
Cationic lipid-mediated transfection of bovine aortic endothelial cells inhibits their attachment

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Abstract: The need for a small-diameter vascular graft for coronary artery and peripheral vascular replacement is great and is projected to increase as the population ages. Synthetic small-diameter vascular grafts fail because of acute thrombosis or chronic intimal hyperplasia leading to restenosis. Endothelial cell seeding has been attempted with limited success in the femoral artery by Zilla and others. However, patency rates have not increased sufficiently to justify large clinical trials. Genetic engineering of endothelial cells before seeding has been proposed to encourage endothelial cell phenotypes that would predispose the graft to patency. In this study, we investigate the effect cationic lipid-mediated transfection of endothelial cells with respect to their attachment to a potential graft material, Fluoropassiv™ (Vas-

cutek). Liposomal transfection was optimized for maximum gene expression. We report that transfection decreases the ability of bovine aortic endothelial cells to attach by approximately 100% as compared with nontransfected control over 18 h. Further, when placed under physiologic shear conditions, this difference is sustained. The effects of gene transfer on endothelial cell adhesion must be included as an important optimization criterion along with gene expression for engineered endothelial cell-seeding applications. © 2002 Wiley Periodicals, Inc. *J Biomed Mater Res* 60: 405–410, 2002; DOI 10.1002/jbm.10062

Key words: vascular grafts; cell seeding; genetic engineering; fluoropolymer; cell adhesion

INTRODUCTION

The development of a synthetic small-diameter vascular graft for coronary and peripheral bypass is a critical need. The challenges confronting the engineering of vascular replacements stem from direct contact of blood with synthetic graft materials.¹ Early materials, such as polyurethanes² and poly(ethylene terephthalate) (PET),³ did not consider hemocompatibility. These grafts served merely as conduits for the blood flow without presenting a surface that would inhibit platelet activation.³ Relatively inert and nonactivating materials such as expanded polytetrafluoroethylene (ePTFE) are largely resistant to the effects of the initiation of thrombosis.⁴ With the use of ePTFE, the effects of acute failure were ameliorated. Chronic failure, based in medial smooth muscle activation, proliferation, and migration into the graft lumen, has

become the major reason for small-diameter graft failure.^{5,6}

Fluoropassiv™, a proprietary material from Vascutek, is a PET weave coated with fluoropolymer thus combining the surface chemistry of ePTFE with the bulk characteristics of PET.⁷ Testing of this material in animal models shows that patency is increased over the use of either of the materials separately.⁸ However, although higher patency is achieved in small-diameter applications when compared with other materials, the increase is not significant enough to justify clinical trials in the coronary bypass application.

Genetic engineering and seeding of endothelial cells has been suggested as a method to create a new vascular graft lining that predisposes the graft to patency.⁹ We have previously reported that gene therapy, particularly the transfection of endothelial cells with the endothelial nitric oxide synthase gene, increases the ability of the endothelial cell to inhibit the early pro-thrombotic platelet aggregation as well as smooth muscle cell proliferation.¹⁰

Retroviral methods have distinct advantages over liposomal methods including stable transduction due to genetic incorporation of the gene of interest into the host genome. Retroviruses have been the method of choice for gene therapy in clinical trials.¹¹ However, their use may decrease transduced cell's adhesive

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properties in attachment-dependent cells.¹² Adenoviruses often produce toxicity, limiting their utility in clinical applications.^{13,14}

Cationic liposomal transfection, a nonviral technique, presents distinct advantages over retroviruses, especially in applications in which the length of expression is not critical. These advantages include the lack of insertional mutagenesis, and a higher level of expression of the desired genes (see Bellamkonda, et al.⁵). Although liposomal transfection may elicit limited toxicity to transfected cells, their use is generally attractive in clinical applications because of the lack of risk of insertional mutagenesis and potential activation of proto-oncogenes. However, the effects of cationic liposome-mediated genetic engineering on cell adhesion have not been fully considered because typically optimization of transfection involves only the expression level of the desired protein, and not attachment.

In the present study, the ability of Fluoropassiv™ graft material to sustain attachment of endothelial cells transfected with cationic liposomes was probed. Green fluorescent protein (GFP) was used as the gene product in this study, because it has no known impact on cellular adhesion. The enhanced GFP (eGFP) vector (GenBank accession number U55762; Clontech) contains a human cytomegalovirus (CMV) promoter that drives the eGFP gene and produces the green fluorescent protein (excitation maximum 488 nm, emission maximum 507).^{15,16} Adhesion studies were conducted in both the presence and absence of physiological shear. Tissue culture polystyrene was used as a control biomaterial.

MATERIALS AND METHODS

Cell culture

Bovine aortic endothelial cells (BAEC) were isolated under standard protocols¹⁷ and used between passages eight and sixteen. Endothelial cell culture was maintained under standard conditions of 5% CO₂, 95% humidity, and 37°C. BAEC were cultured in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone), and 1 µg/mL penicillin-streptomycin (Life Technologies).

Endothelial cell transfection

Transfection of BAEC was mediated by the cationic liposome formulation, 2,3-dioleoyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium-trifluoroacetate and dioleoyl phosphatidylethanolamine (3:1 w/w ratio, Lipofectamine®; Life Technologies). Briefly, endothelial cells were seeded into T-75 flasks (Costar, Cambridge, MA) at 30% confluence. They were allowed to attach

and divide to 50–70% confluence and were transfected using 12 µg/mL Lipofectamine® (Life Technologies) and 15 µg eGFP-N1 plasmid DNA (Clontech) for 6 h in 12 mL of OptiMEM® (Life Technologies). This ratio was found to be optimal in producing the largest percentage of transfected endothelial cells in previous experimentation in our laboratory (30%, data not shown). The transfected endothelial cells were allowed 24 h of recovery before seeding onto test materials.

Light and fluorescence microscopy assessment of morphology was performed using a Nikon Eclipse TE300 microscope. Images were obtained through an Optronics Magnafire digital camera.

Static cell culture on Fluoropassiv™ material

Transfected and nontransfected BAEC were used in the assessment of the ability of Fluoropassiv™ material to support adhesion and growth. Discs of Fluoropassiv™ were placed in 24-well tissue culture plates (Costar) and secured using a ring of silicone rubber tubing (3/8" inner diameter; Cole Palmer, Chicago, IL). The entire plate was subjected to ethylene oxide sterilization. The discs were then coated with human plasma fibronectin (0.5 µg/cm²; Chemicon, Temecula, CA).

Twenty-four hours after transfection, the endothelial cells were removed from their flasks and seeded onto fibronectin-coated Fluoropassiv™ discs. Each well was seeded with 100,000 nontransfected or transfected BAEC. The area of each well was 0.71 cm². At 2, 18, 24, 48, and 72 h after seeding, the medium bathing the wells was removed. This medium containing the detached cells was fixed in a phosphate buffered saline (PBS) containing 2% glutaraldehyde and 3% sucrose. Detached BAEC were counted by particle count (Coulter ZM).

Static cell culture on tissue culture polystyrene

Transfected and nontransfected BAEC were also seeded onto tissue culture polystyrene as a control for the Fluoropassiv™ material described above. Wells of a 24-well tissue culture plate (Costar) were coated with human plasma fibronectin (0.5 µg/cm²; Chemicon). A sterile ring of silicone rubber tubing (3/8" inner diameter; Cole Palmer) was placed into each well to provide equal surface areas as those provided in the section above.

Twenty-four hours after transfection, the endothelial cells were removed from their flasks and seeded. Each well was seeded with 100,000 nontransfected or transfected BAEC. The area of each well was 0.71 cm². Detached BAEC were counted by particle count (Coulter ZM). At 2, 18, 24, 48, and 72 h after seeding, the medium bathing the wells was removed. This medium containing the detached cells was fixed in a PBS containing 2% glutaraldehyde and 3% sucrose.

Application of shear force on seeded Fluoropassiv™ discs

Figure 1 describes the protocol for the experimentation performed. Eighteen hours after seeding, Fluoropassiv™

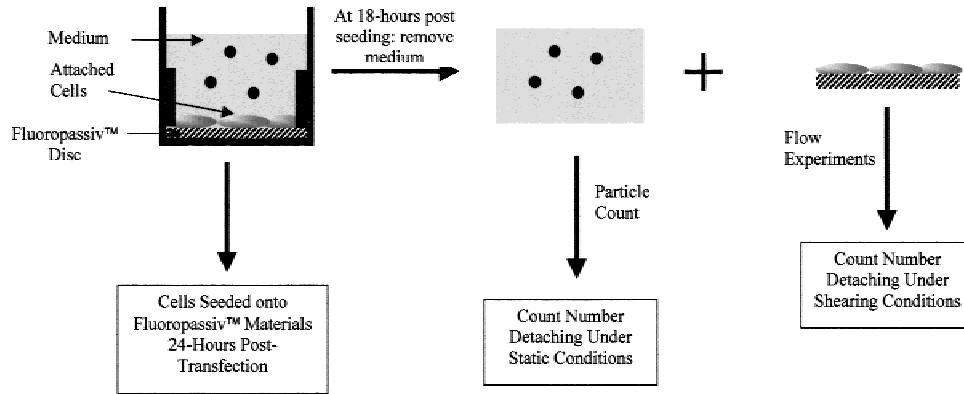


Figure 1. Nontransfected and eGFP-transfected BAEC were seeded and allowed to attach on Fluoropassiv™ discs for 18 h. The supernatant containing the detached cells was removed and counted. Fluoropassiv™ discs with remaining attached cells were subjected to shear stress (4.8 dynes/cm² produced by the flow of PBS at 120 mL/min). BAEC that detached under shear were counted using a Coulter ZM particle counter.

discs with nontransfected and GFP-transfected cells were subjected to physiological shear. Endothelial cells were seeded onto Fluoropassiv™ discs as described previously. Briefly, seeded Fluoropassiv™ discs were carefully placed into a parallel plate flow chamber (courtesy of Dr. Roger Marchant, Case Western Reserve University).¹⁸ The flow chamber was designed to produce laminar flow across the surface of the test material. Silicone tubing (1/4"; Cole Palmer) was used for the circulation of approximately 6 mL of PBS (Life Technologies). A roller pump (model 7519-50; Cole Palmer) was used to produce laminar flow with two cartridges out of phase to dampen pulsatility. The flow rate was determined by fluid displacement over time and shear force applied to the Fluoropassiv™ discs was calculated theoretically.

Shear forces of 4.8 dynes/cm² were applied to the seeded graft material for 5 min. This corresponded to a flow rate of 120 mL/min. After 5 min, the fluid in the flow cell was removed and placed into a 15-mL centrifuge tube (Falcon) and was centrifuged at 910g (IEC MP4R) for 3.5 min to pellet the cells. The pelleted cells were resuspended in 2% glutaraldehyde solution and counted (Coulter ZM).

RESULTS

Endothelial cell transfection produces eGFP expression

A typical transfection of endothelial cells achieved approximately 30% positive transfectants as assessed by count under fluorescence microscopy (Nikon Eclipse TE300). No significant cell death was observed in the transfected flasks as compared with the nontransfected flasks (data not shown). Furthermore, morphology of transfected BAEC resembled that of nontransfected BAEC when observed under light and fluorescent microscopy, as shown in Figure 2.

Static cell culture

Detached BAEC were counted by particle count (Coulter ZM). Detached cells were counted instead of

attached cells to separate the effects of cell division from the absolute counts. To minimize uncounted cells and produce accurate cell counts, the spaces underneath the graft material and the silicone tubing were checked for any cell attachment by trypsinization and counting. No significant cell population was seen in either area (data not shown). The numbers of detached cells stated in this section are based on an initial seeding number of 100,000 BAEC. Table I presents data regarding attachment of nontransfected and GFP-transfected BAEC to both tissue culture polystyrene and Fluoropassiv™ discs.

Attachment time course on Fluoropassiv™ material

The number of nontransfected and transfected cells detached from Fluoropassiv™ discs at 2, 18, 24, 48, and 72 h was assessed by Coulter count. Figure 3 graphically summarizes these data. The time $t = 0$ represents 24 h after transfection and exposure of the cells to lipid and start of seeding onto Fluoropassiv™ discs. Data are presented as mean \pm SEM.

At 2 and 18 h, there was a statistically significant difference in the number of detached cells between nontransfected and GFP-transfected BAEC ($n = 12$ and $p < 0.05$). This difference between nontransfected and GFP-transfected BAEC disappeared from the 24- to the 72-h time points (Fig. 3).

Attachment time course on tissue culture polystyrene

The number of nontransfected and transfected cells detached from tissue culture polystyrene dishes (Costar) at 2, 18, and 24 h was assessed by Coulter count. Figure 4 is a graphical representation of these data.

The differences between nontransfected and GFP-transfected BAEC were statistically significant ($p <$

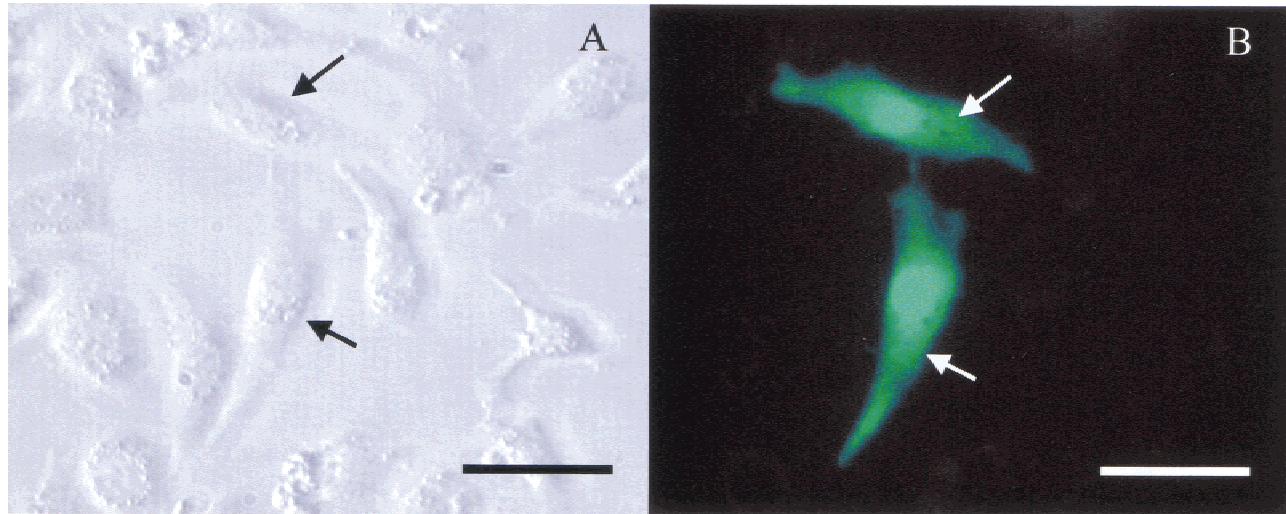


Figure 2. Morphology of BAEC before and after transfection. BAEC were assessed by light and fluorescence microscopy after GFP transfection for morphological changes due to transfection. (A) Both transfected and nontransfected BAEC under light microscopy. Arrows mark two transfected BAEC. (B) GFP-transfected cells under fluorescence microscopy. The arrows mark the same two cells from (A). Note that GFP is a cytosolic protein and therefore does not mark extensions of endothelial cells as brightly as other areas of the cell. Also, note that the morphology of the eGFP expressing cells is similar to that of eGFP nonexpressing cells. Scale bars represent 10 μm . [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

0.05) at the 2- and 18-h time points. Statistical significance between nontransfected and transfected BAEC was lost at 24 h (Table I). However, the number of detached BAEC in either the nontransfected or the transfected sets was small with a maximum of approximately 2000 cells per 100,000 cells seeded. There was no statistically significant increase in detachment between consecutive time periods for either the nontransfected or the transfected cells.

Effects of transfection on BAEC attachment under shear

Medium bathing FluoropassivTM discs was removed at 18 h and counted as described (see above for results). The FluoropassivTM discs containing cells that remained attachment at 18 h were exposed to shear. Figure 5 describes data regarding the detachment of BAEC from FluoropassivTM discs on exposure to shear of 4.8 dynes/cm². Of these attached cells, 4554 \pm 617

nontransfected and 6981 \pm 899 GFP-transfected BAEC were removed because of shear. The difference is statistically significant ($n = 11$ and $p < 0.05$).

DISCUSSION

Genetic engineering for cardiovascular seeding applications is a technique that must be assessed for adverse effects that it may have on the ability of engineered cells to adhere to biomaterials. Although cationic liposomes provide an attractive option for *ex vivo* genetic engineering, this method has been previously optimized for only expression level of the inserted gene. Furthermore, transfection efficiencies with the commercial liposomal techniques are below 50% of exposed cells.¹⁹ Expression levels within positive cells are high, and the duration varies from several days to weeks. For vascular graft restenosis, this level of expression duration may be sufficient.²⁰ In this study,

TABLE I
BAEC Attachment to FluoropassivTM and Tissue Culture Polystyrene

Material	Type of Cell Seeded	Number of Detached Cells				
		2 h	18 h	24 h	48 h	72 h
Fluoropassiv TM disc	Untransfected	2470 \pm 260	4118 \pm 452	14290 \pm 4449	15490 \pm 5287	46759 \pm 12268
	Transfected	5720 \pm 379	11098 \pm 1358	12585 \pm 4447	23002 \pm 5524	51292 \pm 12719
Polystyrene	Untransfected	903 \pm 232	662 \pm 148	891 \pm 169		
	Transfected	1773 \pm 293	1717 \pm 213	1184 \pm 411		

Nontransfected and GFP-transfected BAEC were allowed to attach to FluoropassivTM discs and tissue culture wells for 2, 18, and 24 h. BAEC attachment to FluoropassivTM discs was also assessed at 48 and 72 h. Data are presented as mean \pm SEM.

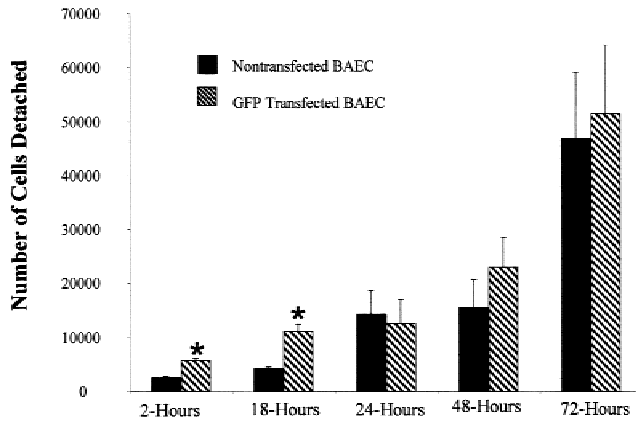


Figure 3. Attachment of BAEC to Fluoropassiv™ material. Both nontransfected (black bars) and GFP-transfected (striped bars) BAEC were seeded onto vascular graft material as described. This figure is a graphical representation of nontransfected and GFP-transfected BAEC detaching from the Fluoropassiv™ substrate at 2- through 72-h time points after seeding. For this experimentation, $n = 12$ and the difference between nontransfected and transfected BAEC was considered statistically significant when $*p < 0.05$ (as assessed by an unpaired Student's t test).

we have assessed the ability of endothelial cells to attach to a vascular graft material surface after cationic liposome-mediated transfection.

At initial time points (2 and 18 h after seeding) GFP-transfected BAEC were about twice as likely to detach from either Fluoropassiv™ discs or tissue culture polystyrene than were nontransfected BAEC. This difference was statistically significant with $p < 0.05$. This would indicate that attachment during this early phase is affected by transfection and not by the substrate, although the absolute numbers of detaching cells depend on the substrate to which they are exposed.

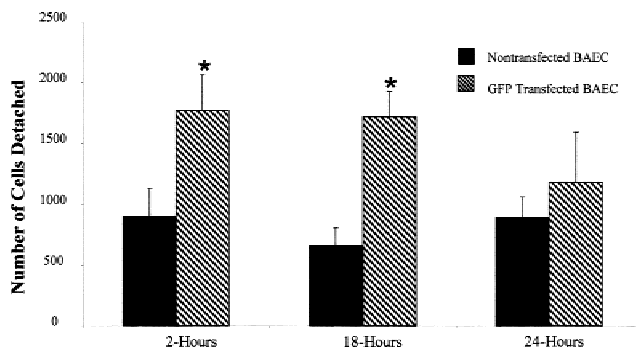


Figure 4. BAEC attachment to tissue culture polystyrene. Assessment of the attachment of nontransfected (black bars) and GFP-transfected (striped bars) BAEC on tissue culture polystyrene was conducted. This figure is a graphical representation of nontransfected and GFP-transfected BAEC detaching from plasma-treated polystyrene (Costar) at 2, 18, and 24 h after seeding. For this experimentation, $n = 6$ and the difference between nontransfected and transfected BAEC was considered statistically significant when $*p < 0.05$ (as assessed by an unpaired Student's t test).

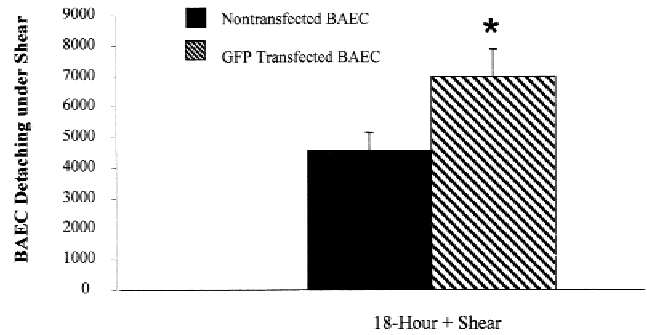


Figure 5. BAEC detachment from Fluoropassiv™ under shear forces. This figure is a graphical representation of nontransfected (black bars) and GFP-transfected (striped bars) BAEC detachment from Fluoropassiv™ under shear. The differences between the groups were statistically significant when $*p < 0.05$, ($n = 12$, as assessed by an unpaired Student's t test).

When examining the detached cells from seeded Fluoropassiv™ discs, the population was composed of approximately the same percentage of transfected cells, as did the attached cells (data not shown). This indicates that expression of eGFP does not predispose cells to detachment and that cells exposed to transfection procedure were equally compromised in the first 48 h after transfection was initiated whether they were expressing eGFP or not.

Others have shown that endothelial cells slough off rapidly under shear conditions leaving 40–60% of the initially seeded cells on the graft.^{21,22} For this reason, the transfected endothelial cells were exposed to shear conditions to simulate the physiological conditions that the graft would experience. On exposure to shear conditions, GFP-transfected BAEC detached from Fluoropassiv™ discs at 153% the rate of nontransfected BAEC. This difference was statistically significant with $p < 0.05$. This would further indicate a diminution of attachment ability by transfected BAEC.

In late-phase attachment (after 24 h), the differences between nontransfected and transfected cells were lost and nontransfected and GFP-transfected BAEC were equally likely to detach from their substrate. This loss of statistically significant difference occurred at the 24-h time point for both Fluoropassiv™ and tissue culture polystyrene.

Furthermore, in late-phase attachment to Fluoropassiv™, increasing numbers of BAEC detached as time progressed (see Fig. 3). There were no statistically significant differences between nontransfected and GFP-transfected BAEC, as described previously. Because transfection was accomplished under the same conditions and of the same population, the later-stage detachment of cells is a phenomenon mediated by the material. The loss of approximately 50% of the seeded cells from Fluoropassiv™ discs at 3 days is striking. It indicates an inability of the Fluoropassiv™ graft mate-

rial to sustain BAEC attachment and growth. In addition, as endothelial cells are attachment dependent, it is highly doubtful that they will divide on or in the graft to any significant degree if they are not attached.^{23,24}

In a recent study by Kaiser and Toborek,²⁵ it was found that the concentration of DNA and lipid used in transfection may cause detachment and death in transfected cells. Whereas no toxicity was seen in our cultures (refer to Fig. 2), our study clearly demonstrates that there are transfection-related stresses that occur at subtoxic concentrations of DNA and lipid.

Late-phase attachment to tissue culture polystyrene was unaffected by transfection. This indicates that transfected cells recover from transfection 48 h after transfection. Finally, the lack of a large number of BAEC detaching from polystyrene as compared with FluoropassivTM suggests that late-phase attachment is substrate dependent.

In conclusion, optimization of transfection must be performed for both expression levels and adhesive abilities. Consideration of the acceptable level of loss of attachment ability is a prerequisite to assessing the desirability of cationic lipid-mediated genetic engineering for endothelial cell-seeding applications.

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