD34⁺ cells are functionally and morphologically heterogeneous, the promoters may not be active in all CD34⁺ cells. Real-time PCR analysis demonstrated that approximately 300 copies of the Ad35 vector genomes per β-actin copy were detected in both GFP-positive and -negative fractions following transduction with the Ad35 vectors containing any type of promoter (Figure 4). These results suggest that Ad35 vectors would infect all CD34⁺ cells, probably via CD46; however, not all infected cells express GFP.

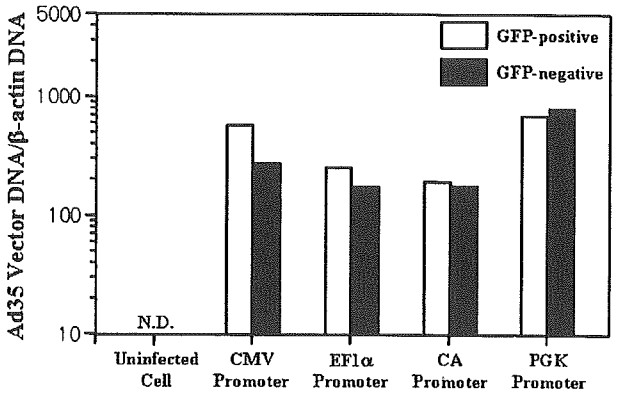

Figure 4 Ad35 vector copy numbers in GFP-positive and -negative cells following Ad35 vector transduction into human bone marrow CD34⁺ cells. The CD34⁺ cells were transduced with Ad35 vectors at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP-positive and -negative cells were sorted and the total DNA was extracted from the cells. The copy numbers of Ad35 vectors and β-actin were analyzed by Taqman PCR. All data represent the means of two independent experiments. ND, not detected (under the limit of detection).

Transduction in immature subpopulations of human bone marrow CD34⁺ cells

Next, to examine the promoter activities in the primitive hematopoietic subpopulations among the CD34⁺ cells, $CD34^+CD38^{low/-}$ cells and $CD34^+AC133^+$ cells were transduced with the Ad35 vectors. $CD34^+CD38^{low/-}$ and $CD34^+AC133^+$ cells are known to be the more primitive subsets among the CD34⁺ cells.[25–27] Transduction experiments demonstrated that the CA, EF1α, and CMVi promoters were clearly superior in $CD34^+CD38^{low/-}$ subsets compared with the CMV, MSCV, and PGK promoters (Figure 5). Similar results were obtained for $CD34^+AC133^+$ subsets (Figure 6). Among these three promoters, use of the CA promoters resulted in the highest transgene expression in both $CD34^+CD38^{low/-}$ and $CD34^+AC133^+$ subsets (57% GFP-positive for $CD34^+CD38^{low/-}$ subsets, 51% GFP-positive for $CD34^+AC133^+$ subsets). These data indicate that the CA, EF1α, and CMVi promoters mediate the higher transduction efficiencies and that the CA promoter is the most efficient in these immature CD34⁺ subpopulations.

CA promoter activity in the colony-forming hematopoietic progenitors

To evaluate the gene expression potential of the CA promoter in colony-forming hematopoietic progenitors, the transduced cells were sorted into GFP-positive and -negative cells following transduction with the Ad35 vector containing the CA promoter, and colony-forming assays were then performed for the sorted GFP-positive and -negative cells. Values indicating the colony-forming unit (CFU) content of the sorted cells 14 days after plating are shown in Table 1. Comparing the total number of colonies derived from the GFP-positive cells with those from the uninfected cells and the GFP-negative cells, it appears that the GFP-positive cells formed almost the same total numbers of colonies as the uninfected cells and the GFP-negative cells. CFU-granulocyte-macrophage (CFU-GM) colonies were grown without significant reduction from the CA promoter-mediated GFP-positive cells, compared with

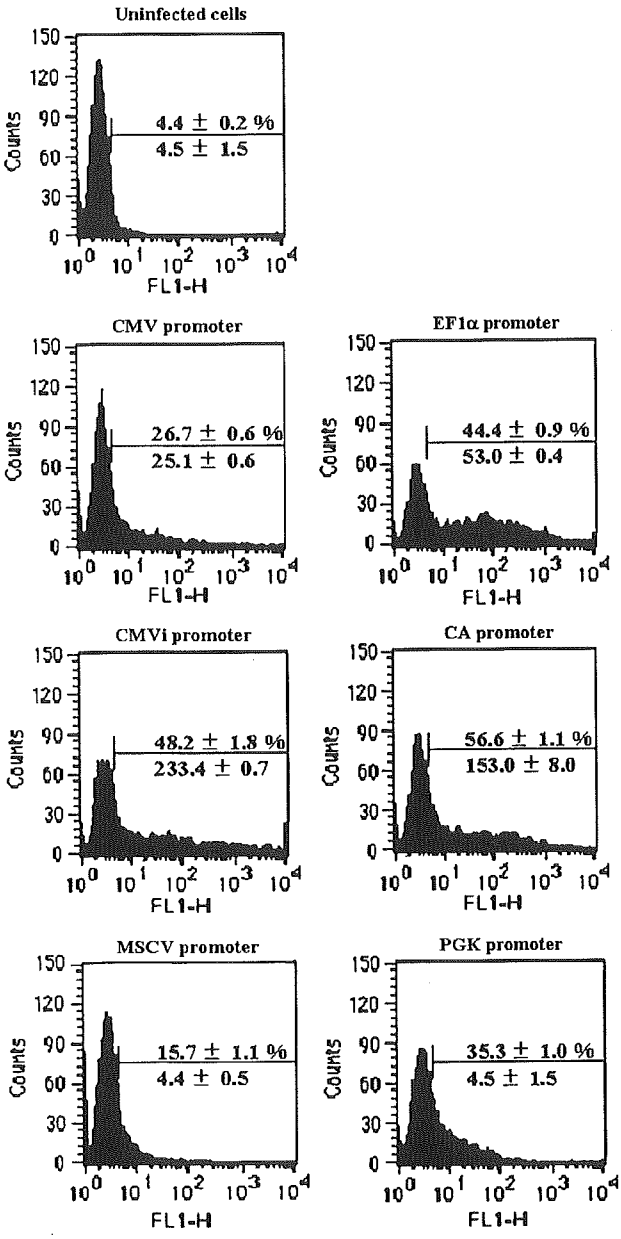

Figure 5 Comparison of promoter activities in the CD34⁺CD38$^{low/-}$ subsets transduced with Ad35 vectors. Results are shown as the percentage of GFP-positive cells (upper) and the MFI (lower) in the panel. The CD34⁺CD38$^{low/-}$ subsets were transduced at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP expression was measured by flow cytometry. All data represent the means±s.d. of two experiments.

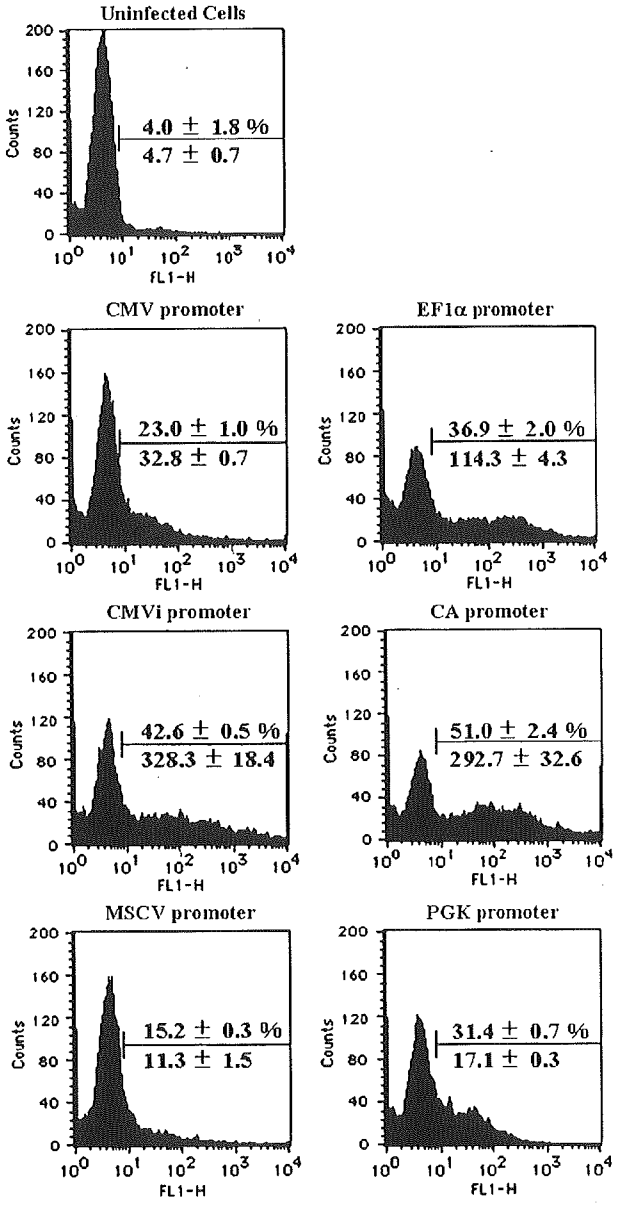

Figure 6 Comparison of promoter activities in human bone marrow CD34⁺AC133⁺ subsets transduced with Ad35 vectors. Results are shown as the percentage of GFP-positive cells (upper) and the MFI (lower) in the panel. The CD34⁺AC133⁺ subsets were transduced at 6000 VP/cell for 6 h, washed, and resuspended in medium. After 48 h, GFP expression was measured by flow cytometry. All data represent the means±s.d. of two experiments.

the GFP-negative cells, although the growth of burst-forming units-erythroid (BFU-E) colonies was slightly impaired in the GFP-positive cells. CFU-granulocyte erythrocyte monocyte macrophage (CFU-Mix) colonies, which are derived from the most primitive hematopoietic progenitors, were also found in GFP-positive cells. These data suggest that the CA promoter would be significantly active in immature colony-forming progenitors. However, the size of colonies from both GFP-positive and -negative cells appeared to be smaller than that in uninfected cells (data not shown), suggesting that exposure to Ad35 vectors slightly impairs the growth of colony-forming bone marrow hematopoietic progenitors under these conditions.

Discussion

The choice of a promoter to drive transgene expression is important in gene transfer experiments. Currently, a few systematic examinations of promoter activities have been carried out in gene transfer experiments *in vivo*[28–30] and

Table 1 Numbers of colonies derived from GFP-positive and -negative cells following transduction with the Ad35 vector containing the CA promoter in human CD34$^+$ cells

CD34$^+$ cells	*Total*	*BFU-E*	*CFU-GM*	*CFU-Mix*
Sample 1				
Uninfected cells	222.8±25.5	72±11.8	150.3±14.2	0.5±0.6
GFP-positive	193.5±29 (86.9%)	61±9.9 (84.7%)	131.5±17.7 (87.5%)	1±1.4
GFP-negative	180.5±13.4 (81%)	24.5±0.7 (34%)	155.5±13.4 (103.5%)	0.5±0.7
Sample 2				
Uninfected cells	124.8±13.5	44.5±6.1	78.8±9.5	1.5±0.6
GFP-positive	115±11.3 (92%)	29±5.7 (65.2%)	85.5±6.4 (108.5%)	0.5±0.7
GFP-negative	158±19.8 (127%)	26±0 (58.4%)	130.5±20.5 (165.6%)	1.5±0.7

The data represent the mean number of colonies±s.d. from duplicate cultures and the percentage of number of colonies/uninfected cells.

in vitro;[19,29,31] however, information regarding the promoter activities in HSCs is limited and controversial. The aim of this study was to identify an Ad35 vector platform for efficient transgene expression in human HSCs by optimizing a promoter that directs transgene expression. For this purpose, we constructed a series of Ad35 vectors in which GFP expression was driven by a variety of promoters and compared the levels of GFP expression in human bone marrow CD34$^+$ cells and immature CD34$^+$ subsets.

In the present study, we examined the following promoter activities in human bone marrow CD34$^+$ cells: the CMV, EF1α, CMVi, CA, MSCV, and PGK promoters, which are widely used in transduction experiments. Comparison of the six types of promoters showed that a significant increase in GFP-positive cells was obtained with the EF1α, CMVi, and CA promoters. Among these three promoters, the CA promoter was the most efficient at transducing human bone marrow CD34$^+$ cells (Figure 3) and immature CD34$^+$ subsets (CD34$^+$CD38$^{low/-}$ and CD34$^+$AC133$^+$ subsets) (Figures 5 and 6) under the condition employed. Furthermore, the CA promoter-mediated GFP-positive cells formed nearly the same numbers of colonies as uninfected cells in the colony assay (Table 1). The powerful activity of the CA promoter has been demonstrated in important target cells for gene therapy, including dendritic cells,[32] lymphocytes,[32] and hepatocytes.[33] In addition, earlier work with the CA promoter noted heightened transgene expression in immature cells. Okabe *et al*[34] have demonstrated that the CA promoter functions in the embryos of transgenic mice. Efficient transgene expression has also been achieved with the CA promoter in murine embryonic stem cells.[35,36] The data described above indicate that the CA promoter would be the promoter of choice for high levels of transgene expression in immature cells, including HSCs/progenitors.

The CMV promoter is one of the strongest promoters and is currently in wide use for transient gene expression experiments. However, the CMV promoter did not mediate high levels of GFP expression in the CD34$^+$ cells (Figure 2b), immature subsets (Figures 5 and 6), and colony-forming CD34$^+$ progenitors (data not shown). Several groups have demonstrated that the CMV promoter does not allow for high transduction efficiencies in human CD34$^+$ cells[3,19] and murine embryonic stem cells,[36,37] suggesting that the CMV promoter might not be appropriate for immature cells. However, the inclusion of intron A into the CMV promoter (CMVi promoter) largely increases GFP expression in the CD34$^+$ cells as well as immature CD34$^+$ subsets. The β-actin intron is also included in the CA promoter. These data suggest that an intron may be a key element for efficient transgene expression in human CD34$^+$ cells, although a detailed mechanism for enhancement of transgene expression by intron A in CD34$^+$ cells has not yet been elucidated.

Recently, the human membrane cofactor protein CD46 has been shown to be a cellular receptor for subgroup B Ad, including Ad35.[11,12] CD46 is a single-chain type I transmembrane glycoprotein that is expressed in almost all human cells.[13,14] The ubiquitous expression of CD46 leads to a broad tropism of Ad35 vectors toward human cultured cells compared with Ad5 vectors.[23] Expression levels of CD46 on the cells appear to correlate with the affinity of Ad35 for the cells, which is similar to the relationship between CAR expression levels on the cells and the transduction efficiencies of Ad5 vectors, as reported below. Segerman *et al*[9] have demonstrated almost 100% binding of Ad35 to human hematopoietic cell lines, Jurkat, K562, and HL-60 cells, which express sufficient levels of CD46.[38,39] The transduction efficiencies of the chimeric Ad5F35 vector, which is an Ad5-based vector containing an Ad35 fiber shaft and knob, have been found to increase progressively with CD46 expression density on a panel of CHO cells stably expressing CD46.[40] Shayakhmetov *et al*[5] have reported the efficient attachment and internalization of Ad35 in human bone marrow CD34$^+$ cells, which express high level of CD46 (Figure 2a). In this study, however, the transduction efficiencies in the CD34$^+$ cells unexpectedly did not increase proportionally with the increased dose of Ad35 vectors, despite the high expression levels of CD46 (Figure 2b). Even the CA promoter, which was the most efficient in this study, did not mediate more than 70% of GFP-positive cells at the increased dose (data not shown). The limitation of the transduction efficiencies in the CD34$^+$ cells is likely due to the promoter activities that direct transgene expression. We have demonstrated that similar amounts of Ad35 vector genome could be detected in both GFP-positive and -negative cells (Figure 4). These results suggest that Ad35 vectors would interact with all of the CD34$^+$ cells via CD46; however, the promoters examined here do not function in all infected CD34$^+$ cells. It is well known that human CD34$^+$ cells are heterogeneous. With the GFP-negative cells

that were infected by the Ad35 vectors, there may not be sufficient enough levels of the transcriptional factors for promoter function. Transduction with Ad35 vector containing a more suitable promoter might result in much higher transduction efficiencies in human CD34$^+$ cells. A similar phenomenon was observed in the study by Shayakhmetov *et al*,[5] in which human CD34$^+$ cells were transduced with the chimeric Ad5F35 vector. The genome of the chimeric Ad5 vector was detected in both GFP-positive and -negative cells.

Segerman *et al*[41] demonstrated that there are two different receptors for Ad35 in human cells. One is CD46 and another receptor is currently unidentified. It remains to be clarified whether human CD34$^+$ cells express the unidentified receptor for Ad35 and whether the unknown receptor plays an important role on Ad35 vector-mediated transduction in the CD34$^+$ cells.

A number of studies have evaluated the transduction efficiencies in CD34$^+$CD38$^{low/-}$ cells by staining the transduced CD34$^+$ cells with anti-CD38 antibody just before flow-cytometric analysis, not before transduction.[6,42,43] However, in this study, sorted CD34$^+$CD38$^{low/-}$ and CD34$^+$AC133$^+$ subpopulations were transduced with Ad35 vectors to evaluate the transduction efficiencies in these immature CD34$^+$ subsets because the expression levels of CD38 in the CD34$^+$ cells would decrease during culture, irrespective of the mature/immature state. Dorrell *et al*[44] and Donaldson *et al*[45] have reported that a dramatic increase in CD34$^+$CD38$^-$ cell frequency occurred during culture; however, these cells lost the potential to repopulate in the NOD/SCID mouse and to form colonies in the colony-forming assay, respectively. We confirmed that the expression levels of CD38 and AC133 decreased after a 2-day culture (data not shown). As such, the CD34$^+$ cells might have to be sorted before transduction to truly evaluate the transduction efficiencies in primitive hematopoietic subsets. In addition, because the CD34$^+$ cells may differentiate during culture and Ad35 vectors may infect differentiated cells efficiently, Ad35 vectors in the present study were incubated with the CD34$^+$ cells for 6 h and the cells were then washed to remove Ad35 vectors. More efficient transduction with Ad35 vectors was achieved when the CD34$^+$ cells were continuously cultured with Ad35 vectors for 48 h (data not shown), as reported previously.[10]

Various methods have been established for feasible generation of Ad vectors.[46] Among these methods, the improved *in vitro* ligation method, which was developed by Mizuguchi and Kay,[20,21] is a simple and efficient method based on plasmid construction. To efficiently construct a series of Ad35 vectors, an improved *in vitro* ligation system was applied to construction of replication-incompetent E1/E3-deleted Ad35 vectors. We have previously constructed Ad35 vector plasmids by gel purification of DNA fragments and two-step *in vitro* ligation.[10] Reddy *et al*[47] have also constructed Ad35 vector plasmids by several steps of *in vitro* ligation of DNA fragments. Vogels *et al*[48] and Gao *et al*[49] have reported construction of recombinant Ad35 vectors by homologous recombination in 293-derived cell lines and PER.C6 cells stably expressing Ad35 E1B 55K, respectively. All of these methods described above are time-consuming and inefficient. Using the improved *in vitro* ligation method, we more rapidly obtained yields of Ad35 vectors similar to those reported previously.[10] In addition, based on the sequence information of the Ad35 E1 and E3 regions, the E1 deletion size has been increased and most of the E3 region has been deleted in pAdMS4, leading to an increase in the packaging capacity. The increase in the deletion size in the E1 and E3 regions did not reduce the transduction efficiencies of Ad35 vectors (data not shown). Further deletions in the E1 and E3 coding regions must make it possible to insert larger foreign genes into the Ad35 vectors.

As the Ad genome does not integrate into the host genome, transgene expression via Ad vectors can occurr transiently, which is suitable for *ex vivo* manipulation of HSCs and the study of gene functions. This property also results in a low risk of insertion mutagenesis, but stable transgene expression is not allowed. To address this limitation, several groups have developed hybrid Ad vectors such as Ad/adeno-associated virus vectors, which can integrate viral genome into the host genome.[50–52] The Ad35 vector would be a promising framework for the development of these improved vectors.

In summary, we have demonstrated that the EF1α, CMV promoter containing intron A, and the hybrid CA promoter is superior at transducing human bone marrow CD34$^+$ cells in the context of Ad35 vector. In particular, the CA promoter functions most efficiently in CD34$^+$ cells and immature CD34$^+$ subsets. The results of our study provide valuable information regarding gene transfer into HSCs.

Materials and methods

Plasmids

Vector plasmids pAdMS2, -3, and -4 were constructed as follows. The *Sbf*I/*Pst*I fragment of pFS2-Ad35-7,[10] which has the left end of the Ad35 genome (bp 1–367 and 2917–3670) with an E1 deletion, was ligated with the *Sbf*I site of pFS2,[10] resulting in pFS2-Ad35-9. (The end of the *Sbf*I site is compatible with a *Pst*I site.) pFS2-Ad35-9 was cut by *Pac*I and *Bam*HI, and ligated with oligonucleotides 1 (5′-TATAACTATAACGGTCCTAAGGTAGCGA*ATTTAAA* **TATCTATGTCGGGTGCGGAGAAAGAGGTAATGAA ATGGCA**-3′) and 2 (5′-GATC**TGCCATTTCATTACCT CTTTCTCCGCACCCGACATAGAT***ATTTAAATT*CGCTA CCTTAGGACCGTTATAGTTATAAT-3′) (I-*Ceu*I, *Swa*I, and PI-*Sce*I recognition sequences are noted by underlining, italics, and bold, respectively), resulting in pFS2-Ad35-10, which contains I-*Ceu*I, *Swa*I, and PI-*Sce*I sites in the E1 deletion site of the Ad35 genome. The *Sbf*I/*Pst*I fragments of pFS2-Ad35-7 and pFS2-Ad35-10 were then ligated, resulting in pFS2-Ad35-11. The *Sbf*I/*Asc*I fragment of pFS2-Ad35-11 was exchanged with the *Sbf*I/*Asc*I fragment of pAdMS1,[10] which clones the whole Ad35 genome, resulting in pAdMS2-1. The *Not*I site of pAdMS2-1 was changed into an *Sbf*I site by using oligonucleotide 3 (5′-GGCCCCTGCAGG-3′) (the *Sbf*I recognition sequence is underlined), resulting in pAdMS2. To delete the E3 region in the Ad35 genome, the *Bam*HI/*Not*I fragment of pHM15-Ad35-1,[10] which has the right end of the Ad35 genome (bp 29732 – right end of the genome), was cloned into *Bam*HI/*Not*I sites of a shuttle plasmid pFS1, which contains multicloning sites composed of *Sal*I/*Sbf*I/*Eco*RI/*Bam*HI/*Swa*I/*Not*I sites, creating pFS1-Ad35-1. pFS1 was constructed by ligation of *Xba*I/*Sac*I-digested pGEM7Zf(+) (Promega

Corp., MA, USA) with the oligonucleotides containing the multi-cloning sites. The *SalI*/*Eco*RI fragment of pHM15-A35-1 (bp 23583-27760) was inserted between the *SalI*/*Eco*RI sites of pFS1-Ad35-1, resulting in pFS1-Ad35-2. pFS1-Ad35-2 was then cut by *Eco*RI/*Bam*HI and ligated with oligonuclotide 4 (5′-AATTGGCCACG TAGGCC-3′) and 5 (5′-GATCGGCCTACGTGGCC-3′) (*Sfi*I recognition sequence is underlined), resulting in pFS1-Ad35-9. The *SalI*/*Not*I fragment of pFS1-Ad35-9 was ligated with the *SalI*/*Not*I fragment of pHM14-Ad35-1, creating pHM14-Ad35-3. pHM14-Ad35-1 was constructed by cloning of the *Eco*RI/*Kpn*I fragment (bp 21945–29545) of the Ad35 genome into the *Eco*RI/*Kpn*I sites of pHM14.[53] The *Eco*RI/*Not*I fragment of pHM14-Ad35-3 was ligated with the *Eco*RI/*Not*I fragment of pAdMS1, resulting in pAdMS3-1. The *Not*I site of pAdMS3-1 was changed into an *Sbf*I site by using oligonucleotide 3, resulting in pAdMS3. The *Sbf*I/*Asc*I fragment of pFS2-Ad35-11 was ligated with the *Sbf*I/*Asc*I fragment of pAdMS3-1, creating pAdMS4-1. The *Not*I site of pAdMS4-1 was changed into a *Sbf*I site by using oligonucleotide 3, resulting in pAdMS4. pAdMS2 and -4 have I-*Ceu*I, *Swa*I, and PI-*Sce*I sites in the E1 deletion region (ΔE1: bp 368–3374). pAdMS3 and -4 have an *Sfi*I site in the E3 deletion region (ΔE3: bp 27 761–29 731). The E1a and E1b coding regions of Ad35 are located from bp 569 to 1441 and from bp 1611 to 3400, respectively, according to the Ad35 genome sequence (GenBank Accession No. AY271307). The E3a and E3b coding regions of Ad35 are located from bp 27 199 to 29 496 and from bp 29 538 to 30 622, respectively. pAdMS2, -3, and -4 have *Sbf*I sites at both ends of the Ad genome.

Shuttle plasmids containing a variety of promoters were constructed by changing the CMV promoter of pHMCMV5[21] into another type of promoter, including the EF1α promoter, the CA promoter, the mouse PGK promoter, the MSCV promoter, and the CMVi promoter. The EF1α promoter is derived from pEF1α/myc/nuc (Invitrogen, Carlsbad, CA, USA). The CMVi promoter is derived from pGeneGrip (Gene Therapy Systems, San Diego, CA, USA). The composite CA promoter and PGK promoter were kindly provided by Dr J Miyazaki (Osaka University, Osaka, Japan) and Dr MA Kay (Standford University, CA, USA), respectively. The MSCV promoter was a kind gift of Dr RG Hawley (American Red Cross, MD, USA).

E1/E3-deleted Ad35 vectors expressing enhanced GFP

To construct the plasmid for a recombinant E1/E3-deleted Ad35 vector containing a CMV promoter-driven GFP expression cassette, pHMCMV-GFP1[54] and pAdMS4 were digested with I-*Ceu*I and PI-*Sce*I. The digested pAdMS4 was ligated with the I-*Ceu*I/PI-*Sce*I fragment of pHMCMV-GFP1 containing a GFP expression cassette, resulting in pAdMS4-CMVGFP. pAdMS4-CMVGFP was linearized by the digestion with *Sbf*I. The linearized DNA was transfected into VK10-9 cells (kindly provided by Dr V Krougliak),[22] which are 293 cells expressing the E4 proteins of Ad5 as well as the E1 proteins. A cytopathic effect (CPE) was observed 10–14 days after transfection, and the virus was then amplified in VK10-9 cells and purified by the conventional method for Ad5 vector preparation. For the preparation of recombinant E1/E3-deleted Ad35 vectors containing various types of promoters, a *GFP* gene was cloned into multi-cloning sites in the shuttle plasmids containing various types of promoters, and the Ad35 vectors were then prepared by methods similar to those described above.

Transduction experiment

Human bone marrow CD34$^+$ cells were purchased from Biowhittaker, Inc., Walkersville, MD, USA. The cells were recovered from the frozen stock, suspended in StemSpan™ 2000 containing cytokine cocktail StemSpan™ CC100 (human Flt-3 ligand (100 ng/ml), human stem cell factor (100 ng/ml), human interleukin (IL)-3 (20 ng/ml), and human IL-6 (20 ng/ml)) (StemCell Technologies Inc., Vancouver, BC, Canada), and were seeded into a 48- or 96-well plate (1–5 × 10^4 cells/well). The cells were transduced with the GFP-expressing Ad35 vectors at the indicated VP/cell 16–18 h after seeding. At 6 h after incubation, the cells were washed to remove the Ad35 vectors and resuspended in the medium. At 48 h after transduction, 10^4 cells per sample were analyzed for GFP expression by flow cytometry on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, Tokyo, Japan).

Flow-cytometric analysis of CD46 expression

Human bone marrow CD34$^+$ cells were suspended in staining buffer containing fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD46 antibody (Pharmingen, San Diego, CA, USA). After washing with the sorting solution, the stained cells (10^4 cells) were analyzed using a FACSCalibur and CellQuest software (Becton Dickinson). For simultaneous analysis of GFP and CD46 expression, the transduced cells were incubated with mouse anti-human CD46 antibody (Pharmingen). Subsequently, the cells were washed and incubated with phycoerythrin (PE)-conjugated goat anti-mouse IgG second antibody (Pharmingen). After washing with the sorting solution, the analysis was performed as described above.

Real-time quantitative PCR

Human bone marrow CD34$^+$ cells were incubated with the Ad35 vectors at 6000 VP/cell, and control cells were incubated without the Ad35 vectors. After a 6-h incubation, the medium was changed to remove the Ad35 vectors. At 48 h after transduction, the cells were harvested, pelleted, and washed gently. The cells were then sorted into GFP-positive and -negative fractions using a FACSVantage SE (Becton Dickinson). Sort purities were greater than 90% for both GFP-positive and -negative fractions. The sorted cells were treated with trypsin and DNase, followed by washing to remove the extracellular vector genome. Total DNA, including the Ad35 vector DNA, was extracted from the GFP-positive and -negative cells using a Tissue DNeasy Kit (Qiagen, Valencia, CA, USA). The quantitative real-time PCR was performed with 2.5 ng of sample DNA, 0.5 μM each primer, 0.16 μM TaqMan probe, and 25 μl of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA) in a final volume of 50 μl using the ABI Prism 7000 sequence detection system (Applied Biosystems). The PCR was initially denatured at 95°C for 10 min and then subjected to cycles of 95°C for 15 s and

60°C for 1 min. The reaction was carried out for 50 cycles. Primers for amplification were located in the pIX region of Ad35 genome. The sequences of the primers and probe used were as follows: forward, 5′-TGGATGGAAG ACCCGTTCAA-3′; reverse, 5′-CGTCCAAAGGTGAAG AACTTAAAGT-3′; probe, 5′-FAM-CGCCAATTCTTC AACGCTGACCTATGC-TAMRA-3′. These sequences were designed using Primer Express softeware version 1.0 (Applied Biosystems), and it was confirmed that they amplified the products of desired size. The Ad35 vector plasmid pAdMS4 was used as a standard. For human β-actin quantification, β-actin control reagent (Applied Biosystems) was used.

Purification of immature CD34⁺ subpopulations

Human bone marrow $CD34^+$ cells were incubated with PE-conjugated mouse anti-human AC133 monoclonal antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) or FITC-conjugated mouse anti-human CD38 monoclonal antibody (eBioscience, San Diego, CA, USA) immediately after the cells were recovered from the frozen stock. After washing, cell sorting was performed using a FACSVantage SE (Becton Dickinson). Sorting gates were set to sort the $CD38^{low/-}$ and $AC133^+$ subpopulations. Sort purities were greater than 80% for both $CD38^{low/-}$ and $AC133^+$ subpopulations. The sorted $CD34^+CD38^{low/-}$ and $CD34^+AC133^+$ cells were transduced with the Ad35 vectors at 6000 VP/cell, as described above.

Colony-forming assay

The GFP-positive and -negative cells were recovered 48 h after transduction with the Ad35 vector containing the CA promoter, as described above. In all, 1000 cells of each fraction were then plated in a 35-mm dish containing Methocult H4444 methylcellulose medium (erythropoietin; 3 U/ml, stem cell factor; 50 ng/ml, GM-CSF; 10 ng/ml, IL-3; 10 ng/ml) (Stem Cell Technologies). After 14 days of incubation at 37°C in a 5% CO_2 incubator, CFU-GM, BFU-E, and CFU-Mix colonies were enumerated under a microscope. The experiments were performed in duplicate. Uninfected cells were also sorted into a GFP-negative fraction and treated as described above.

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