

ORIGINAL ARTICLE

# Directing adenovirus across the blood–brain barrier via melanotransferrin (P97) transcytosis pathway in an *in vitro* model

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Adenovirus serotype 5 (Ad5) is widely used in the development of gene therapy protocols. However, current gene therapy strategies involving brain are mostly based on intracranial injection. A major obstacle for systemically administered vectors to infect brain tissue is the blood–brain barrier (BBB). One strategy to cross the BBB is transcytosis, a transcellular transport process that shuttles a molecule from one side of the cell to the other side. Recently, melanotransferrin (MTf)/P97 was found to be able to cross the BBB and accumulate in brain. We thus hypothesize that re-directing Ad5 vectors to the MTf transcytosis pathway may facilitate Ad5 vectors to cross the BBB. To test this hypothesis, we constructed a bi-specific adaptor protein containing the extracellular domain of the coxsackie-adenovirus receptor (CAR) and the full-length melanotransferrin (sCAR-MTf), and

investigated its ability to re-direct Ad5 vectors to the MTf transcytosis pathway. We found this adaptor protein could re-direct Ad5 to the MTf transcytosis pathway in an *in vitro* BBB model, and the transcytosed Ad5 viral particles retained their native infectivity. The sCAR-MTf-mediated Ad5 transcytosis was temperature- and dose dependent. In addition, we examined the directionality of sCAR-MTf-mediated Ad5 transcytosis, and found the efficiency of apical-to-basal transcytosis was much higher than that of basal-to-apical direction, supporting a role of this strategy in transporting Ad5 vectors towards the brain. Taken together, our study demonstrated that re-directing Ad5 to the MTf transcytosis pathway could facilitate gene delivery across the BBB.

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**Keywords:** adenovirus; melanotransferrin; transcytosis; blood–brain barrier; gene therapy

## Introduction

Human adenovirus serotype 5 (Ad5) is widely explored as a gene delivery vector for a variety of tissues/organs including brain, largely owing to its high gene delivery efficiency, easy manipulation, high titer production *in vitro*, well-characterized biology and minor pathological effect in humans. However, for brain-related gene therapy strategies, such as brain cancer gene therapy, and gene therapy for genetic diseases involving central nervous system pathology, current protocols under development are mainly based on intra-cranial injections.<sup>1–3</sup> This is not only clinically difficult because it requires high risk surgical procedures, but also inefficient in gene delivery, as localized injections do not allow Ad5 vectors to access all diseased cells. Therefore, it is essential to develop a strategy that allows systemically administered Ad5 vectors to infect the brain cells.

A major obstacle for intravascular-administered Ad5 vectors to enter the brain cells is the blood–brain barrier (BBB).<sup>2,3</sup> The BBB is formed by brain capillary endothelial cells, which are closely sealed by tight junctions and contain few fenestrae and few endocytic vesicles. The BBB restricts the transport of most small polar molecules and macromolecules from the cerebrovascular circulation into the brain tissue, including therapeutic agents such as adenoviral vectors.

One strategy to cross the endothelial barrier is transcytosis, a transcellular transport process that shuttles a molecule from one side of the cell to the other side. It involves endocytosis, intracellular transport, and exocytosis of the molecule. Typical examples of this pathway include the transport of albumin and lipids, hormones and peptides that bind avidly to albumin,<sup>4,5</sup> and transferrin receptor (TfR)-mediated transcytosis.<sup>6,7</sup> Transcytosis pathways are not only employed by blood components, but also adopted by viruses such as human immunodeficient viruses, adeno-associated virus and poliovirus.<sup>8–10</sup>

Recent studies discovered that human melanotransferrin (MTf, also named P97), a transferrin homolog originally identified in human melanoma, was highly accumulated into the mouse brain following intravenous injection.<sup>11,12</sup> These studies demonstrated that MTf could

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cross the brain endothelial cells without affecting the integrity of the BBB and with a much higher rate than what is seen with transferrin and albumin.<sup>11,12</sup> These studies suggest that MTf could be a good candidate for drug delivery to brain. It should be noted that these studies were performed with human MTf in mouse brain, and in *in vitro* BBB models established with bovine brain microvascular endothelial cells (BBMVEC), suggesting MTf functions without significant species discrepancy.

MTf is a glycosylated protein, and exists in two forms including a cell membrane bound form and a soluble, secreted form.<sup>13</sup> The exact function of both forms remains largely unknown. The membrane bound MTf is composed of 719 amino-acid residues, which comprises two homologous extracellular domains of 342 and 352 amino-acid residues, respectively, and a C-terminal 25-residue stretch of predominantly uncharged and hydrophobic amino-acid residues, which is believed to form a glycosylphosphatidylinositol (GPI) membrane anchor.<sup>13</sup> It has been demonstrated that transendothelial transport of MTf occurs via receptor-mediated endocytosis, and low-density lipoprotein (LDL) receptor-related protein (LRP) appears to be involved in MTf trans-endothelial transport.<sup>11</sup>

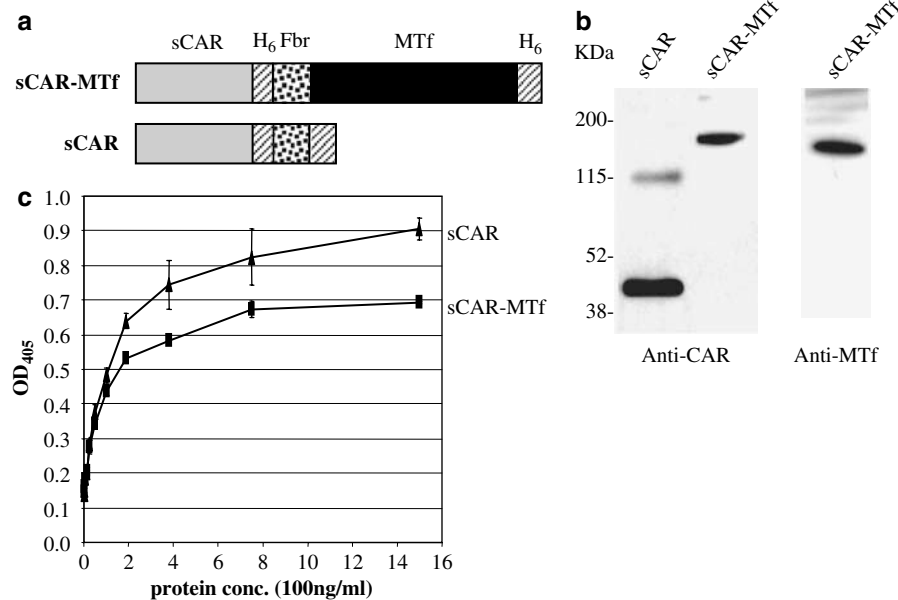
In this study, we attempted to re-direct Ad5 vectors to the MTf transcytosis pathway so that they can deliver transgenes across the BBB in an *in vitro* model system. Infection of Ad5 is initiated by attachment of its capsid protein fiber to the cell surface coxsackie-adenovirus receptor (sCAR), followed by interaction of another capsid protein, penton base, with cell surface  $\alpha_v$  integrins that triggers the internalization of the viruses.<sup>14-16</sup> Based on this information, investigators have utilized bi-

specific adaptor proteins that bind to both Ad5 vectors and alternative receptors expressed on the surface of the target cells to re-direct Ad5 tropism.<sup>17-19</sup> In our study, we attempted to re-direct Ad5 across the BBB using the bi-specific adaptor protein strategy. We constructed a bi-specific adaptor protein containing the extracellular domain of CAR (sCAR) and the full-length MTf, namely sCAR-MTf, and demonstrated its ability to re-direct Ad5 vectors across the BBB using an *in vitro* BBB model. This work represents the first study that employs transcytosis pathway to re-direct Ad5 vectors across the BBB.

## Results

### Generation of bi-specific adaptor protein sCAR-MTf

Previous studies have shown that bi-specific adaptor proteins that bind both Ad5 vector and alternative receptors can be used to re-direct Ad5 vector entering cells through the alternative receptors.<sup>17-19</sup> In order to re-direct Ad5 vector to MTf transcytosis pathway, we constructed a bi-specific adaptor protein containing the extracellular domain of CAR and the GPI anchor-deleted full-length MTf (termed sCAR-MTf) (Figure 1a). In the adaptor protein, sCAR that binds to Ad5 fiber is located at the N-terminal end, whereas MTf that targets to MTf transcytosis pathway is located at the C-terminal part. The GPI-deleted MTf was employed because deletion of the GPI anchor has been shown to allow production of the soluble form of MTf.<sup>20</sup> In addition, a bacteriophage T4 fibrin trimerization domain was used to connect the two binding domains, as fiber exists as trimer in Ad5 virions and trimerized sCAR is expected to bind fiber



**Figure 1** Generation of bi-specific adaptor protein sCAR-MTf. (a) Diagram of the bi-specific adaptor protein sCAR-MTf and control sCAR. H<sub>6</sub>: His<sub>6</sub> epitope; Fbr: T4 Fibrin trimerization domain. (b) Western blotting assay showing the adaptor proteins were expressed and purified. In the assay, about 500 ng of each purified protein were used for SDS-PAGE and subsequent Western blotting assay with anti-CAR and/or anti-MTf antibody. Note: the upper fainter band in sCAR lane appears to be the trimeric form of sCAR. We often observed this when a large amount of protein was loaded. (c) ELISA binding assay suggesting sCAR-MTf can efficiently bind to Ad5 vectors. In the assay, Ad5 vectors were immobilized on the wells of a 96-well ELISA plate, and then incubated with different amount of sCAR or sCAR-MTf. The binding activity was analyzed by anti-His<sub>6</sub> antibody.

better than sCAR monomer. Two His<sub>6</sub> epitopes (in the middle and at the C-terminal end) were included to ensure protein purification. Similarly, a control protein sCAR that contains the sCAR, His<sub>6</sub>, and fibrin trimerization domains, but not MTf domain was constructed. The expression of the adaptor proteins, which were driven by CMV promoter, was accomplished using Ad5 as gene delivery vector in mammalian HeLa cells as described in the Materials and methods. Proteins were purified from the media, as the adaptor proteins were designed as secreted proteins, and confirmed by Western blotting analysis using both anti-CAR and anti-MTf antibodies (Figure 1b).

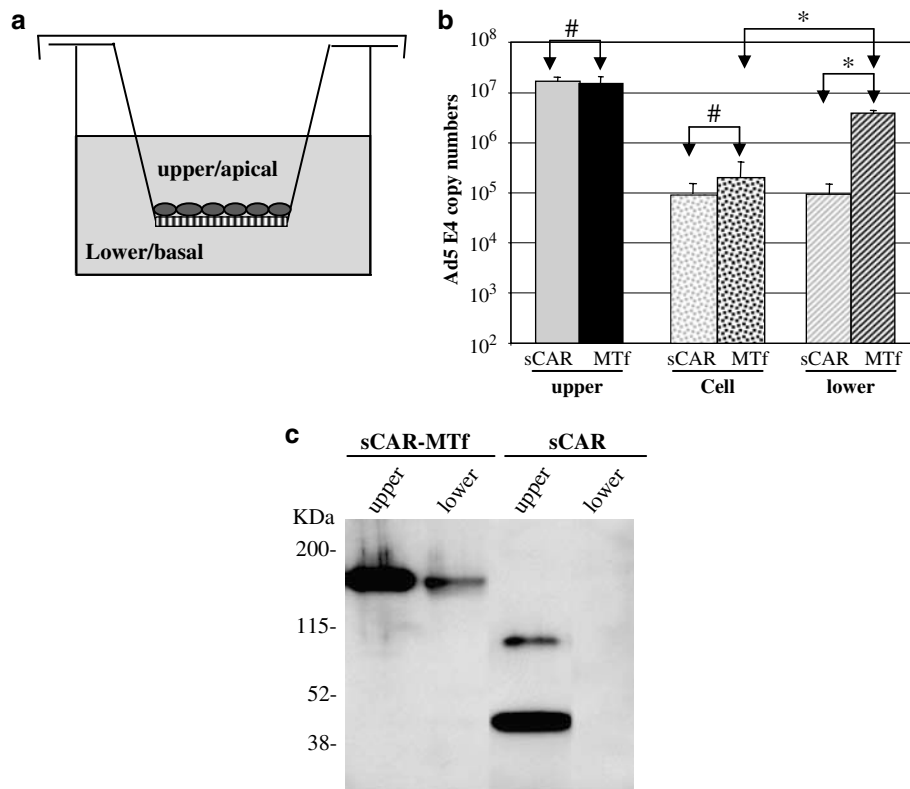
Next, we examined whether the adaptor proteins retained their ability to interact with Ad5 vectors. *In vitro* ELISA (enzyme-linked immunosorbent assay) binding assay was performed in this regard. In the assay, unmodified Ad5 viral particles (VPs) were immobilized to each well of a 96-well ELISA plate, and incubated with different amount of fusion protein sCAR-MTf or control sCAR. After extensive wash, the binding activity was detected with anti-His<sub>6</sub> antibody, followed by incubation with alkaline phosphatase (AP)-conjugated secondary antibody. The binding activity was assessed based on the AP activity. The results indicated that sCAR-MTf was capable of binding to Ad5 vectors, and the maximal

binding could reach ~80% of that between sCAR and Ad5 vectors (Figure 1c).

#### Ad5 transcytosis mediated by sCAR-MTf

To test whether the bi-specific sCAR-MTf adaptor protein was able to re-direct Ad5 to MTf transcytosis pathway, we employed an *in vitro* BBB model system that has been widely used. The BBB model was established with BBMVEC cells in a transwell system, in which the BBMVEC cells were grown on the transwell inserts (polyester, 3.0 μm pore size, 12 mm diameter; Figure 2a). To establish a well-sealed endothelial barrier, the cells were cultured for 5–7 days following confluency, with media refreshed every other day. In the meantime, the transendothelial electrical resistance (TEER) was monitored. TEER reflects impedance of the passage of small ions through the physiological barrier, and has been widely used to measure BBB integrity.<sup>21–25</sup> Transcytosis assays were performed when the TEER of each BBB model reached the maximal value, which mostly were in the range of 100–150 Ω cm<sup>2</sup>.

As previous studies have shown that MTf prefers apical-to-basal transcellular transport,<sup>11</sup> in the transcytosis assay, we pre-mixed Ad5 VPs with sCAR-MTf or control sCAR, and added the complexes to the upper chamber (apical side of the barrier). The cells were then



**Figure 2** sCAR-MTf-mediated Ad5 transcytosis in the *in vitro* BBB model. (a) Diagram of the *in vitro* BBB model. BBMVEC cells were cultured on the transwell inserts. (b) Ad5 vectors were re-directed to transendothelial transport pathway in the presence of sCAR-MTf. Following transcytosis assay, samples were collected from both the apical and basal chambers. The BBMVEC cells were also collected by trypsin treatment. DNA isolation was performed for each sample and processed for quantitative PCR to detect Ad5 copy numbers using primers in the E4 region. Apical-to-basal transendothelial transport of Ad5 VPs were significantly increased in the presence of sCAR-MTf, suggesting Ad5 vectors were re-directed to transcytosis pathway by sCAR-MTf. \*Indicates  $P < 0.01$ , and #indicates  $P > 0.05$ , as analyzed by Student's *t*-test. (c) Western blotting assay showing sCAR-MTf, but not sCAR, was transported to the basal chamber together with the Ad5 vectors, which further confirmed the transendothelial transport of the Ad5 vectors was mediated by sCAR-MTf.

incubated for 6 h in a 37°C, 5% CO<sub>2</sub>-containing humidified incubator to allow transcytosis to occur. At the end of the experiments, we collected the samples from both the apical and basal chambers, and analyzed the presence of Ad5 virions and adaptor proteins. In addition, we also collected the BBMVEC cells by trypsin treatment to analyze the presence of Ad5 VPs that were endocytosed and remained inside the cells. Presence of the Ad5 particles was examined with quantitative real-time polymerase chain reaction (PCR) that measures the Ad5 E4 copy number. Western blotting assay was performed to detect the presence of the adaptor proteins after concentrating the samples using protein concentrator. As shown in Figure 2, we found that in the presence of sCAR-MTf, over 50 times more Ad5 particles, which accounted for about 5% of total Ad5 particles, were transported to the basal chambers compared to that in the presence of sCAR ( $P < 0.01$ ; Figure 2b). Consistent with this, we detected sCAR-MTf protein in the basal chamber samples, but not sCAR, although similar amounts of adaptor proteins were detected in the upper chambers, which indicated that similar amounts of the proteins were used in the assay (Figure 2c). Furthermore, our results showed that the amount of Ad5 particles that were endocytosed and remained inside the cells in the presence of sCAR-MTf was similar to that of in the presence of sCAR ( $P = 0.42$ ), and was significantly less than what was transcytosed to the basal chamber in the presence of sCAR-MTf ( $P < 0.01$ ) (Figure 2b). This suggests most Ad5 vectors were re-directed by sCAR-MTf to transcytosis pathway, not endocytosis pathway.

It should be noted that we have repeated the experiments many times, and often there were 40–50 times more transcytosed Ad5 particles in the presence of sCAR-MTf than that of sCAR, but the range could be as low as 10 times higher or as high as 100 times higher. The variation was probably due to variable cell culture/differentiation conditions and different batches of purified proteins. In addition, following the transcytosis assay, the TEER of each BBB model was checked and no significant difference from before the assay was found (Table 1), suggesting the BBB integrity was maintained during the transcytosis process. Together our data suggested the bi-specific adaptor protein sCAR-MTf was capable of re-directing Ad5 vectors to the transcytosis pathway.

#### Functionality of the transcytosed Ad5 VPs

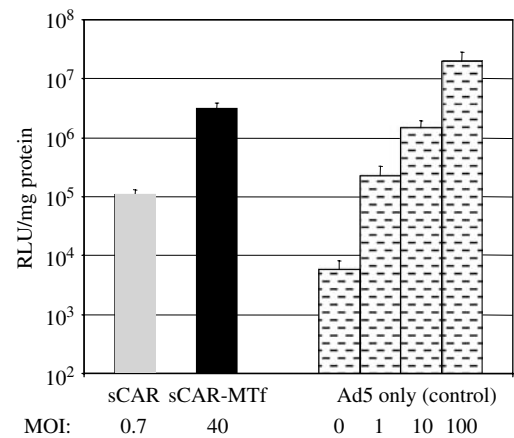
We further investigated whether the transcytosed Ad5 VPs maintained their functional integrity during the transcytosis process. We evaluated the functionality of these Ad5 particles by examining their gene transfer efficacy. In the experiments, 50 µl of each sample collected from the basal chambers after transcytosis assay were used to infect a high-CAR cell line A549 cells, and their gene transfer efficacy was evaluated using the luciferase reporter gene that was incorporated in the E1 region of the Ad5 vectors. The actual multiplicity of infections (MOIs) were calculated based on the Ad5 copy numbers in the transcytosed samples, which were obtained by quantitative real-time PCR. Ad5 vectors that did not undergo transcytosis assay were used at various MOIs as control. Our results showed that the transcytosed Ad5 VPs retained their gene transfer efficacy

**Table 1** A representative set of TEER data before and after transcytosis assay

Group	TEER ( $\Omega \text{ cm}^2$ )		P-value
	Before transcytosis	After transcytosis	
Ad5+sCAR	124±8.5	113±12.2	0.27
Ad5+sCAR-MTf	130±15.0	112±4.7	0.13

Abbreviations: sCAR-MTf, soluble coxsackie-adenovirus receptor and the full-length melanotransferrin; TEER, transendothelial electrical resistance.

Three wells were used in the transcytosis assay for each group. Student's *t*-test was used to determine the *P*-value of the TEER before and after transcytosis.  $P < 0.05$  was considered statistically significant.

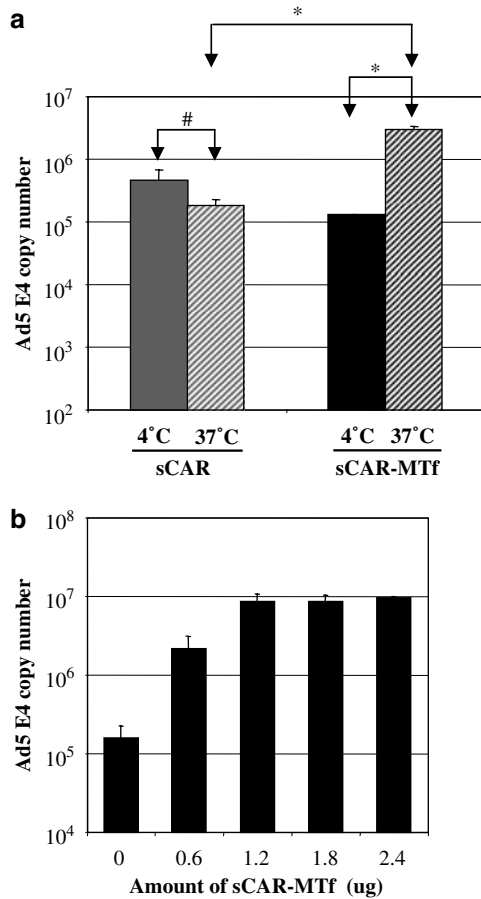


**Figure 3** Infectivity of the transcytosed Ad5 VPs. Fifty microliters of each sample obtained from the basal chambers in the transcytosis assay were used to infect A549 cells. The actual MOIs of the infections as shown were calculated based on the corresponding quantitative PCR results. Freshly thawed Ad5 vectors that did not go through transcytosis assay were used to infect cells at MOI of 1, 10 and 100 as positive control, whereas uninfected cells (MOI = 0) as negative control. The relative light units per mg protein indicating the activity of the luciferase reporter that was incorporated in the Ad5 E1 region suggest the transcytosed Ad5 VPs maintained their infectivity/gene transfer efficacy.

(Figure 3), suggesting the transcytosis process did not harm the integrity of the Ad5 viruses.

#### Temperature-dependence of sCAR-MTf-mediated Ad5 transcytosis

Transcytosis is an active transcellular transport process. Temperature dependence is thus one characteristic of transcytosis. This temperature dependence was demonstrated for recombinant MTf transcytosis.<sup>11</sup> To confirm Ad5 vectors were indeed transported across the endothelial barrier via transcytosis pathway, we examined temperature dependence of this process by performing the transcytosis assay at either 4 or 37°C. As shown in Figure 4a, sCAR-MTf-mediated transcytosis was completely inhibited at 4°C ( $P < 0.01$ ), whereas in the control experiment with sCAR, there was no significant difference between 4 and 37°C ( $P > 0.05$ ).



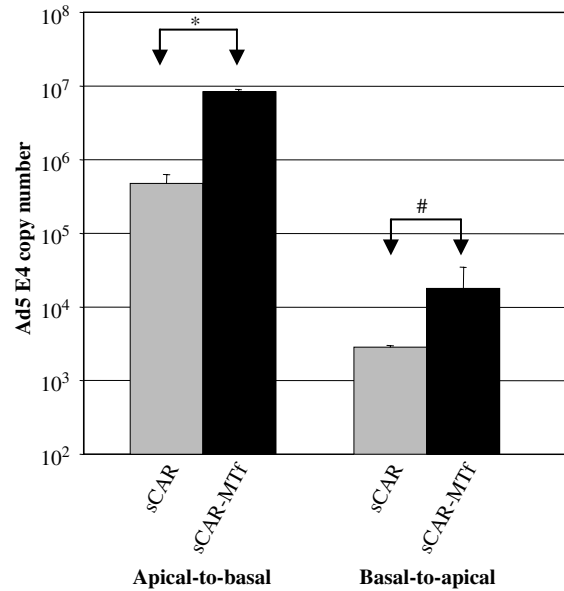
**Figure 4** Characterization of the temperature- and dose dependence of sCAR-MTf-mediated Ad5 transcytosis. (a) The transcytosis assay was performed at either 4 or 37°C as described above. sCAR-MTf-mediated Ad5 transcytosis was completely inhibited at 4°C. \*Indicates  $P < 0.01$ , and # indicates  $P > 0.05$ , as assessed by Student's *t*-test. (b) The transcytosis assay was performed with fixed amount of Ad5 ( $10^8$  VPs) and various amount of sCAR-MTf protein at 37°C for 6 h as described above. Transendothelial transport of Ad5 was saturated when more than 1.2 µg of the adaptor protein were used.

#### Dose dependence of sCAR-MTf-mediated Ad5 transcytosis

MTf transcytosis is a receptor-mediated process, sCAR-MTf-mediated Ad5 transcytosis is thus expected to be dose dependent, and may be saturated. To test this and to determine the optimal dose for the transcytosis assay, we performed transcytosis assays using different amount of sCAR-MTf protein (Figure 4b). Fixed amount of Ad5 viruses ( $10^8$  VPs) were used in each assay. After the transcytosis assay, the samples from the lower chambers were collected, viral DNA was isolated and subsequently used for quantitative PCR analysis. Our results suggested that efficacy of sCAR-MTf-mediated Ad5 transcellular transport was dependent on the dose of the adaptor protein, and the process was saturated in the presence of 1.2 µg sCAR-MTf adaptor protein when  $10^8$  Ad5 VPs were used (Figure 4b).

#### Directionality of sCAR-MTf-mediated Ad5 transcytosis

Recombinant MTf has been shown to prefer apical-to-basal transcytosis, although basal-to-apical could also



**Figure 5** Directionality of sCAR-MTf-mediated Ad5 transcytosis. Ad5 ( $10^8$  VPs) and sCAR-MTf protein (1.0 µg) were pre-mixed and added to either the apical or the basal chamber of each transwell containing the BBB model. Presence of Ad5 VPs in the opposite chamber was examined by quantitative PCR. \*Indicates  $P < 0.01$ , and # indicates  $P > 0.05$ , as assessed by Student's *t*-test.

occur.<sup>11</sup> To examine whether the sCAR-MTf adaptor protein maintained the apical-to-basal preference, which is essential for our purpose of directing Ad5 vectors into the brain, we performed transcytosis assay in both directions using the same amount of Ad5 vectors and the adaptor proteins. We did not detect significant basal-to-apical transcytosis of Ad5 vectors in the presence of sCAR-MTf, as the amount of transcytosed VPs was not significantly different from that of control experiment using sCAR, although sCAR-MTf-mediated Ad5 transcytosis occurred efficiently in apical-to-basal direction (Figure 5). These results suggest the adaptor protein has strong apical-to-basal preference in directing Ad5 vectors across the endothelial barrier, supporting its potential role of transporting Ad5 vectors towards the brain.

## Discussion

In this study, we attempted to re-target Ad5 vectors to MTf transcytosis pathway, so that Ad5 vectors can traverse the BBB. We designed and constructed a bi-specific adaptor protein sCAR-MTf, and examined its ability to re-direct Ad5 across the BBB using an *in vitro* BBB model system established with BBMVEC cells. The adaptor protein was able to re-direct Ad5 vectors to traverse the BBB, often with an efficiency of 40–50 times higher than that of control. The Ad5 VPs undergone transcellular transport maintained their functionality/ infectivity, as assessed by their gene transfer efficacy. In addition, the adaptor protein mediated Ad5 transcytosis was temperature- and dose dependent, which are the characteristics of receptor-mediated transcytosis, in accordance with previous studies.<sup>11,12</sup> Importantly, sCAR-MTf-mediated Ad5 transcytosis showed strong

apical-to-basal preference, arguing for its potential utility in transporting Ad5 vectors into brain tissue.

Employment of an *in vitro* model is essential to study whether the bi-specific adaptor protein sCAR-MTf was capable of re-directing Ad5 vectors traverse the BBB. In this work, the BBB model was established with BMVEC cells that were obtained from Cell Applications Inc. (San Diego, CA, USA), at second passage (P2), and used at  $P \leq 6$ . The maximal TEER of each BBB model was often in the range of 100–150  $\Omega \text{ cm}^2$ , which was reached around 5–7 days following confluency. There was no significant change in TEER when we cultured the BBB model up to 10 days after the cells became confluent. The TEER values of the BBB model in our study are comparable to other studies using BMVEC,<sup>22,26</sup> similar to rat BMVEC cells,<sup>24</sup> slightly lower than human BMVEC,<sup>21</sup> but higher than feline BMVEC.<sup>23</sup> Clearly, the TEER is different for different cell types. In addition, it has been shown that TEER can be modulated by many factors such as cytokines, cAMP, cGMP, nitric oxide, heat and co-culturing with other cells.<sup>21,22,24–26</sup>

Currently, no specific receptor has been identified for MTf. Although human MTf shares 39% homology with human serum transferrin,<sup>13</sup> TfR is found not to be responsible for MTf transcytosis. Instead, a member of LDL receptor family, LRP, may play an essential role in this regard.<sup>11</sup> In fact, LRP may be a common mediator for its binding partners to traverse the BBB. In addition to MTf, LDL, lactoferrin and LDL receptor-associated protein (RAP) have been found to cross the BBB with high efficiency and the receptor involved in their transcellular transport appears to be LRP.<sup>11,12,27–30</sup> *In vivo* transport across the BBB of these proteins may also help explain the observations that MTf, lactoferrin and LRP accumulate in the brain of patients with neurological diseases such as Alzheimer's disease.<sup>31–34</sup> Interestingly, LRP-mediated transcytosis may only be a feature of endothelial cells, as in other cell types or organs, majority of these proteins, once internalized, are found to be degraded or recycled.<sup>35–37</sup> Differentiation stage of endothelial cells also appears to play a role in determining what pathway the protein uptake is taken. For example, in growing brain capillary endothelial cells, LDL is classically internalized by the clathrin pathway, and directed to lysosomes for degradation. However, when the cells are fully differentiated, even though the classic degradation pathway (via lysosomes) is functional, LDL is mostly directed to non-degradation transcytosis pathway.<sup>27</sup> In accordance, our data showed that in the presence of sCAR-MTf, the majority of internalized Ad5 VPs were directed to the transcytosis pathway in the BBB model that is formed by differentiated cells, and very little remained inside the cells (Figure 2b). Apparently, the transcytosis pathway adopts a different trafficking mechanism from the classical endocytosis pathway, as it can bypass the lysosomal degradation. In this regard, caveolae, a type of vesicles that contain enriched caveolin and are non-clathrin coated, has been implicated in LRP-mediated LDL transcytosis through the brain microvascular endothelial cells.<sup>27</sup> The precise mechanism for transcytosis of the proteins across the BBB, however, remains to be investigated.

Adenovirus retargeting has been widely explored in terms of specific and effective gene delivery into certain target cells. It has been demonstrated that genetic

incorporation of alternative targeting motifs into Ad5 fiber improves gene transfer efficacy and specificity.<sup>38–41</sup> However, genetic incorporation has a size limit. Epitopes less than 100 amino-acid residues may be incorporated into fiber knob domain without affecting viral assembly.<sup>42</sup> Alternatively, bi-specific adaptor proteins composed of sCAR and the alternate targeting motif such as epidermal growth factor, human transferrin and a single-chain antibody against carcinoembryonic antigen have been successfully employed to retarget Ad5 vectors.<sup>17–19</sup> *In vivo* stability of this strategy has also been demonstrated.<sup>43,44</sup> Our study adopted this adaptor strategy, as MTf is a large protein, and the domain responsible for its transcytosis is not defined thus far.

In our study, about 5% of Ad5 particles could be transcytosed across the BBB in the presence of sCAR-MTf. The percentage, however, may vary from 3 to 10% depending on cell condition and the batches of proteins. In addition, we observed about 0.1% Ad5 transendothelial transport in all of the negative control transcytosis assays, which include experiments with sCAR (Figures 2, 4a and 5) or Ad5 alone (Figure 4b). These apparently represented nonspecific Ad5 crossing the BBB, presumably owing to leakiness through the paracellular space or occasional escape of endosomes and exocytosis to the other side of the barrier.

Systemic gene delivery into brain is one of the most challenging problems faced by gene therapy investigators. The highly defensive BBB turns out to be the major hurdle. Engineering gene delivery vector so that it can traverse the BBB is thus a rational and attractive direction. In this study, we took advantage of the high efficiency of MTf transcytosis, and applied it in combination with Ad5 vector re-targeting strategy. Another potential advantage using sCAR-MTf for gene delivery into brain is that the major components of the adaptor protein, the extracellular domain of CAR and MTf, are endogenously expressed in human, therefore, the host immune response against the adaptor protein sCAR-MTf is expected to be minimal for *in vivo* applications. Although the data presented here were obtained *in vitro*, the proof-of-principle study appears to be very promising. *In vivo* utility of sCAR adaptor protein-mediated Ad5 retargeting strategy has been directly explored and demonstrated in an earlier study, in which an adaptor protein composed of sCAR and anti-carcinoembryonic antigen single chain antibody, sCAR-MFE, has been shown to re-direct Ad5 to the lungs of a transient transgenic mouse model overexpressing carcinoembryonic antigen in the pulmonary vasculature.<sup>19</sup> Our further efforts will be focused on investigation of the *in vivo* utility of sCAR-MTf-mediated Ad5 transcytosis, and the means of improving the efficiency of this strategy.

## Materials and methods

### Antibodies

The rabbit polyclonal antibody against CAR was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The mouse anti-His-Tag monoclonal antibody (Penta.His) was purchased from Qiagen (Valencia, CA, USA). To generate mouse monoclonal antibody against MTf, M-19 hybridoma cells that were raised against P97 antigen (i.e., MTf) were purchased

from American Type Culture Collection (ATCC). Antibody generation and purification were accomplished in the hybridoma core facility at University of Alabama at Birmingham. Secondary antibodies including AP-conjugated donkey anti-mouse and horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

#### Cells and cell culture

The human embryonic kidney cells transformed with Ad5-E1 DNA (293), the human lung carcinoma cell line A549, and the human melanoma cell line MeWo cells were purchased from ATCC. All of these cells were cultured in Dulbecco's modified Eagle medium-Ham's F12 medium that contains 10% fetal calf serum (FCS) and 2 mM L-glutamine, and grown in a 37°C, 5% CO<sub>2</sub> humidified incubator. The BBMVEC cells were purchased from Cell Applications Inc., and cultured in the complete BBMVEC growth media (also from Cell Applications Inc.). The cell culture flasks or transwell inserts were coated with attachment factor solution (Cell Applications Inc.) before cell seeding, as described in the manufacturer's protocol. The cells were maintained in a 37°C, 5% CO<sub>2</sub> humidified incubator, and the culture media were refreshed every other day until experimental execution.

#### Generation of Ad5 vector that expresses sCAR-MTf fusion protein

In order to express sCAR-MTf fusion protein using Ad5 vector, we first generated a shuttle vector containing the expression cassette, which is composed of (in order) CMV promoter, sCAR ectodomain that consists of its own leader sequence (amino-acid residues 1–236), a 5-amino-acid residue peptide link (GGPGS), a His<sub>6</sub> epitope in the middle, a bacteriophage T4 fibrin trimerization domain, full-length soluble MTf, and a C-terminal His<sub>6</sub> epitope. Construction of this cassette was carried out sequentially as described below. First, an extra His<sub>6</sub> epitope was cloned into the parent vector pcDNA3sCAR6hfibrin containing sCAR ectodomain, the peptide link (GGPGS), a His<sub>6</sub> epitope and T4 fibrin trimerization domain,<sup>45</sup> to generate pcDNA3sCAR6hf6h, in which the extra His<sub>6</sub> epitope was introduced into the C-terminal end of the expression cassette. The expression cassette then contained all components designed except the MTf fragment. This cassette including the CMV promoter and SV40pA was then amplified by PCR, and subcloned into pShuttle (Stratagene, La Jolla, CA, USA) vector, resulting in pShuttle.sCAR6hf6h.

Next, we obtained human MTf cDNA from human melanoma cell line MeWo cells. This was accomplished by reverse transcription (RT)-PCR. Briefly, the total RNA from MeWo cells was extracted using Qiagen RNeasy mini kit according to the manufacturer's manual. The RT reaction was then performed with universal primer oligo dT, and about 1 µg of total RNA was used as template. The full-length soluble form of MTf lacking the C-terminal GPI anchor (27 amino-acid residues) was subsequently amplified using the RT reaction as template. This MTf full-length fragment was then inserted into pShuttle.sCAR6hf6h in-frame at the position of between the fibrin trimerization domain and the

C-terminal His<sub>6</sub> epitope. The resultant plasmid was named pShuttle.sCAR-MTf.

To incorporate the expression cassette of sCAR-MTf into the Ad5 vector, homologous recombination was performed between pShuttle.sCAR-MTf and Adeasy vector (Stratagene). This was accomplished by co-transformation of the linearized shuttle vector pShuttle.sCAR-MTf and backbone pAdeasy into *Escherichia coli* BJ5183. The recombinants were initially screened by DNA isolation and restriction digestions. The plasmid DNA of positive candidates were then transformed into bacteria DH10B, and more DNA was isolated and screened. The final positive recombinants were confirmed by sequencing analysis, and the resultant Ad5 vector was named pAdeasy.sCAR-MTf.

To rescue the viruses encoding sCAR-MTf, pAdeasy.sCAR-MTf was digested with Pac I, and transfected into the Ad-E1 expressing 293 cells with Superfect (Qiagen). After plaques were formed, they were collected and processed for large-scale amplification in 293 cells. The viruses (named Ad5.sCAR-MTf) were then purified by standard CsCl gradient centrifugation.<sup>46</sup>

#### Protein expression and purification

We employed HeLa cells to express and purify the adaptor protein. Twenty flasks (185 cm<sup>2</sup>) of HeLa cells that were grown to 80% confluency were infected with the Ad5 viruses encoding the adaptor protein at MOI of 500. The infected cells were then cultured for 48 h in a 37°C, 5% CO<sub>2</sub> humidified incubator before protein purification. As the adaptor protein was designed as secreted protein, we collected the culture media for protein purification. Purification of the protein was performed based on the His<sub>6</sub> epitopes that were incorporated in the adaptor protein, and the Qiaexpressionist™ system (Qiagen) was used to purify the His<sub>6</sub>-tagged protein. For purification, we first concentrated the media to ~50 ml using protein concentrator columns (10- or 50-kDa MWCO, Millipore, Billerica, MA, USA) according to the manufacturer's manual. Then, 1/10 volume of 10 × supplemental buffer (500 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0, 1.5 M NaCl, 100 mM imidazole) was added to the media to adjust salt and pH condition of the sample. Next, 1 ml of Ni-NTA agarose (Qiagen) that was pre-washed with phosphate-buffered saline (PBS) was added into the media and the sample was incubated at 4°C for 2 h on an end-over-end shaker. The Ni-NTA agarose beads were then collected by centrifuging the sample at 1000 r.p.m. for 5 min. After washing the beads twice with washing buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole, 0.05% Tween-20, pH. 8.0), the adaptor protein was eluted with elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 250 mM imidazole, 0.05% Tween-20, pH. 8.0). The protein concentration was determined using Bio-Rad DC protein assay kit.

#### Enzyme-linked immunoabsorbent assay

*In vitro* ELISA binding assay was performed similarly as described previously.<sup>39</sup> In brief, 10<sup>9</sup> VPs of Ad5 was immobilized on wells of a 96-well ELISA plate (Maxisorp; Nunc, Roskilde, Denmark) by overnight incubation at 4°C. The wells were then washed four times with Tris-buffered saline (TBS) containing 0.05% Tween 20, blocked with blocking solution (2% bovine serum albumin (BSA)+0.05% Tween 20 in TBS) for 1 h, and

incubated with different concentrations of purified adaptor proteins in binding buffer (0.5% BSA+0.05% Tween 20 in TBS) overnight at 4°C. The binding of adaptor protein to Ad5 viruses was detected by incubating with anti-His tag antibody and AP-conjugated corresponding secondary antibody, followed by color reaction that detects AP activity. The color reaction was performed using *p*-nitrophenyl phosphate (Sigma, St Louis, MO, USA) as recommended by the manufacturer, and absorbance at 405 nm (OD<sub>405</sub>) was obtained using a microplate reader (Molecular Devices, Menlo Park, CA, USA).

#### Western blotting assay

In the assay, protein samples were boiled in Laemmli sample buffer, separated on 4–15% polyacrylamide gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was then incubated with blocking solution containing 5% skim milk and 0.05% Tween 20 in TBS for 1 h at room temperature and processed for incubation with primary antibody against CAR or MTf. After washing with TBS and re-blocking, the membrane was incubated with HRP-conjugated secondary antibody for 2 h at room temperature. After extensive washing, the immunoreactive bands were detected by enhanced chemiluminescence plus Western blotting detection system as recommended by the manufacturer (Amersham Biosciences, Piscataway, NJ, USA).

#### In vitro BBB model and transcytosis assay

The *in vitro* BBB model was established by culturing BBMVEC cells in a transwell system, in which the BBMVEC cells were grown on the transwell inserts (polyester, 3.0 μm pore size and 12 mm diameter, Costar, Corning Incorporated, Acton, MA, USA). To establish a well-sealed endothelial barrier, the cells were continued culturing for 5–7 days after confluency, with media refreshed every other day. In the meantime, the TEER was monitored using millicell-ERS apparatus (Millipore). As the TEER is inversely proportional to the area of the tissue, the standard practice is to report TEER as the product of the resistance (Ω) and the growth area (cm<sup>2</sup>). In this study, the TEER of each BBB was obtained by subtracting the background resistance (inserts without cells) from the measured barrier resistance, then multiplying by the growth area of 12-mm inserts (1.13 cm<sup>2</sup>). Transcytosis assay was performed when TEER reached 100–150 Ω cm<sup>2</sup>, the maximal TEER the BBB models could reach in our study.

The transcytosis assay was performed as following, which was modified from previous studies.<sup>11,18</sup> In the assay, the BBMVEC cells grown in the transwell system were pre-incubated in Ringer-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) solution for 2 h at 37°C, 5% CO<sub>2</sub>. The virus–adaptor protein complex was formed by mixing 10<sup>8</sup> VPs of Ad5 with 1 μg of adaptor proteins in 250 μl of Ringer-HEPES solution and incubating at 37°C for 15 min. The virus–protein complex was then added to the apical chamber of each well containing the BBB model, in which the basal chamber contains 700 μl of Ringer-HEPES solution. The cells were incubated again in a 37°C, 5% CO<sub>2</sub> humidified incubator for

6 h. At the end of the experiments, the samples from basal chambers were collected to analyze transcytosed VPs and proteins. Samples from the apical chambers and cell layers were also collected and analyzed.

To analyze the presence of VPs, 200 μl of the samples were used to extract DNA, and processed for real-time quantitative PCR that assessed the Ad5 E4 copy number. For Western blotting assay, the samples were concentrated to about 100 μl with protein concentrator (10-kDa MWCO, Sartorius, Vivascience, Edgewood, NY, USA), then processed for Western blotting assay as described above.

#### Gene transfer assay

Gene transfer efficacy of the transcytosed Ad5 vectors was assessed in A549 cells by measuring luciferase activity, essentially as described previously.<sup>46</sup> In brief, 50 μl of each sample from the basal chambers after transcytosis assay were diluted in 100 μl culture media containing 2% FCS, and used to infect A549 cells plated in a 48-well cell culture plate. Two hours later, 150 μl of complete culture media containing 10% FCS were added into each well, and the cells were continued in culture for 24 h in a 37°C, 5% CO<sub>2</sub> humidified incubator. To measure the luciferase activity, the cells were washed with PBS, and lysed by one freeze-thaw cycle in 100 μl of reporter lysis buffer (Promega, Madison, WI, USA). Ten microliters of each sample were used to measure the luciferase activity using a luciferase assay kit (Promega) and a luminometer (Berthold, Gaithersburg, MD, USA). The total amount of protein in each sample was determined by Bro-Rad DC protein Assay kit (Bio-Rad).

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