Sorting and Function of the Human Folate Receptor Is Independent of the Caveolin Expression in Fisher Rat Thyroid Epithelial Cells

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Caveolae are small, flask-shaped, non-clathrin coated invaginations of the plasma membrane of many mammalian cells. Caveolae have a coat that includes caveolin. They have been implicated in numerous cellular processes, including potocytosis. Since the human folate receptor (hFR) and other glycosyl-phosphatidylinositol (GPI)-tailed proteins have been co-localized to caveolae, we studied the caveolin role in the hFR function by transfecting hFR and/or caveolin cDNA into Fischer rat thyroid epithelial (FRT) cells that normally do not express detectable levels of either protein. We isolated and characterized stable clones as follows: they express (1) high levels of caveolin alone, (2) hFR and caveolin, or (3) hFR alone. We discovered that hFR is correctly processed, sorted, and anchored by a GPI tail to the plasma membrane in FRT cells. No difference in the total folic acid binding or cell surface folic acid binding activity were found between the FRT cells that were transfected with hFR, or cells that were transfected with hFR and caveolin. The hFR that was expressed on the cell surface of clones that were transfected with hFR was also sensitive to phosphatidylinositol-specific phospholipase C (PI-PLC) release, and incorporated radiolabeled ethanolamine that supports the attachment of a GPI-tail on hFR. We conclude that the processing, sorting, and function of hFR is independent on the caveolin expression in FRT cells.

Keywords: Caveolin, Folate receptor, Fischer rat thyroid epithelial (FRT) cell, GPI tailed protein

Introduction

The hFR is an essential component of one of two different transport systems that have been described for folate and antifolate transport (Elwood et al., 1986; Antony, 1992; Chung et al., 1993). The human KB cell hFR is the best-characterized isoform of folate receptors (Elwood et al., 1986); it is the subject of this paper. The hFR is a 40 kDa membrane glycoprotein that contains a unique high-affinity ligand binding site (Elwood, 1989) that is attached to the membrane by a GPI tail to directly participate in clathrin-mediated endocytosis. It has been hypothesized that the hFR and other GPI-tailed receptors may require another protein in the plasma membrane in order to function (Anderson et al., 1992; Miotti et al., 2000). However, no such protein has been identified. In this study, we examined the possibility that the hFR works in conjunction with caveolin.

Caveolae, first described 50 years ago, are small, flask-shaped, non-clathrin-coated invaginations of the plasma membrane of many mammalian cells (Yamada, 1955). Caveolae have a coat that includes caveolin, as well as other cytoplasmically-oriented signaling molecules. This includes heterotrimeric guanosine triphosphatase (GTPase), nonreceptor-type protein-tyrosine kinases, and calcium channels and pumps. In addition, Ca-ATPase, IP3-receptor like protein, PLD1, and GPI-anchored proteins are associated with caveolae (Bagnoli et al., 2000; Lee et al., 2001; Park et al., 2001). Caveolae are enriched with GM1 ganglioside, and cholesterol appears to be necessary for their structure and function (Bist et al., 2000; Bridges et al., 2001; Oh and Schnitzer, 2001). Caveolae function is reportedly regulated by kinase activity and requires an intact network of microtubules (Parton et al., 1994).

Caveolin is a 22 kDa tyrosine phosphoprotein that is enriched within the caveolae (Schnitzer et al., 1995; Engelman et al., 1999; Fujimoto et al., 2000; Galbiati et al., 1999). Caveolin decorates the cytoplasmic surface of caveolae and has been proposed to have a structural role in maintaining...
caveolae, perhaps analogous to the clathrin coat in coated pits. Caveolae were normal, however, even after caveolin was translocated to the Golgi in the presence of cholesterol oxidase (Smart et al., 1994). It is unknown whether caveolin is involved in targeting GPI-tailed proteins to the plasma membrane, or whether caveolin is necessary for the function of GPI-tailed proteins.

Based on the activity of many proteins that are associated with caveolae, these structures have been implicated in signal transduction, calcium regulation, and nonclathrin-dependent receptor-mediated transport processes, such as transcytosis and endocytosis (Scherer et al., 1994; Kim et al., 1999; Shu et al., 2000). However, the exact function of caveolae has not been determined. Parton et al (1994) reported that alkaline phosphatase that is clustered in caveolae may detach from the cell surface, and that a small fraction of the caveolae endocytoses are regulated by kinase activity. Conflicting reports have been published regarding the possible role for caveolae in the vesicular transport of a glucose transporter (Glut4) to the plasma membrane from intracellular sites in adipocytes (Kandror et al., 1995; Ryu and Jung, 2001). Another function, a process termed potocytosis, was proposed for caveolae in the endocytosis of small molecules (Anderson et al., 1992). As originally proposed, the potocytosis model requires the clustering of receptors in caveolae that results in a high concentration of ligand within the caveolae. Although hFR and other GPI proteins are clustered in caveolae, the clustering is dependent on cross-linking by polyclonal secondary antibodies. In the absence of antibodies, the hFR and other GPI proteins are diffusely distributed on the plasma membrane. This observation is inconsistent with the potocytosis model.

To investigate the role of the caveolin expression in sorting and function of hFR, we transfected the hFR cDNA, caveolin cDNA, or both into FRT cells that do not express endogenous caveolin (Sargiacomo et al., 1993).

Materials and Methods

Materials The $^{32}$P-labeled pteroylglutamic acid (histamine derivative folic acid) was purchased from New England Nuclear (Boston, USA). The $[^3$H]folic acid and $[^3$H]5MTFH (dl-N-5-methyltetrahydrofolate) were purchased from Moravek Biochemicals (Brea, USA). Folic acid and 5MTHF were purchased from the Sigma Chemical Co. (St. Louis, USA). The $[^3$S]methionine and $[^3$S]cytochrome c were purchased from ICN (Costa Mesa, USA). Scintillation fluid was purchased from Research Products International (Mt. Prospect, USA). Acrylamide and low molecular weight markers were purchased from Bio-Rad (Richmond, USA). All of the restriction enzymes and reagents for radiolabeling cDNA probes were purchased from Promega (Madison, USA). The $[^3$P]dCTP and $[^3$H]dGTP (each with a specific activity of >800 Ci/mmol) were obtained from the Amersham Corporation (Arlington Heights, USA). All of the other chemicals were of reagent grade or higher, and were purchased from the Sigma Chemical Co. (St. Louis, USA), or Fisher Scientific (Pittsburgh, USA).

Cells and tissue culture reagents Reagents for cell culture and genetinysulfate (antibiotic G418) were purchased through Gibco Laboratories (Grand Island, USA). The penicillin/streptomycin/ fungizone solution (PSF, 100X) and fetal calf serum (FCS) were purchased from Biologics (Rockville, USA).

Plasmid construction of hFR The pRC/CMV vector (Invitrogen, San Diego, USA) contains a cytomegalovirus (CMV) promoter and neomycin resistance gene. The cDNA that encoded hFR was isolated from a placental cDNA library and subcloned at the EcoRI multiple cloning site of pGEM4Z (Promega, Madison, USA). The Hind III-linearized pRC/CMV expression vector was ligated with a blunt-ended hFR cDNA insert using T4 DNA ligase. Competent E. coli (JM109) was transformed with the ligation mixture. Recombinant plasmids were isolated by the Qiagen maxiprep kit (Chatsworth, USA). We designated the plasmid construct that contained the pRC/CMV vector and human KB cell folate receptor cDNA as pRC/FR. The sense orientation of the folate receptor cDNA in pRC/CMV vector (pRC/FR) was verified by restriction enzyme digests and DNA sequencing using $[^3$S]dATP.

Cloning of the human caveolin cDNA The caveolin sequence was previously published (Glenney, 1992). Human lung mRNA was purchased from Clontech (Palo Alto, USA), and reverse transcribed using the Perkin Elmer first-strand kit (Boston, USA). Human caveolin was amplified from human lung total RNA using Amplitaq (Perkin Elmer, Boston, USA). The 5' primer corresponded to bp 13-34 of the caveolin sequence, and the 3' antisense primer corresponded to bp 931-905 of the caveolin antisense sequence. HindIII and XbaI sites were included in the 5' and 3' primers, respectively, to subclone the caveolin cDNA into the HindIII and XbaI sites within the pRC/CMV vector. We designated the plasmid construct as pRC/caveolin. The sequence of the caveolin was verified by DNA sequencing. Based on the sequence analysis of 3 independent bacterial clones, we observed that each human caveolin recombinant sequence has a G rather than a C at the bp 271 position, and is identical to the canine caveolin sequence. Therefore, in contrast to the previous report (Glenney, 1992), the human caveolin sequence does not appear to be different from the canine caveolin, but retains an Asp rather than a His at amino acid 82.

Tissue culture For our experiments, FRT cells were cultured in Ham’s F12 media with 10% FCS and PSF. Cells were grown in monolayers in 10 cm tissue culture dishes that contained 10 ml media at 37°C under a humidified atmosphere of 5% CO$_2$. They were subcultured weekly. To harvest the cells, the medium was decanted, and 1 ml of a 2.5% trypsin solution was added to the dishes. The cells were incubated at room temperature (rt) for 2 min, the excess solution was decanted, and the cells were immediately resuspended in media.

Stable transfection For stable transfection of FRT cells with 10 µg of pRe/FR or pRC/caveolin, we used the electroporation method (Bio-Rad, Hercules, USA) with 4 x 10$^6$ cells in 0.4 ml PBS at 300 Volt and 960 mF settings. Stable transfectants were selected.
in 25 mg/ml G418 and maintained in 125 μg/ml G418. We designated the stable clone that was transfected with pRc/FR alone as clone F; pRc/FR and pRe/caveolin as clone FC, and pRe/caveolin alone as clone C.

Protein estimation The protein concentration was determined using the Micro BCA protein assay reagent kit (Pierce, Rockford, USA) in the presence of 0.1% (v/v) Triton X-100. The bovine serum albumin fraction V was used as a standard.

Western blot Stable transfectants of the FRT cells (2×10^6 cells/35 mm well) were plated overnight. The cells were washed twice with PBS and scraped into 1 ml PBS and 20 mM EDTA, pH 7.4. The cells were pelleted in a microcentrifuge and solubilized with PBS and 1% TX-100 in a well, and supernatant (500 μl) was added to air-dried samples. The samples were vortexed, boiled for 10 min, electrophoresed on a 12.5% SDS-PAGE, and transferred to nitrocellulose filter paper. Western blots for caveolin were incubated with a 1:200 dilution of a mouse anti-rabbit IgG, and analyzed by the ECL system.

PI-PLC releasability studies The cells (2×10^5) were incubated in 35 mm wells overnight. Cell monolayers were rinsed once with 3 ml ice-cold PBS. The cells were incubated in 50 mM Tris, 150 mM NaCl, pH 7.4 with or without PI-PLC (100 μU/ml/well), for 60 min at 37°C. Cell surface binding assays using [3H]ethanolamine labeling Stable transfectants of the FRT cells (10^6 cells/35 mm well) were grown, then labeled with [3H]ethanolamine in a R-media that contained FCS (50%) and [3H]ethanolamine (100 Ci). The cells were solubilized and immunoprecipitated, described in Materials and Methods.

Folate transport experiments Stable transfectants of FRT cells (5×10^4 cells/10 cm plate) were passaged twice for 7 days in DMEM and 10% FCS and PSF that contained a final folate concentration of 1-10 nM (Chung et al., 1994) in order to reduce intracellular folates. After 2 weeks, the 2×10^5 cells were plated at 35 mm well for 1 day. Cell monolayers were washed two times with an ice-cold acid saline pH 4.5 and neutralized by rinsing the monolayer twice with ice-cold PBS, pH 7.4. To measure the internalized ligand, the cells were incubated at 37°C in 1 ml of prewarmed DMEM with 50 μg/ml BSA, and 100 nM of ligand ([H]H6olic acid or [3', 5', 7, 9-3H]SMTHF) for various times in a 37°C waterbath, as previously published (Chung et al., 1995). To determine specific folate internalization, parallel experiments were performed in the presence of 500-fold excess cold ligand. The monolayer was washed twice with 2 ml of acid saline (pH 4.5) in order to remove the surface-bound ligand, and with 2 ml of ice-cold PBS, pH 7.4. The cells were solubilized by adding 1 ml PBS with 1% TX-100 in a well, and supernatant (500 μl) was added to 10 ml of a liquid scintillation cocktail and counted in a liquid scintillation counter (Downers Grove, USA) at 50% efficiency.

Immunoprecipitation and EndoH digestion Stable transfectants of the FRT cells (10^6 cells/35 mm well) were carried out as previously described (Chung et al., 1995; Bae et al., 2001). After immunoprecipitation and Endo H digestion, the samples were electrophoresed on a 12.5% SDS-PAGE. The gels were treated three times with 50% methanol and 10% acetic acid, rinsed once with H2O, and incubated for 1 h in Enlightening. After drying, the gels were autoradiographed at –70°C in cassettes with two intensifying screens.

Growth studies Stable transfectants of the FRT cells (5×10^5 cells/35 mm well) were plated for two passages of 7 days each in DMEM that contained FCS and PSF. This resulted in a final folate concentration of approximately 1-10 nM (Chung et al., 1993). Photographs of the cells were taken after 14 days.

Results Stable transfection of FRT cells with hFR and/or caveolin Following electroporation of hFR cDNA, caveolin cDNA, or both into FRT cells, we selected clones that stably express easily detectable levels of hFR (F clones), caveolin (C clones), or both (FC clones), as determined by a Western blot analysis. Figure 1 shows duplicate Western blots of total cellular protein that was probed with an antibody to caveolin (Fig. 1A) and an antibody to hFR (Fig. 1B). Clone C and FC (Fig. 1A, lanes 2, 3) express caveolin protein. Wild-type FRT cells and clone F do not express detectable levels of the caveolin protein (Fig. 1A, lanes 1, 4), Clones FC and F express hFR protein (Fig. 1B, lanes 3, 4); whereas, wild-type FRT cells and clone C do not express detectable levels of the hFR protein (Fig. 1B, lanes 1, 2). Therefore, three different phenotypes of stably transfected clones were selected as follows: (1)clone C expresses only caveolin protein, (2) clone F expresses only the hFR protein; (3) and clone FC expresses both hFR and caveolin proteins. These three clones were used to study the potential interaction between hFR and caveolin.
Characterization of FRT cells transfected with hFR (clones FC and F) FRT cells transfected with hFR compared to the wild-type FRT cells, or to clone C cells; solubilized total cellular extracts of clones FC and F contained a significant increase in their total folic acid binding capacity (Table 1). To determine whether the caveolin expression is a determinant of hFR sorting to the plasma membrane, we next determined whether hFR binds folic acid at the cell surface. Table 2 shows that clones FC and F bind high levels of radiolabeled folic acid at their cell surface. Table 2 also demonstrates that parental FRT cells and clone C do not contain detectable levels of cell surface folic acid binding. These results confirm and extend the results of total folic acid binding assays (Table 1). Since the levels of folic acid binding are comparable between clones FC and F, these results show that hFR is expressed at the cell surface and binds folic acid independently of caveolin.

Clone F internalizes [³H]folic acid and [³H]5MTHF at the cell surface To show that hFR is functional at the cell surface in the transfected FRT cells, we measured the specific internalization of [³H]folic acid (Fig. 2A) and [³H]5MTHF (Fig. 2B). Clones FC and F that express elevated levels of hFR also show increased [³H]folic acid and [³H]5MTHF uptake relative to wild-type FRT cells. Wild-type FRT cells do not transport detectable amounts of folates. Since the kinetics and levels of internalization of folic acid are comparable between clones FC and F, these results demonstrate that hFR can function independently of caveolin.

The hFR is correctly processed in clone F To study potential effects of the caveolin expression on hFR synthesis and processing, we compared the post-translational processing of oligosaccharides on hFR in clones FC and F. Figure 3 contains a SDS-PAGE analysis of the immunoprecipitates of pulse-labeled hFR at different time points with or without Endo H. Fig. 3 demonstrates that FRT cells that are transfected with hFR alone (clone F, Fig. 3B) and with hFR and caveolin (clone FC, Fig. 3A) show normally processed hFR of the same apparent molecular weight. This suggests that they are identical in their time course of acquiring Endo H resistance and in their synthesis of hFR. The hFR is not detectable in wild-type FRT cells or in clone C (Fig. 1). In order to demonstrate that a typical GPI tail anchors hFR, we studied the co-translational addition of GPI tails to hFR by FRT cells using PI-PLC, and also by endogenously labeling with [³H]ethanolamine. Table 2 shows that hFR that is expressed on the cell surface of clones FC and F is sensitive to PI-PLC release. Following an 18-hour labeling with [³H]ethanolamine, immunoprecipitated hFR contained

### Table 1. Total [¹²⁵I]folic acid and [³H]folic acid binding

<table>
<thead>
<tr>
<th>Cells</th>
<th>[¹²⁵I]folic acid cpm/µg</th>
<th>[³H]folic acid cpm/µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRT</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>FRT-C</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>FRT-CF</td>
<td>1.1 × 10⁶</td>
<td>3.2 × 10⁴</td>
</tr>
<tr>
<td>FRT-F</td>
<td>1.2 × 10⁶</td>
<td>3.0 × 10⁴</td>
</tr>
</tbody>
</table>

Total folic binding assays of 10 cm confluent plates of FRT cells that were stably transfected with caveolin and/or hFR. Folic acid binding assays on detergent-solubilized protein extracts were carried out as previously described (Elwood et al., 1986) using [¹²⁵I]-labeled pteroylglutamic acid (histamine derivative of folic acid) or [³H]folic acid. The folic acid binding activity is expressed as cpm per µg protein.

### Table 2. Cell surface [¹²⁵I]folic acid binding

<table>
<thead>
<tr>
<th>Cells</th>
<th>[¹²⁵I]folic acid cpm/10⁶ cells</th>
<th>PI-PLC released %</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRT</td>
<td>423</td>
<td>n.d.</td>
</tr>
<tr>
<td>FRT-C</td>
<td>303</td>
<td>n.d.</td>
</tr>
<tr>
<td>FRT-CF</td>
<td>36,891</td>
<td>89</td>
</tr>
<tr>
<td>FRT-F</td>
<td>34,343</td>
<td>93</td>
</tr>
</tbody>
</table>

FRT cells (10⁶ cells) that were stably transfected with caveolin and/or hFR were plated in 35 mm plates. After dissociating surface-bound folates from cell surface folate receptors with acid saline (described in “Materials and Methods”), the cells were incubated with radio-labeled folic acid for 10 min at 10°C with or without excess cold ligand. Aliquots of the solubilized cells were counted and the results are expressed as cpm per 10⁶ cells per 30 min. Cell surface folate binding that was released with PI-PLC was determined as described in “Materials and Methods”. (n.d.=not determined)
Sorting and Function of the Human Folate Receptor

Two latter observations confirm that hFR is GPI-linked following transfection into FRT cells. FRT cells expressing hFR have a growth advantage in physiologic concentrations of folates

Stably transfected clones were plated in physiologic concentrations of folates. Under these conditions, the wild-type FRT cells (Fig. 5A) and clone C cells (Fig. 5B) were detached after two passages, while the transfected clones FC (Fig. 5C) and F (Fig. 5D) survived in long-term culture. These results show that transfecting hFR into FRT cells confers a growth advantage in physiologic folic acid concentrations; this growth advantage is independent of caveolin. These results, therefore, extend the observations that cells, which stably express high hFR levels, have a growth advantage in physiologic concentrations of folates (Chung et al., 1993), and confirms the [3H]folic acid and [3H]MTHF transport data (Fig. 2).

Discussion

In these experiments, we explored the role of caveolin in the expression and function of hFR. We transfected hFR cDNA and/or caveolin cDNA into FRT cells. The wild-type FRT and FRT cells that were transfected with caveolin alone did not express detectable levels of caveolin or hFR. In FRT cells that were transfected with hFR, we observed that recombinant hFR is expressed and attached to the plasma membrane by a GPI anchor, based on the release of hFR from the plasma

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Fig. 2. [3H]folic acid and [3H]5MTHA transport study. Folic acid (FA) (A) and 5MTHA transports (B) in FRT cells were transfected with hFR and caveolin (Clone FC), or with hFR alone (clone F). The cells (2×10⁶ cells/35 mm well) were plated one night after two passages of 7 days each in DMEM that contained 10% FCS and PSF. After dissociating surface-bound folates from the cell surface folate receptor (described in “Materials and Methods”), the cells were allowed to internalize [3H]folic acid (100 nM) for various times at 37°C. Aliquots of the solubilized cells were counted, and the results are expressed as pmole/mg total protein.

Fig. 3. Endo H sensitivity of human folate receptor. The hFRs were immunoprecipitated from the FRT clone FC (A) that was transfected with hFR and caveolin cDNA, and the FTR clone F (B) that was transfected with hFR cDNA only. Clones were plated in 35 mm wells and pulse-radiolabeled with [35S]methionine and [35S]cysteine for 60 min at 37°C, then chased for the incubated times in complete media. At the end of the chase period, hFRs were immunoprecipitated, digested with or without Endo H, and resolved on a 12.5% SDS-PAGE (described in “Materials and Methods”).

[3H]ethanolamine (Fig. 4). Two latter observations confirm that hFR is GPI-linked following transfection into FRT cells.
membrane by PI-PLC (Table 2) and incorporation of $[^3]$H$^{\text{H}}$ethanolamine (Fig. 4). We observed that total folate binding (Table 1), cell surface folate binding (Table 2), or folate transport (Fig. 2) between FRT cells that were transfected with hFR alone or FRT cells that were transfected with both hFR and caveolin are equivalent. In contrast, wild-type FRT or FRT cells that were transfected with caveolin alone did not contain folate binding activity, or transport folates under these conditions. In addition, the synthesis rate and the post-translational modification of hFR are identical in the absence or presence of caveolin in these transfected cells (Fig. 3). FRT cells that were transfected with hFR alone or with hFR and caveolin had a growth advantage in media that contained physiologic concentrations of folates, when compared to wild-type FRT cells that lacked hFR or FRT cells that were transfected with caveolin alone (Fig. 5). We reported that the Endo H digestion, folate binding, and growth advantage for CHOD10 transfected hFR into wild-type CHO that express caveolin (Chung et al., 1994). Others reported this growth advantage for other types of cells that stably transfected and expressed high levels of hFR (Matsue et al., 1992; Chung et al., 1995). We conclude that the synthesis, processing, sorting, and function of hFR is independent on the caveolin expression in FRT cells.

The role of caveolae is ambiguous and the function of caveolin is not well known. The hFR and other GPI proteins cluster in caveolae only upon crosslinking with polyclonal secondary antibodies. However, in the absence of a clustering agent, GPI proteins are diffusely distributed on the cell surface (Mayor et al., 1995; Wu et al., 1997). The latter observation does not support the potocytosis model of folic acid uptake. The authors suggest that internalization of the folate receptor is by endocytosis that is mediated by clathrin-dependent and independent pathways. However, GPI-anchoring is important for intracellular signaling for several different proteins; cross-linking of the protein is a prerequisite for signaling in most cases (Miotti et al., 2000). Although the physiological activators of clustering hFR are unknown, cross-linking may be involved in the hFR function. Rijnboutt et al (1996) showed that in KBD10 cells, hFR is not clustered in caveolae, which suggests that the hFR function is unrelated to caveolae. Cain et al (1995) also reported that neutrophils, monocytes, lymphocytes, and platelets in peripheral blood express caveolin, but they have few caveolae structures. This raises doubts about the role of caveolin in the caveolae structure and function.

In this study, we discovered that the hFR function is independent of the caveolin expression in FRT cells. Our results indicate that the potocytosis model of folate internalization by hFR needs to be modified. We are further exploring the mechanism of hFR internalization of folates in order to understand how GPI-linked receptors function.

![Fig. 4](image4.png)

Fig. 4. hFR labeled with $[^3]$H$^{\text{H}}$ethanolamine. The cells were metabolically labeled for 18 h with $[^3]$H$^{\text{H}}$ethanolamine in R-media that contained 50% FCS and 100 Ci $[^3]$H$^{\text{H}}$ethanolamine. The cell membrane proteins were solubilized from wild-type FRT (lane 1), clone C that was transfected with caveolin cDNA only (lane 2), clone FC that was transfected with hFR and caveolin cDNA (lane 3), and clone F that was transfected with hFR cDNA only (lane 4). The hFRs were immunoprecipitated (described in “Materials and Methods”).

![Fig. 5](image5.png)

Fig. 5. Determination of growth. The $5 \times 10^4$ cells of wild-type FRT (A), FRT cells that were stably transfected with caveolin alone (B), with hFR and caveolin (C), and with hFR alone (D) were plated after two passages of 7 days, each in DMEM that contained 10% FCS and PSF. Photos of the cells were taken at 14 days after plating.
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References


