Adenovirus Type 5 Substituted with Type 11 or 35 Fiber Structure Increases its Infectivity to Human Cells Enabling Dual Gene Transfer in CD46-dependent and -independent Manners

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Abstract. *Infectivity of adenovirus type 5 (Ad5) to cells depends primarily on its fiber-mediated binding to the coxsackievirus and adenovirus receptor (CAR) on target cells. Down-regulated CAR expression, often found in human tumors, hampered Ad5-mediated gene transfer. Ad 11 and Ad 35, belonging to a subtype B group, use CD46 as their cellular receptors; accordingly, chimeric Ad5 whose fiber structure was substituted with that of the type 11 or 35 (Ad5/11 or Ad5/35) could infect human cells in a different manner from Ad5. We found that Ad5/35 infected human tumors, including pancreatic and breast cancer, and human fibroblasts better than Ad5 and Ad5/11. Infectivity of Ad5/35 to these cells was correlated with that of Ad5/11, but efficacy of Ad5/35- and Ad5/11-mediated transduction was not directly correlated with the expression level of CD46 in the target cells. Infection of human hepatoma cells with measles virus, whose cellular receptor is CD46, down-regulated the CD46 expression and reduced subsequent infectivity of Ad5/35 but not Ad5/11. Infection of Ad5 suppressed subsequent gene transfer by Ad5 but not by Ad5/11 or Ad5/35. Likewise infection of Ad5/35 decreased following gene transduction by Ad5/35 and Ad5/11, but to a lesser extent by Ad5. These data collectively showed that combinatory use of Ad5 and the chimeric Ad enables dual gene transfer into target cells and suggest that infectivity of subtype B Ad does not completely depend on CD46 expression and that other receptors possibly influence the infectivity.*

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Adenovirus (Ad) vector can efficiently transduce target cells with an exogenous gene and in particular Ad type 5 (Ad5), belonging to subtype C, has been widely used for gene therapy. Attachment of Ad5 to target cells is mediated primarily by the binding of Ad fibers, which include the shaft and the knob portion, to a cellular receptor, the coxsackievirus and adenovirus receptor (CAR), and secondly by the interaction between Ad penton base and integrins on target cells (1). Since the cellular tropism of Ad5 depends essentially on the interaction between the fiber and CAR, the expression level of CAR influences the specificity and the efficiency of Ad5-mediated gene transduction (2). CAR is ubiquitously expressed in epithelial cells; however, CAR expression is often down-regulated in various types of clinical tumor specimens partly due to activation of the RAF-MEK-ERK pathway in tumors (3, 4). Previous studies also suggested that progression and aggressiveness of various tumors were correlated with suppressed CAR expression (4, 5). The down-regulated CAR expression in tumors therefore hampered efficient Ad5-mediated transduction and compromised the utility of Ad5 vector as gene therapy for cancer.

Ad11 and Ad35, which belong to the subgroup B and infect respiratory and urinary tracts in humans, attach cells in a CAR-independent manner (6); thereby, the repertoire of Ad11- or Ad35-infected cells can be different from that of Ad5-infected cells. Previous studies showed that chimeric Ad5 vector whose fiber portion was replaced by that of Ad11 or Ad35 (Ad5/11 and Ad5/35) infected target cells with the same efficacy as Ad11 or Ad35 (7, 8). The chimeric Ad can thus be an alternative vector for transducing human cells, in particular low CAR-expressing cells, which are resistant to Ad5 infection. In fact, the chimeric Ad could infect human $CD34^+$ cells more efficiently than Ad5 and is a possible vector for hematopoietic stem cells with low CAR expression (7, 8). Recent reports also demonstrated that the cellular

receptor of Ad11 and Ad35 is CD46 (9, 10). CD46 is a complement-regulating protein serving as a cofactor for factor I-mediated degradation of C3b/C4b complement and is also a receptor for human herpesvirus 6 and measles virus (11). Since CD46 is expressed in a variety of human cells, Ad11 and Ad35-derived vector can deliver aimed gene(s) to many types of target tissues. However, the infectivity of Ad11 and Ad35 to human tumors, particularly in comparison with normal cells, has not been extensively analyzed. In this study, we compared the infectivity of Ad5 with that of the chimeric Ad5 bearing either the Ad11 or the Ad35 fiber to human pancreatic and breast cancer cells, and normal fibroblasts. We also investigated the relationship between the CD46 expression and infectivity of the chimeric Ad and examined whether down-regulated CD46 expression with measles virus (12) could modulate infectivity of the chimeric Ad.

Materials and Methods

Cells. Human embryonic kidney 293 cells, human pancreatic cancer MIA-PaCa-2, BxPC-3, Panc-1 and AsPC-1 cells, human breast cancer MCF-7, OCUBM, MDA-MB-231, MDA-MB-436 and T47D cells, human normal fibroblasts HFF, OUMS-24 and MRC-5 cells, and human hepatoma HuH-7 cells were cultured with RPMI-1640 medium supplemented with 10% fetal calf serum.

Northern blot analysis. Extracted RNA (20 μg) was subjected to electrophoresis on a formaldehyde-agarose gel and transferred to a nylon filter. The filter was hybridized with [α-^{32}P]dCTP-labeled CAR cDNA in a PerfectHyb solution (Toyobo, Osaka, Japan) at 68˚C for 1 h, and then washed with a solution of 0.2 X SSC/0.1%SDS at 65˚C. The filter was rehybridized with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA as a control.

Infection with Ad and flow cytometry. E1- and E3-deleted Ad5, Ad5/11 and Ad5/35 in which the green fluorescent protein (GFP) gene was activated with cytomegalovirus promoter (Ad5GFP, Ad5/11GFP and Ad5/35GFP) (7, 8) were provided by Avior Therapeutics (Seattle, WA, USA). Cells were infected with Ad at a multiplicity of infection (MOI) of 3 or 30 for 30 min and were washed to remove Ad. They were cultured for 2 days and then analyzed for their GFP intensity with FACScan (Becton Dickinson, Mountain View, CA, USA) with CellQuest software (Becton Dickinson). Expression of CD46 was examined with fluorescein isothiocyanate (FITC)-conjugated anti-human CD46 antibody (PharMingen, San Diego, CA, USA). The expression level of CD46 was expressed as the mean fluorescence value subtracted from that of cells stained with FITC-conjugated, isotype-matched control antibody. Cells whose fluorescence was greater than the brightest 5% of uninfected cells were judged as positively stained. Correlation coefficiency was calculated based on the percentages of GFP-positive cells and arbitrary units of mean fluorescence intensity of CD46.

Blocking of Ad infection. For Ad-mediated inhibition, HuH-7 cells were infected with E1- and E3-deleted Ad5 bearing β-galactosidase gene (Ad5LacZ) at an MOI of 30 or Ad5 with type 35 fiber and β-galactosidase gene (Ad5/35LacZ) at an MOI of 100 for 30 min and then incubated with Ad5GFP, Ad5/11GFP or Ad5/35GFP at an MOI of 10 for 30 min. For blocking of CD46-mediated infection, HuH-7 cells were incubated with 10 mg/ml anti-CD46 antibody (PharMingen) and then infected with Ad5GFP, Ad5/11GFP or Ad5/35GFP at an MOI of 10 for 30 min. For measles virus-mediated inhibition of Ad infection, HuH-7 cells were incubated with the Edmonston strain of measles virus (11) at an MOI of 1 for 2 days and then the measles virus was removed. HuH-7 cells were then incubated with Ad5GFP, Ad5/11GFP or Ad5/35GFP at an MOI of 10 for 30 min. After the infection with three types of Ad, HuH-7 cells were washed to remove the Ad and were analyzed for the GFP fluorescence intensity 2 days later.

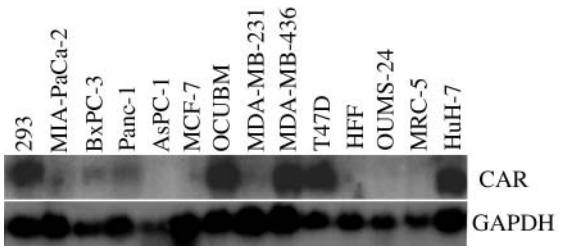

Figure 1. *Northern blot analysis of the CAR expression in human pancreatic and breast cancer cells, and normal fibroblasts. HuH-7 and 293 cells were used as representative CAR-positive cells. The same filter was also hybridized with GAPDH cDNA as a control.*

Results

Expression of CAR in human cells. We examined the expression of CAR in human pancreatic and breast cancer cells, and fibroblasts (Figure 1). The expression level varied among the cells tested and the expression in all the pancreatic cancer cell lines and fibroblasts was relatively low compared with that of 293 and HuH-7 cells, both of which expressed CAR at a high level. Three kinds of breast cancer cells, OCUBM, MDA-MB-436 and T47D, expressed CAR at a similar level as 293 and HuH-7 cells, but the expression level of MCF-7 and MDA-MB-231 cells was as low as that of pancreatic cancer cells and fibroblasts.

Infectivity of Ad5 and chimeric Ad. We compared the transduction efficiency of Ad5GFP, Ad5/11GFP and Ad5/35GFP with fluorescence intensity, since the promoter used for GFP expression was identical and the fluorescence intensity was thereby influenced only by Ad infectivity (Figure 2). HuH-7 cells were used as representative targets of Ad5-mediated infection. All the pancreatic cancer cells tested were relatively resistant to Ad5GFP-mediated infection but were susceptible to Ad5/35GFP- and to lesser extent to Ad5/11GFP-mediated infection. Infectivity of Ad5GFP to breast cancer cells was in general greater than that to pancreatic cancer cells, and was not precisely concordant with the CAR expression level (Figure 1). Infectivity of Ad5GFP

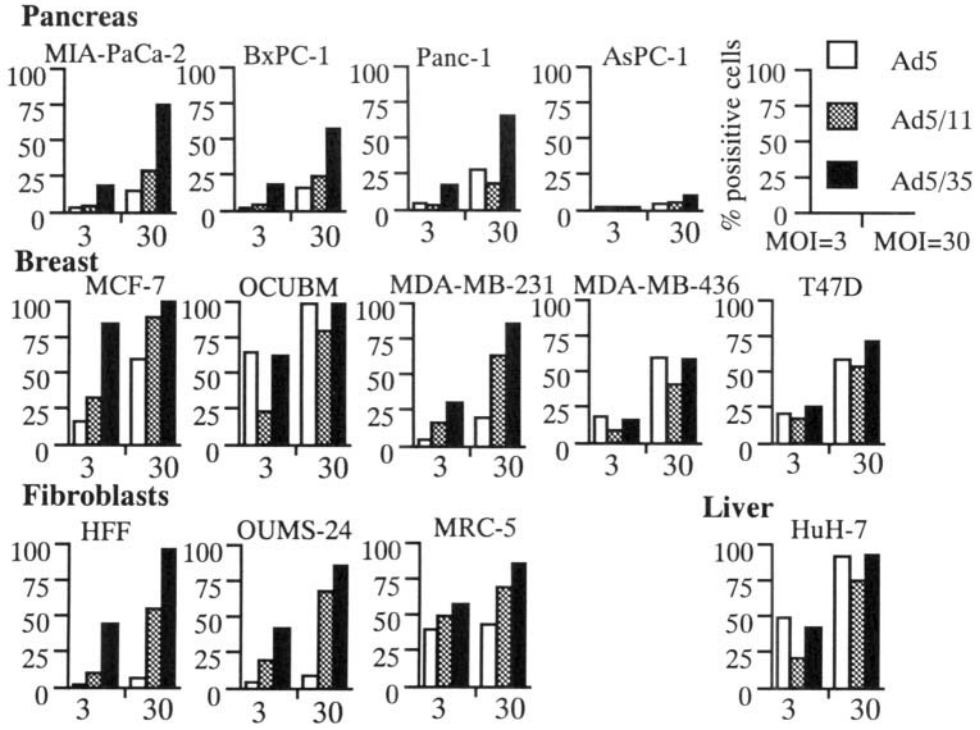

Figure 2. *Percentage of GFP-positive cells as examined with flow cytometry. Pancreas and breast cancer cells, hepatoma cells and fibroblasts were infected with Ad5GFP, Ad5/11GFP or Ad5/35GFP at a MOI of 3 or 30.*

and Ad5/35GFP was not different among CAR-high OCUBM, MDA-MB-436 and T47D cells. In normal fibroblasts, both Ad5/11GFP and Ad5/35GFP infected better than Ad5GFP, in particular in HFF and OUMS-24 cells. The infectivity of Ad5/35 was greater than Ad5/11 in all the cells tested. These data collectively showed that the chimeric Ad infected pancreatic cancer cells and fibroblasts better than Ad5 did, and that infectivity of the chimeric Ad to breast cancer cells was comparable to that of Ad5.

Relationship between Ad infectivity and CD46 expression. We examined the correlation of infectivity among the Ad vectors by comparing the GFP brightness in respective cells (Figure 3). The transduction efficiency of Ad5GFP was weakly related to that of Ad5/11GFP ($p=0.032$) but not to Ad5/35GFP ($p=0.097$) and the efficacy of A5/11GFP was strongly related to that of Ad5/35GFP ($p<0.01$). A flow cytometrical analysis also showed that all the cells tested were positive for CD46 expression to different degrees (data not shown). The CD46 expression level was not correlated with infectivity of any kind of Ad (Ad5: $p=0.20$; Ad5/11: $p=0.46$; Ad5/35: $p=0.14$).

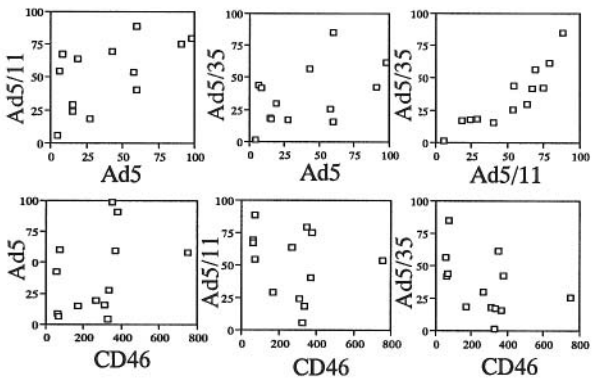

Figure 3. *Correlation of Ad infectivity and CD46 expression. Expression level of CD46 was expressed as an arbitrary unit of mean fluorescence intensity and infectivity of Ad was shown as percentage of GFP-positive cells (MOI for Ad5/35GFP: 3, MOI for Ad5GFP and Ad5/35GFP: 30). Squares represent MIA-PaCa-2, BxPC-3, Panc-1, AsPC-1, MCF-7, OCUBM, MDA-MB-231, MDA-MB-436, T47D, HFF, OUMS-24, MRC-5 and HuH-7 cell lines.*

Blocking of Ad infectivity. We examined whether Ad5 or Ad5/35 infection would inhibit subsequent gene transfer mediated by Ad5, Ad5/11 or Ad5/35. Infection with Ad5LacZ reduced GFP-positive HuH-7 cells that were subsequently infected with Ad5GFP but not Ad5/11GFP or Ad5/35GFP (Figure 4A). Simultaneous infection of HuH-7

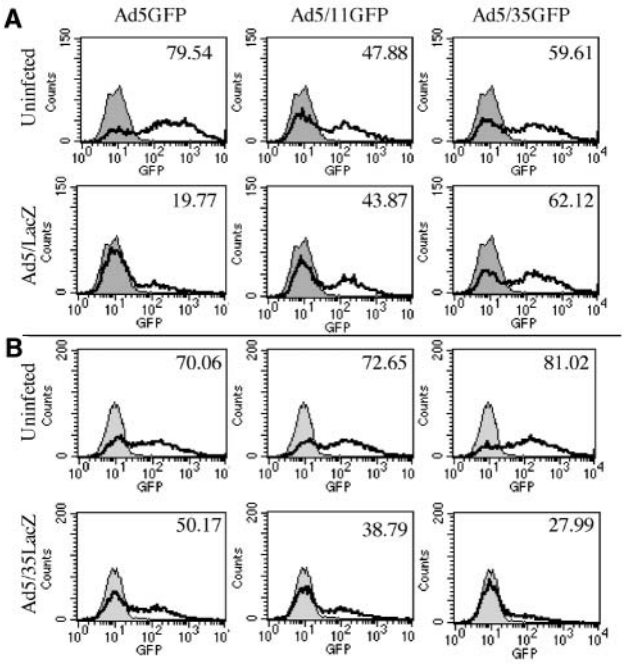

Figure 4. *Influence of Ad infection on subsequent gene transduction by Ad5LacZ or Ad5/35LacZ. HuH-7 cells were infected with (A) Ad5LacZ (MOI=30) or (B) Ad5/35LacZ (MOI=100), or uninfected, and then infected with Ad5GPF, Ad5/11GFP or Ad5/35GFP (MOI=10). Uninfected HuH-7 cells are shown as shaded areas and the percentage of GFP-positive cells is also shown.*

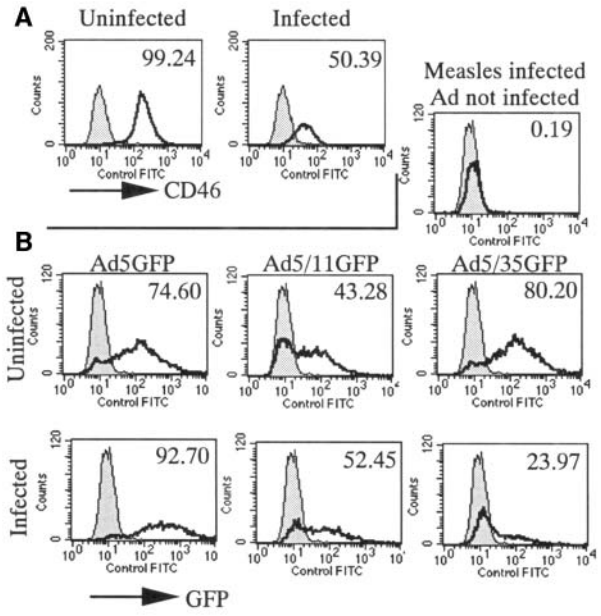

Figure 5. *Influence of measles virus infection on (A) CD46 expression and (B) subsequent Ad infectivity. HuH-7 cells were infected with measles virus (MOI=1) and then (A) stained with anti-CD46 antibody or control antibody (shaded) or (B) subsequently infected with Ad5GFP, Ad5/11GFP or Ad5/35GFP (MOI=10). Uninfected cells are shown as shaded areas and the percentage of CD46- or GFP-positive cells is shown.*

cells with both Ad5LacZ and Ad5GFP also reduced GFP-positive cell numbers (data not shown) while infection with Ad5LacZ alone did not affect autofluorescence of HuH-7 cells (data not shown). Precedent infection with Ad5/35LacZ inhibited subsequent gene transfer by Ad5/11 and Ad5/35, but the inhibition of Ad5-mediated gene transfer was not as great as that of Ad5/11 or Ad5/35 (Figure 4B). Treatment of HuH-7 cells with adequate amounts of anti-CD46 antibody to inhibit measles virus infection (11), however, did not influence GFP-positive cell numbers irrespective of Ad types infected (data not shown).

We infected HuH-7 cells with the Edmonston strain of measles virus, whose cellular receptor is CD46, and then examined the subsequent CD46 expression level (Figure 5A). Infection with measles virus down-regulated CD46 expression. We then infected HuH-7 cells with the three kinds of Ad under this condition (Figure 5B). The number of GFP-positive cells was greater with Ad5GFP or Ad5/11GFP infection but significantly lower with Ad5/35GFP infection.

Discussion

In this study, we investigated the infectivity of Ad5 and fiber-modified chimeric Ad to human pancreatic and breast cancer cells, and fibroblasts. Although Ad5-mediated gene transduction to these tumors has been reported (13, 14), Ad5 infectivity to the tumors in relation to their CAR expression was not extensively examined. Moreover, transduction of the tumors with such retargeting Ad has not been well investigated except in a few studies in which Ad5/35 type vector transduced a gene in a breast cancer cell line better than Ad5 vector (15). The present study demonstrated that all the pancreatic cancer cells tested expressed CAR at a relatively low level but some breast cancer cell lines expressed CAR at a comparable level to 293 and HuH-7 hepatoma cells. The CAR expression level did not directly correlate with the infectivity of Ad5 in the respective cell lines; however, Ad5-mediated transduction was in general greater in breast cancer than in pancreatic cancer cells. Susceptibility of fibroblasts to Ad5 was variable and was not correlated with their CAR expression. Previous studies showed that expression of CAR transcripts was concordant with CAR protein level (16) and a correlation between CAR expression level and Ad5 infectivity was generally observed (4). However, our present results did not support such a correlation probably because the contribution of CAR-fiber independent pathways to Ad5-mediated infectivity varied in the tumors tested.

Ad5/35GFP infected tumors and fibroblasts better than Ad5GFP did, and Ad5/11GFP showed that the transduction efficacy was comparable to that of Ad5GPF in most of the cell lines tested. Use of the Ad11 or Ad35 fiber structure could increase the infectivity to CAR-low or CAR-negative tumors; however, it also enhanced the infectivity to normal fibroblasts. This raises a safety issue regarding damage of normal tissues in the vicinity of tumors when the chimeric Ad is administered into tumors *in vivo*. Recombinant Ad5 bearing the Ad11 or Ad35 fiber structure thereby needs transcriptional regulation of an exogenous gene to improve the safety and consequently the efficacy of Ad-mediated gene therapy for cancer.

Recently, CD46 was reported as a cellular receptor for Ad11 and Ad35 because forced expression of the *CD46* gene rendered non-permissive cells susceptible to Ad11- and Ad35-mediated infection (9, 10); however, our results suggested that CD46 was not a unique receptor for both types of Ad. Firstly, a significant relation between the CD46 expression level and infectivity of Ad5/11 or Ad5/35 was not observed. This could be due to differential transcriptional activity of cytomegalovirus promoter in cells and to differential threshold levels of Ad infectivity. Secondly, infection with measles virus, which down-regulated the CD46 expression, suppressed Ad35- but not Ad11-mediated transduction. Although the meales virus-mediated down-regulation of CD46 was not complete, the down-regulated CD46 level was concordant with the previous observation (12). Thirdly, treatment of HuH-7 cells with anti-CD46 antibody, which inhibits the binding of measles virus (12), did not influence the infectivity of the chimeric Ad. The correlation analysis of Ad infectivity, however, also suggested the complexity of their cellular receptors. We observed positive correlation between Ad5- and Ad5/11-mediated and between Ad5/11- and Ad5/35-mediated but not between Ad5- and Ad5/35-mediated infectivity. Moreover, the present study showed that measles virus possibly shared the receptor with Ad5/35 but not with Ad5 or Ad5/11. These data collectively suggested that Ad5, Ad11 and Ad35 might in part share putative common receptors, although they have unique receptors besides the shared receptor(s). CD46 is indeed one of the cellular receptors for Ad11 and Ad35, and CD46 has been reported to associate with other molecules to constitute the CD46 complex (17, 18). Viral cellular receptors are relatively complex; Ad5 has an additional receptor besides CAR (19) and the Edmonston strain can also bind target cells through CD150 as well as CD46 (12).

Although properties of the receptor complexes for Ad11 and Ad35 need further investigations, the present study demonstrated possible gene transduction with dual viral infection. Ad5-mediated transduction did not inhibit subsequent Ad5/11 or Ad5/35 infection, and measles virus infection did not disturb subsequent Ad5- and Ad5/11-mediated transduction. Precedent Ad5/35 infection down-regulated A5/11- and Ad5/35-mediated gene transfer but to a lesser extent Ad5-mediated transduction. These data suggest possible multiple gene delivery to target cells with combinatory use of Ad5 and the chimeric Ad. Preexisting immunity to Ad5 may restrict the use of the vector for gene therapy; thereby, the chimeric Ad, even though the fiber structure does not constitute major epitopes for immunogenicity, can circumvent immune responses to some extent which decrease the efficacy of Ad-mediated gene delivery. In fact, the population positive for anti-Ad11 or anti-Ad35 antibody was much smaller than that bearing anti-Ad5 antibody (20). Since recombinant measles virus can also be a possible vector (21), the present study implies a possible combinatory usage of Ad5 or Ad5/11 and measles virus. Recent studies suggested that replication-competent Ad, whose early immediate genes are regulated by tumor promoters, can specifically destroy tumors (22). Replication-competent oncolytic Ad whose fiber structure is modified to type 11 or type 35 can infect tumors better than Ad5 and consequently has greater cytotoxic potency with lower MOIs. The fiber-modified Ad, designed to replicate specifically in tumors with tumor- or tissue-specific transcriptional activation units, would be a potential therapeutic tool for local cancer treatment.

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