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Efficiency of Membrane Protein Expression Following Infection with Recombinant Adenovirus of Polarized Non-Transformed Human Retinal Pigment Epithelial Cells

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Abstract

Transient expression of exogenous proteins facilitates studies of molecular mechanisms and utility for transplantation of retinal pigment epithelial (RPE) cells in culture. Here, we compared expression of the membrane protein $\beta 5$ integrin-GFP ($\beta 5$ -GFP) in two recently established models of differentiated human RPE, adult RPE stem cell-derived RPE and primary fetal RPE, upon infection with recombinant adenovirus or transfection with DNA in liposomes. We varied viral titer and duration of virus incubation and examined $\beta 5$ -GFP and the tight junction marker ZO-1 in manipulated cells by confocal microscopy. Fewer than 5 % of cells expressed $\beta 5$ -GFP after liposome-mediated transfection. The percentage of cells with detectable $\beta 5$ -GFP exceeded 90 % after adenovirus infection for as little as 1 h. Decreasing virus titer two-fold did not alter the fraction of cells expressing $\beta 5$ -GFP but increased variability of $\beta 5$ -GFP level among cells. In cells with low expression levels, $\beta 5$ -GFP localized mostly to the apical plasma membrane like endogenous $\alpha \nu \beta 5$ integrin. In cells with high expression levels, $\beta 5$ -GFP localized to the cytoplasm in addition to the apical surface suggesting accumulation in trafficking compartments. Altogether, adenovirus delivery yields efficient exogenous membrane protein expression of correct polarity in differentiated human RPE cells in culture.

Keywords

$\beta 5$ integrin-GFP; Infectivity; Primary human fetal RPE; Protein expression; Recombinant adenovirus; RPE; RPESC-RPE

97.1 Introduction

Post-mitotic retinal pigment epithelial (RPE) cells form a polarized monolayer epithelium that fulfills numerous functions each one of which supports photoreceptor long-term function and viability. These include light absorption, transepithelial transport, re-isomerization of all-*trans* retinal, polarized secretion of growth factors, retinal adhesion and the diurnal clearance phagocytosis of shed photoreceptor outer segment tips (Strauss 2005). Impaired RPE-photoreceptor interactions cause retinal dysfunction or retinal degeneration in experimental animal models and contribute to inherited human retinal diseases and age-related macular degeneration.

The availability of RPE cells in culture facilitates studies of RPE functionality and molecular mechanisms otherwise limited by lack of access and sufficient yield to RPE tissue (Mazzoni et al. 2014). Over the past decades several groups have reported protocols to establish and grow polarized non-transformed human RPE cells that retain many characteristics of the RPE in the human eye (Sonoda et al. 2009; Hu and Bok 2010). Among these, adult retinal pigment epithelial stem cell-derived-RPE cells (RPESC-RPE) and primary human fetal RPE cells (hfRPE) are established using stringent, published protocols and seeded for studies at passage 1 or 2 followed by differentiation over several weeks, during which post-confluent monolayers generate pigment, polarize and acquire RPE specific marker proteins (Maminishkis et al. 2006; Blenkinsop et al. 2013).

Mechanistic studies of these novel high quality RPE models greatly benefit from efficient genetic manipulation. Adenovirus vectors are known to infect RPE cells without significant cytotoxicity and recombinant adenovirus-mediated gene transfer has long been used to manipulate gene expression of RPE cells *in vivo* and in culture (Trapani et al. 2014). Utility of virus transduced cells for functional studies requires (1) a large fraction of cells expressing exogenous protein, (2) low variability in exogenous protein expression level among transduced cells, and (3) correct subcellular localization of the exogenous protein. Here, we assess these parameters for differentiated, polarized RPESC-RPE and hfRPE cells infected with recombinant adenovirus encoding the transmembrane protein $\beta 5$ integrin-GFP ($\beta 5$ -GFP).

97.2 Materials and Methods

97.2.1 Human RPE Cell Cultures

RPESC-RPE cells (Salero et al. 2012) were seeded at passage-2 on 6.5-mm Transwell® filters with 0.4 μ m pore size (Corning Costar) (Blenkinsop et al. 2013). RPESC-RPE cells were maintained according to published procedures for 6–7 weeks before being used for experiments.

HfRPE cells at passage-0 were provided by Dr. Sheldon Miller (National Eye Institute, National Institutes of Health, Bethesda, MD) and maintained and re-seeded according to published protocols (Maminishkis et al. 2006). HfRPE cells of passage-2 were maintained on glass cover slips in 96-well plates for 4 weeks before being used for experiments.

97.2.2 Adenovirus-Mediated Transduction

Generation of replication-defective, recombinant adenovirus encoding GFP-tagged human $\beta 5$ integrin was described previously (Nandrot et al. 2012). Adenovirus stock was diluted to 5, 2.5, or 1.25×10^{10} virus particles (vp)/mL in serum-free DMEM and applied to cells for 1 or 15 h followed by incubation in complete medium for 23 or 9 h, respectively, before fixation.

97.2.3 Liposome-Mediated Transfection

pEGFP-N2 expression plasmid encoding $\beta 5$ -GFP was described previously (Nandrot et al. 2012). Cells were transfected with plasmid DNA in the presence of Lipofectamine 2000 as suggested by the manufacturer (Life Technologies). Cells were fixed 24 h after transfection.

97.2.4 Immunofluorescence Staining and Microscopy

RPE cells were fixed with ice-cold methanol for 5 min. Tight junctions were labeled with ZO-1 antibodies and AlexaFluor594-conjugated secondary antibodies (Life Technologies). Nuclei were counterstained with DAPI. X-y image stacks were acquired on a Leica TSP5 laser-scanning confocal microscopy system) and were compiled using Adobe Photoshop CS4.

97.3 Results

97.3.1 Infectivity of RPESC-RPE Cells

To optimize efficiency of exogenous protein expression following infection with adenovirus in RPESC-RPE cells were exposed to adenovirus particles at different concentrations and for different durations. We used a recombinant, replication defective adenovirus encoding human $\beta 5$ integrin with a C-terminal GFP tag ($\beta 5$ -GFP). We previously found that this adenovirus promotes expression of $\beta 5$ -GFP protein that forms heterodimeric receptors with endogenous human or rat αv integrin subunits that localize to the cell surface in fibroblasts, RPE cell lines and primary rat and mouse RPE in culture (Nandrot et al. 2012). Moreover, $\beta 5$ -GFP expression rescues the POS recognition deficiency of primary RPE derived from ITGB5^{-/-} mice indicating that $\alpha v\beta 5$ -GFP receptors function like $\alpha v\beta 5$ integrin (Nandrot et al. 2004; Nandrot et al. 2012). Importantly, $\beta 5$ -GFP shows robust green fluorescence that is largely maintained even after cell fixation and indirect immunofluorescence staining procedures.

We first exposed RPESC-RPE cells for 15 h to adenovirus at different concentrations and used confocal microscopy to assess GFP fluorescence in cells fixed 24 h after the start of infection. Figure 97.1a–c illustrates that most RPESC-RPE cells expressed $\beta 5$ -GFP regardless of virus titer. In comparison, delivery of $\beta 5$ -GFP expression plasmid via liposomes was very inefficient (Fig. 97.1d). Quantification of the fraction of RPESC-RPE cells with detectable $\beta 5$ -GFP fluorescence revealed that exposure to 5×10^{10} or 2.5×10^{10} vp/mL resulted in $\beta 5$ -GFP expression by 97 % of RPESC-RPE cells (Fig. 97.1e). Exposure to 1.25×10^{10} vp/mL was slightly less efficient yielding 90 % of RPESC-RPE cells with visible GFP fluorescence (Fig. 97.1e). However, in cells transduced with adenovirus at 2.5×10^{10} or 5×10^{10} vp/mL fluorescent cells showed uniformly high levels of integrin $\beta 5$ -GFP.

Display of x-z confocal sections revealed that $\beta 5$ -GFP in these brightly fluorescent cells localized to sites in the cytoplasm and to the apical surface (Fig. 97.1a and b, x-z displays). In contrast, cells transduced with adenovirus at 1.25×10^{10} vp/mL resulted in a heterogeneous pattern with fluorescence varying significantly among $\beta 5$ -GFP-positive RPESC-RPE cells. Notably, in cells with low or moderate levels of fluorescence, most $\beta 5$ -GFP appeared to localize to the cells' apical surface, while highly fluorescent cells showed cytoplasmic $\beta 5$ -GFP like cells transduced with adenovirus at higher concentration (Fig. 97.1c, x-z display).

We next tested if exposure to adenovirus for a shorter time period would decrease efficiency of transduction of RPESC-RPE cells. Limiting adenovirus exposure to only 1 h did not significantly reduce the percentage of RPESC-RPE cells expressing $\beta 5$ -GFP regardless of virus titer (Fig. 97.1d).

97.3.2 Infectivity of hfrPE Cells

Finally, we tested if highly differentiated, non-transformed hfrPE cells share the high infectivity of RPESC-RPE. Indeed, 93 % of hfrPE cells were fluorescent following 15-h exposure to 1.25×10^{10} vp/mL and most cells were brightly fluorescent (Fig. 97.2a, and c). In contrast, only 3.5 % of hfrPE cells were $\beta 5$ -GFP-positive after liposome-mediated transfection and their fluorescence was uniformly dim (Fig. 97.2b and c).

97.4 Discussion

Our experiments reveal that the two distinct post-confluent, highly differentiated, non-transformed human RPE cell strains we studied, RPESC-RPE and hfrPE, are highly susceptible to adenovirus infection. The finding that exposure to adenovirus for 1 h was as efficient in transducing cells as exposure for 15 h was unexpected. An earlier study found that transduction of confluent human primary RPE cells increased in a linear fashion with infection times of 16–70 h and was negligible if adenovirus was added for only 4 h (da Cruz et al. 1996). It is possible that adenovirus enters RPE cells more efficiently after extended periods of differentiation and polarization as induced in the two model systems we studied.

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Abbreviations

$\beta 5$ -GFP	$\beta 5$ integrin-GFP
hfrPE	Primary human fetal RPE
RPE	Retinal pigment epithelium

RPESC-RPE	Adult retinal pigment epithelium stem cell-derived-RPE
Vp	Virus particles

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Fig. 97.1.

$\beta 5$ -GFP expression by RPESC-RPE cells following adenovirus infection or liposome-mediated plasmid transfection. **a–d**: Images show fluorescence microscopy of $\beta 5$ -GFP (green), ZO-1 (red), and cell nuclei (blue) in RPESC-RPE cells after adenovirus infection (adv) or liposome-mediated transfection (lipo). **a–c** $\beta 5$ -GFP in RPESC-RPE cells after adenovirus infection (adv) for 15 h at 5×10^{10} vp/mL (**a**) 2.5×10^{10} vp/mL (**b**) or 1.25×10^{10} vp/mL (**c**), or 24 h after liposome-mediated transfection (**d**). The top of each panel shows a maximum projection of a representative image stack, the bottom of each panel shows a select x-z plane. Microscopy settings were adjusted to optimize the dynamic range for each image. Scale bar: 20 μ m. **e**: Quantification of RPESC-RPE expressing $\beta 5$ -GFP at any detectable level after exposure to adenovirus for 1 hour (white bars) or 15 hours (black bars) or after liposome-mediated transfection (black bar), as indicated. Bars show mean $\pm$ SD, $n = 3$. **f**: Relative intensity of fluorescence of single cells after infection or transfection as in e and as indicated.

Fig. 97.2.

β5-GFP expression by hfRPE cells following adenovirus infection or liposome-mediated plasmid transfection. Images show fluorescence microscopy of β5-GFP (*green*), ZO-1 (*red*), and cell nuclei (*blue*) in hfRPE cells after infection for 15 h with adenovirus at 1.25×10^{10} vp/mL (**a**) or liposome-mediated transfection (**b**). Maximum projections of representative image stacks are shown. Microscopy settings were adjusted to optimize the dynamic range for each image. Scale bar: 20 μm. **c** Quantification of hfRPE expressing β5-GFP at any detectable level after exposure to adenovirus for 15 h (adv) or after liposome-mediated transfection (lipo). Bars show mean ± SD, $n = 3$.