

requiring sustained, long-term expression of therapeutic proteins. In this study, we generated novel lentiviral vectors with a tet-off system, and demonstrated that our lentiviral vector systems were significantly effective and strictly regulated in hADMPCs, without affecting their stem cell properties.

Gene silencing is of considerable importance where stable, long-term expression is required. Researchers have reported that transgene silencing occurred when the CMV promoter was used in some cell types, especially in embryonic stem cells [15–17]. Since Kawabata et al. also demonstrated that virus-derived promoters inefficiently functioned in embryonic stem cells in gene transfer experiments [37], down-regulation and unsuitability of promoters in stem cells should be considered. Therefore, transduction efficacy and durability of transgene expression in hADMPCs is also an important issue to be determined. Qin et al. reported that the human EF-1 $\alpha$  promoter and the TRE promoter are more efficient than the CMV promoter to drive lentiviral mediated transgene expression in rat bone marrow-derived MSCs [18]. McGinley et al. also showed that EF-1 $\alpha$  and human phosphoglycerate kinase-1 (PGK) promoters have a clear advantage over the CMV promoter in transducing rat bone marrow-derived MSC transduction with lentivirus [19]. Consistent with their findings, our data also demonstrated that the EF-1 $\alpha$  promoter was more efficient than the CMV promoter to drive EGFP expression in hADMPCs (Figure 1A, B). Moreover, a significant decrease in fluorescent intensity was observed by 28 days after transduction with lentiviral vector CSII-CMV-EGFP (Figure 1C), suggesting that the CMV promoter might be silenced in hADMPCs. We also demonstrated the intriguing finding that most (>90%) of the hADMPCs transduced with pTRE-EGFP-EF-tTA-2A-Bsd strongly expressed EGFP in the absence of Dox, whereas >50% of the cells transduced with pTRE-EGFP-CMV-tTA-2A-Bsd were EGFP negative, regardless of their blasticidin resistance (Figure 3A, B). Our data demonstrated that the inhibitor of histone deacetylation trichostatin A (TSA) re-induced the expression of EGFP (Figure 3C, D), suggesting that “promoter suppression” might occur by histone deacetylation, not by DNA methylation of CpG sites within the TRE tight promoter. “Promoter suppression” is a transcript repression of a 5' transcriptional unit by a 3' unit when 2 transcriptional units lie adjacent in head-to-tail tandem on a chromosome [28,29]. In this study, it is possible that the downstream unit of CMV-tTA-2A-Bsd repressed the upstream unit of TRE-EGFP because (1) resistance to blasticidin implies the transcriptional unit of CMV-tTA-2A-Bsd is active, and (2) reactivation of EGFP expression by TSA implies the transcriptional unit of TRE-EGFP is epigenetically silenced. In order to eliminate the promoter suppression or transcriptional interference between 2 transcriptional units, some researchers have been trying to separate the 2 units by polyadenylation, terminator, and insulator sequences [28,38]. However, these sequences extend the lentiviral vector size, which may affect the lentiviral titers produced from the vector. From this point of view, our finding that the transcriptional unit driven from the TRE tight promoter is resistant to gene silencing when arranged in tandem with the EF-tTA-2A-Bsd transcriptional unit (Figure 3) is of interest in the fields of both basic and clinical research, although the underlying mechanism remains elusive.

In general, large numbers of cells displaying the appropriate phenotypes are required for tissue engineering. Moreover, fully differentiated cells do not proliferate [39]. Therefore, in order to obtain enough cells to perform a transplant from genetically modified MSCs, it is important to develop a system in which the gene of interest is tightly regulated and inducible, and in which stably expressing transgenic cell lines can be obtained without

affecting their stem cell properties. Using the system, MSCs transduced with lentiviral vectors can be selected and increased in numbers from a limited number of MSCs, before the target genes are induced. After obtaining an adequate number of gene-manipulated MSCs, the target genes could be induced in order to start differentiation. According to our data, hADMPCs transduced with pTRE-EGFP-EF-tTA-2A-Bsd were successfully selected by blasticidin, could proliferate, maintain their stem cell properties, and regulate EGFP expression tightly by Dox (Figure 4, 5), demonstrating that this all-in-one lentiviral vector is a promising gene delivery system for generating the material for artificial organs.

A major advantage of using the 2A cleavage factor in the construction of multi-cistronic vectors is its small size compared to internal promoter entry site (IRES) sequences. Because the titer of the lentivirus decreases with increasing size of the lentiviral vector, it is important to minimize the length of the sequences. In addition, linkage of 2 genes by 2A peptide resulted in efficient co-expression of the genes, whereas a gene placed downstream of an IRES is expressed at 2- to 3-fold lower levels than a gene placed upstream [40,41]. In this study, tTA-2A-Bsd cassette driven from CMV or EF-1 $\alpha$  promoter showed ~90% cleavage (Figure 3). However, the point that should be considered is the effect of residual 2A peptide on the protein. As the processing occurred at the end of the 2A peptide, the 2A tag remains attached at the tTA C-terminus. Our data demonstrated that the presence of this extra 2A peptide did not seem to interfere with the activity of tTA since Dox strictly regulated the expression of EGFP under the control of TRE-tight promoter (Figure 2D, 3A, 3B and 5). Moreover, when Bsd is cleaved, an additional proline is attached at the N-terminus. We demonstrated that this did not affect a function of Bsd because hADMPCs transduced with either pTRE-EGFP-CMV-tTA-2A-Bsd or pTRE-EGFP-EF-tTA-2A-Bsd could survive and proliferate in medium containing blasticidin at a concentration at which all of the parental hADMPCs died.

Another advantage of our lentiviral system is the availability of a restriction enzyme treatment/ligation independent cloning system, called the Gateway system (Invitrogen). In general, the construction of lentiviral vectors using a conventional restriction enzyme/ligation cloning method has poor efficiency due to the large sizes and the lack of proper cloning sites. In our hands, cloning efficiency into our new lentiviral vectors pTRE-Rfa-CMV-tTA-2A-Bsd or pTRE-Rfa-EF-tTA-2A-Bsd using LR recombination reaches nearly 100%, saving time and effort in construction of the vectors. In addition, there are several resources available that take advantage of the Gateway vector. For example, CCSB Human ORFeome Collection (Dana-Farber Cancer Institute, Center for Cancer Systems Biology) represents almost 12,000 fully-sequenced cloned human ORFs which can be readily transferred to Gateway compatible destination vectors for various functional proteomics studies [42]. Block-iT pol II miR RNAi system from Invitrogen, which is designed to express artificial miRNAs, also enables compatibility with Gateway destination vectors for gene knock-down experiments [43].

In conclusion, our new single tet-off lentiviral vector system provides powerful tools not only for applied research on hADMPCs and other stem cells, but also basic research on a variety of cell lines and primary cells.

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## Author Contributions

Conceived and designed the experiments: HM MM. Performed the experiments: HM MM KS HO AM. Analyzed the data: HM MM KS.

Contributed reagents/materials/analysis tools: HM MM HO AI AM. Wrote the paper: HM MM TH.

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