Autocrine production of prostaglandin F$_{2\alpha}$ enhances phenotypic transformation of normal rat kidney fibroblasts


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Harks, E. G. A., P. H. J. Peters, J. L. J. van Dongen, E. J. J. van Zoelen, and A. P. R. Theuvenet. Autocrine production of prostaglandin F$_{2\alpha}$ enhances phenotypic transformation of normal rat kidney fibroblasts. Am J Physiol Cell Physiol 289:C130–C137, 2005. First published March 9, 2005; doi:10.1152/ajpcell.00416.2004.—We have used normal rat kidney (NRK) fibroblasts as an in vitro model system to study cell transformation. These cells obtain a transformed phenotype upon stimulation with growth-modulating factors such as retinoic acid (RA) or transforming growth factor-β (TGF-β). Patch-clamp experiments showed that transformation is paralleled by a profound membrane depolarization from around −70 to −20 mV. This depolarization is caused by a compound in the medium conditioned by transformed NRK cells, which increases intracellular Ca$_{2+}$ levels and thereby activates Ca$_{2+}$-dependent Cl$^{-}$ channels. This compound was identified as prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) using electrospray ionization mass spectrometry. The active concentration in the medium conditioned by transformed NRK cells as determined using an enzyme immunoassay was 19.7 ± 2.5 nM (n = 6), compared with 1.5 ± 0.1 nM (n = 3) conditioned by nontransformed NRK cells. Externally added PGF$_{2\alpha}$ was able to trigger NRK cells that had grown to density arrest to restart their proliferation. This proliferation was inhibited when the FP receptor (i.e., natural receptor for PGF$_{2\alpha}$) was blocked by AL-8810. RA-induced phenotypic transformation of NRK cells was partially (−25%) suppressed by AL-8810. Our results demonstrate that PGF$_{2\alpha}$ acts as an autocrine enhancer and paracrine inducer of cell transformation and suggest that it may play a crucial role in carcinogenesis in general.

NOMINAL RAT KIDNEY (NRK) FIBROBLASTS provide an attractive model system for studying density-dependent growth and transformation of cells (1, 25). For their proliferation, NRK cells strictly depend on externally added growth factors, and when cultured in the presence of specific combinations of epidermal growth factor (EGF) with other polypeptide growth factors or modulating factors such as retinoic acid (RA) or transforming growth factor-β (TGF-β), these nontransformed cells can reversibly acquire a transformed phenotype (32).

Depending on the combination of growth factors used, three different growth states of cultured NRK fibroblasts can be distinguished. First, confluent NRK monolayers can be made quiescent by serum deprivation. Under these conditions, all cells in the monolayer are in the resting phase of the cell cycle but are still responsive to growth factor stimulation. Upon addition of EGF as the only growth-stimulating agent, quiescent NRK cells can be triggered to restart their proliferation until the monolayer has reached a critical cell density and has become density arrested (32). Density-dependent growth inhibition is one of the main features that distinguishes normal cells from tumor cells and prevents excessive cell growth. However, this protection mechanism can be overcome in NRK cells upon additional application of RA or TGF-β and results in a transformed phenotype after ≥2 days (34). In contrast to quiescent and density-arrested NRK monolayers, only phenotypically transformed NRK cells can proliferate under anchorage-independent conditions and form cellular multilayers when cultured in dishes (18). Anchorage-independent growth is known to be an in vitro correlate of tumorigenic behavior of cells in vivo (5, 28), and phenotypic transformation of NRK cells can thus be considered an in vitro analog for tumorigenesis.

Previous studies have shown that the membrane potential and intracellular Ca$_{2+}$ concentration of quiescent and density-arrested NRK cells are different. In contrast to quiescent monolayers, which have a stable, low membrane potential, monolayers of density-arrested NRK fibroblasts repetitively fire action potentials (8, 9). Such action potentials are accompanied by transient increases in intracellular Ca$_{2+}$ levels. The ionic basis for the excitability of NRK fibroblasts has been identified in patch-clamp experiments (14). Voltage-clamp experiments showed the presence of three prominent membrane conductances in NRK cells: inwardly rectifying K$^+$ conductance, L-type Ca$_{2+}$ conductance, and Ca$_{2+}$-dependent Cl$^{-}$ conductance. The contribution of each individual conductance to action potential firing in NRK cells became evident in current-clamp experiments and was further established by mathematical modeling of NRK cells (31). In summary, activation of the L-type Ca$_{2+}$ conductance is responsible for the initiation of the action potential, subsequent activation of the Ca$_{2+}$-dependent Cl$^{-}$ conductance sets the plateau phase, and the inwardly rectifying K$^+$ conductance mediates repolarization and generates the resting potential around −70 mV. From mathematical modeling, it became evident that Ca$_{2+}$ release from internal stores is probably of major importance for the regenerative firing (31). L-type Ca$_{2+}$ channels in muscle cells are functionally coupled to ryanodine receptors on Ca$_{2+}$ stores, and Ca$_{2+}$ release from these stores significantly contributes to global Ca$_{2+}$ signaling in these cells (2). The role of ryanodine and inositol 1,4,5-trisphosphate (IP$_3$)-sensitive Ca$_{2+}$ release channels in periodic action potential firing by NRK cells still has to be established.

In the present study, we examined the electrophysiological properties of phenotypically transformed NRK fibroblasts and

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found that these cells are depolarized because of a release of prostaglandin F2α (PGF2α). We explored the possible involvement of this prostaglandin in RA-induced phenotypic transformation of NRK cells, as well as its potential to act as a paracrine trigger for uncontrolled cell proliferation. The results are discussed in light of the role of prostaglandins and cyclooxygenase (COX) enzymes in cell transformation.

**MATERIALS AND METHODS**

**Cell culturing.** NRK fibroblasts (clone 49F) were cultured in HCO₃⁻-buffered Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Paisley, UK) supplemented with 10% newborn calf serum (HyClone Laboratories, Logan, UT). Confluent cultures were made quiescent using a subsequent 1- to 3-d incubation in serum-free DF medium (DMEM-Ham’s F-12 medium, 1:1; Gibco/Life Technologies, Paisley, UK) supplemented with 30 nM Na₂SeO₃ and 10 μg/ml human transferrin. Density-arrested monolayers were obtained using the subsequent incubation for 2 days with 5 ng/ml EGF (Collaborative Biomedical Products, Bedford, MA) in combination with 5 μg/ml insulin (Sigma, St. Louis, MO). Phenotypically transformed NRK cells were obtained 2–3 days after the addition of 50 ng/ml RA to density-arrested monolayers. Instead of RA, TGF-β (2 ng/ml) was also used as a growth-modulating factor. Conditioned media were obtained from NRK cells cultured in flasks (175 cm²) containing 50 ml of culture medium and harvested 2–3 days after addition of RA.

**Electrophysiology.** Whole cell current-clamp experiments were performed with NRK cells that were perfused at room temperature with serum-free DF medium equilibrated with 75% CO₂ to pH 7.4. Borosilicate patch pipettes (GC150-15; Clark, Reading, UK) with resistances of 4–6 MΩ were used, and intracellular pipette solution was made quiescent using a subsequent 1- to 3-d incubation in serum-free DF medium (DMEM-Ham’s F-12 medium, 1:1; Gibco/Life Technologies, Paisley, UK) supplemented with 30 nM Na₂SeO₃ and 10 μg/ml human transferrin. Density-arrested monolayers were obtained using the subsequent incubation for 2 days with 5 ng/ml EGF (Collaborative Biomedical Products, Bedford, MA) in combination with 5 μg/ml insulin (Sigma, St. Louis, MO). Phenotypically transformed NRK cells were obtained 2–3 days after the addition of 50 ng/ml RA to density-arrested monolayers. Instead of RA, TGF-β (2 ng/ml) was also used as a growth-modulating factor. Conditioned media were obtained from NRK cells cultured in flasks (175 cm²) containing 50 ml of culture medium and harvested 2–3 days after addition of RA.

**Electrophysiology.** Whole cell current-clamp experiments were performed with NRK cells that were perfused at room temperature with serum-free DF medium equilibrated with 75% CO₂ to pH 7.4. Borosilicate patch pipettes (GC150-15; Clark, Reading, UK) with resistances of 4–6 MΩ were used, and intracellular pipette solution contained (in mM) 25 NaCl, 120 KCl, 1.0 CaCl₂, 1.0 MgCl₂, 3.5 EGTA, and 10 HEPES-KOH (pH 7.4). Data analysis was performed offline using Microcal Origin software version 6.0 (Microcal Software, Northampton, MA). Membrane potentials (expressed in millivolts) of quiescent and transformed NRK cells were determined by averaging records over at least 5 min. Peak potential, plateau potential, and frequency (min⁻¹) of action potential firing in density-arrested monolayers were derived from at least five action potentials per trace.

**Intracellular Ca²⁺ measurements.** Coverslips with monolayers of NRK fibroblasts were placed in a Leiden cell chamber and loaded for 30 min at room temperature with 2 μM fura-2 AM (Molecular Probes, Eugene, OR) in Hanks’ medium containing (in mM) 137 NaCl, 5.4 KCl, 1.0 CaCl₂, 0.5 MgCl₂, 0.40 MgSO₄, 4.2 NaHCO₃, 0.34 NaH₂PO₄, 0.35 KH₂PO₄, and 5.6 glucose (pH 7.4), supplemented with 0.025% Pluronic F-127 and 3% FCS. Dynamic video imaging was performed as described elsewhere (7). Excitation wavelengths of 340 and 380 nm (bandwidth 8–15 nm) were provided by a 150-W xenon lamp (UXL S150 MO; Ushio, Tokyo, Japan), and fura-2 fluorescence emission was monitored using a 440-nm cutoff DCLP mirror in front of a CoolSNAP fx monochrome digital camera (Roper Scientific, Tucson, AZ). Image acquisition, using camera pixel binning of 4, and computation of ratio images (F₃₄₀/F₃₈₀) were performed every 6 s using MetaFluor software version 4.6 (Universal Imaging, Downingtown, PA). Camera acquisition time was 100 ms per excitation wavelength.

**Purification procedure.** The biologically active compound secreted by transformed NRK fibroblasts was purified from the culture medium by applying several separation steps. First, solid-phase extraction (Sep-Pak C18; Waters, Milford, MA) was performed in which the activity was eluted with 96% ethanol supplemented with 0.1% acetic acid. Aliquots of the active fraction were dried under N₂, resuspended in 10% acetonitrile with 0.1% trifluoroacetic acid (TFA) and subsequently loaded onto an Ultrasphere ODS 5-μm silica [inner diameter (ID), 4.6 mm; length (L), 25 cm] reverse-phase HPLC column (Beckman, San Ramon, CA). A linear gradient was applied from 10–90% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. Active fractions were analyzed along the purification procedure by testing them for their ability to depolarize confluent monolayers of quiescent NRK cells in patch-clamp experiments (see Electrophysiology). The active HPLC fraction was loaded onto a Superoxide Peptide HR 10/30 gel permeation column (Pharmacia, Peapack, NJ) and eluted with 27% acetonitrile in 0.1% TFA at a flow rate of 0.1 ml/min. The purified fraction was loaded onto a second reverse-phase HPLC (100 μl of direct loop injection) at a flow rate of 0.1 ml/min using an Alltima C18 5-μm silica column (ID 2.1 mm, L 150 mm; Alltech, Deerfield, IL). A linear gradient was applied from 10–90% acetonitrile in 0.1% TFA. The fraction obtained after the final HPLC step that was active is referred to as TransX throughout this article.

**Enzyme immunoassay.** The PGF2α concentration was determined in the conditioned media of NRK cells cultured under different growth conditions and in TransX using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s protocol. In the medium conditioned by density-arrested monolayers, the concentration was determined 2 days after addition of EGF-insulin to quiescent monolayers. In the medium conditioned using phenotypically transformed monolayers, the concentration was determined 2 days after addition of EGF-insulin-RA to density-arrested monolayers.

**Growth-stimulation assays.** Proliferation of NRK cells was investigated in growth stimulation assays in which cumulative incorporation of [³H]thymidine (0.5 μCi/ml added; Amersham International) was measured between 24 and 40 h after addition of the growth factors to density-arrested NRK monolayers (35). When AL-8810 (Cayman Chemical) was used to block the FP receptor, it was added 1 h before addition of the growth factors. Dose-response curves for TransX and PGF₂α were created using sigmoidal curve fitting with Microlab Origin software version 6.0. EC₅₀ and Hill coefficients were derived from these curves.

**Statistics.** Student’s t-test was used for statistical comparisons. Numeric data are presented as means ± SE throughout this article, with n representing the number of replicates of each experiment.

**RESULTS**

**Growth state-dependent membrane potential of NRK cells.** We investigated the effect of phenotypic transformation on the membrane potential of NRK cells by performing whole cell current-clamp experiments on NRK fibroblasts that had been transformed in the presence of growth-stimulating factors. The membrane potential of NRK cells at certain growth conditions is summarized in Table 1.

Quiescent monolayers exhibit a stable resting membrane potential around −70 mV (Fig. 1A), which is maintained by inwardly rectifying K⁺ conductances (14). Although fibroblasts are classic examples of nonexcitable cells, monolayers of density-arrested NRK fibroblasts repetitively fire action potentials (Fig. 1B). This phenomenon was first described by De Roos et al. (9), and to date NRK fibroblasts are the only...
Table 1. Membrane potential of normal rat kidney fibroblasts under different conditions

<table>
<thead>
<tr>
<th>Cell Conditions</th>
<th>No. of Samples</th>
<th>Membrane Potential, mV</th>
<th>Action Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quiescent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No compound added</td>
<td>39</td>
<td>-73.1±0.7</td>
<td></td>
</tr>
<tr>
<td>CM alone</td>
<td>30</td>
<td>-19.4±0.6</td>
<td></td>
</tr>
<tr>
<td>CM + 50 μM BAPTA-AM</td>
<td>3</td>
<td>-67.3±2.2</td>
<td></td>
</tr>
<tr>
<td>CM + 250 μM flufenamic acid</td>
<td>3</td>
<td>-73.7±0.7</td>
<td></td>
</tr>
<tr>
<td>Density arrested</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting potential, mV</td>
<td>18</td>
<td>-60.6±1.1</td>
<td></td>
</tr>
<tr>
<td>Peak AP, mV</td>
<td>18</td>
<td>+4.7±2.4</td>
<td></td>
</tr>
<tr>
<td>Plateau AP, mV</td>
<td>18</td>
<td>-21.3±0.7</td>
<td></td>
</tr>
<tr>
<td>AP duration, s</td>
<td>18</td>
<td>37.9±3.5</td>
<td></td>
</tr>
<tr>
<td>AP frequency, min⁻¹</td>
<td>18</td>
<td>-0.24±0.5</td>
<td></td>
</tr>
<tr>
<td>Transformed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA 25</td>
<td>-22.0±1.8*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β 3</td>
<td>3</td>
<td>-16.7±3.3*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. CM, conditioned medium of phenotypically transformed normal rat kidney cells; AP, action potential; RA, retinoic acid; TGF-β, transforming growth factor-β. *P > 0.1, not significantly different.

fibroblasts known to generate action potentials. The action potentials are based on the sequential activation of L-type Ca²⁺, Ca²⁺-dependent Cl⁻, and inwardly rectifying K⁺ conductances (14). Typically, they were of long duration (between 18 and 72 s), and firing occurred at a low frequency (between 0.1 and 0.83 min⁻¹). Mathematical modeling revealed that periodic firing most likely involves complex intracellular Ca²⁺ dynamics (31).

In contrast to their nontransformed counterparts, transformed NRK cells are strongly depolarized around −20 mV (Fig. 1C), regardless of whether RA or TGF-β was used to induce transformation (Table 1). In other words, phenotypic transformation of NRK fibroblasts is accompanied by a strong membrane depolarization.

Conditioned medium from phenotypically transformed NRK cells has depolarizing activity. The membrane of phenotypically transformed NRK cells slowly started to repolarize upon continuous perfusion with fresh serum-free medium, and within 45 min the cells fired action potentials that were similar to those observed in density-arrested monolayers (n = 11; Fig. 2A). Because RA, EGF, and insulin by themselves or in combination did not affect the membrane potential of the quiescent cells in short-term incubations when used to induce phenotypic transformation, we hypothesized that phenotypically transformed NRK cells secrete a compound that has depolarizing activity.

To test this hypothesis, we harvested the culture medium of phenotypically transformed NRK cells and applied it to quiescent monolayers. This conditioned medium (CM) reversibly depolarized NRK cells in such monolayers from −73.0±0.5 mV to −19.4±0.6 mV (n = 30) without any sign of homologous desensitization (Fig. 2B). Because this depolarization is close to the equilibrium potential for Cl⁻ in NRK cells (8), we investigated whether the effect of CM on the membrane potential was mediated by activation of Ca²⁺-dependent Cl⁻ channels (see Table 1), which are known to be present in NRK cells (14). Under conditions in which intracellular Ca²⁺ was buffered by incubation with BAPTA-AM, the strong CM-induced depolarization of quiescent monolayers was pre-

![Image](http://ajpcell.physiology.org/DownloadedFrom/10.220.33.1.onSeptember22.2017)

![Image](http://ajpcell.physiology.org/DownloadedFrom/10.220.33.1.onSeptember22.2017)
vented. In addition, flufenamic acid, a widely used blocker of Ca\(^{2+}\)-dependent Cl\(^{-}\) channels (36), relieved the depolarization induced by CM almost instantly. The CM of NRK cells that had been transformed by TGF-β also strongly depolarized quiescent NRK cells. The membrane potential after addition of this medium was \(-25.3 \pm 3.1\) mV (n = 4) and was not different from the potential observed after addition of the medium conditioned by RA-transformed cells (P > 0.05).

Taken together, these data seem to indicate that phenotypically transformed NRK cells secrete a compound in their medium that depolarizes NRK cells by the activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels.

Identification of the active compound with mass spectrometry and enzyme immunoassay. To unravel the chemical structure of the active compound, we purified it from the medium that depolarizes NRK cells by the activation of Ca\(^{2+}\)-activated Cl\(^{-}\) channels using a combination of reverse-phase chromatography and gel permeation chromatography as described in MATERIALS AND METHODS. After each separation step, the collected fractions were tested for their ability to depolarize quiescent NRK monolayers. The active fraction from the final HPLC separation, provisionally referred to as TransX, was subsequently analyzed using ESI mass spectrometry (ESI-MS) in combination with TOF mass separation.

Figure 3A shows the positive ion ESI-MS of TransX. By performing fragmentation of the compound corresponding to the major peak at m/z 377.3 using positive ion ESI-MS/MS, we obtained a fingerprint of the chemical structure of this molecule (Fig. 3B). The fragmentation pattern showed peaks at m/z 377.3, m/z 359.3, m/z 341.3, and m/z 315.3, as well as isotope peaks corresponding to m/z 377.3 and m/z 359.3 shown in Fig. 3, C and D, respectively. On the basis of this fingerprint, we could identify the compound as PGF\(_{2\alpha}\). To verify this conclusion, we measured the mass spectrum of commercially produced PGF\(_{2\alpha}\) (1,000 pmol; Cayman Chemical), which has a molecular weight of 354.5 g/mol. Figure 3E shows that PGF\(_{2\alpha}\) (see inset for structure) was measured as a sodium adduct at m/z 377.3, and when this peak was further analyzed with ESI-MS/MS, the fragmentation pattern observed was the same as that observed for TransX (Fig. 3F), with similar isotope peaks (Fig. 3, G and H). The fragmentation peaks resulted from the sequential losses of water (from m/z 377.3 to m/z 359.3 to m/z 341.3) and both water and carboxyl (from m/z 377.3 to m/z 315.3) from the parent compound. These results demonstrate that TransX contains PGF\(_{2\alpha}\) and that the depolarizing activity of TransX can be ascribed to this prostaglandin (13).

We also measured the mass spectra of two other prostaglandins with known Ca\(^{2+}\)-mobilizing activity (38). Prominent

![Figure 3A](image-url)
peaks were measured at m/z 375.3 when commercially produced prostaglandin E2 (PGE2; mol wt 352.5 g/mol) or prostaglandin D2 (PGD2; mol wt 352.5 g/mol) was injected (both 1,000 pmol; Cayman Chemical). However, no peaks were found at m/z 377.3, indicating that TransX does not contain detectable amounts of PGE2 and PGD2.

To confirm the presence of elevated PGF2α concentrations in the medium conditioned by phenotypically transformed NRK fibroblasts, we used a PGF2α-specific enzyme immunoassay. Quiescent NRK monolayers (cf. Fig. 1A) did not secrete detectable amounts of PGF2α, while density-arrested NRK cells (cf. Fig. 1B) secreted PGF2α at a very low concentration in medium (1.5 ± 0.1 nM; n = 3). In contrast, the PGF2α concentration in the medium conditioned by transformed NRK cells was at least 10-fold (19.7 ± 2.5 nM; n = 6) that released by nontransformed NRK cells. After removal of the culture medium from phenotypically transformed NRK cells and replacement by fresh serum-free medium, the PGF2α concentration after 8 h of incubation was 3.6 ± 1.4 nM (n = 3). Apparently, NRK cells continue the secretion of PGF2α once they have obtained a transformed phenotype, also in the absence of externally added growth factors.

Thus phenotypically transformed NRK cells exhibit an enhanced secretion of PGF2α, as the depolarizing activity in culture medium.

Role of COX activity in the production of the active compound. Because COX mediates the production of prostaglandins, including PGF2α, we investigated whether inhibiting COX activity abolishes the depolarizing activity of the medium conditioned by transformed NRK cells.

For that purpose, we applied a cocktail of COX inhibitors (indomethacin, flurbiprofen, and ibuprofen; all 5 μM) before and after RA-induced phenotypic transformation of NRK cells and measured whether the CM could still depolarize quiescent NRK monolayers. Media conditioned by NRK cells that had been transformed in the absence of COX inhibitors could reversibly depolarize the cells to −19.4 ± 0.6 mV (n = 30), but the membrane potential was hardly affected (−68.9 ± 0.9 mV; n = 11) when the cocktail of COX inhibitors was applied 1 h before addition of the growth factors, and the CM was harvested 2 days later. Similar results were obtained when phenotypically transformed NRK cells were incubated in fresh serum-free medium. Media harvested after 8-h incubation depolarized quiescent NRK cells to −20.1 ± 1.5 mV (n = 5), but the membrane potential remained unchanged (−72.6 ± 1.2 mV; n = 3) when the cocktail of COX inhibitors was applied 1 h before replacement of the culture of the fresh medium. In agreement with the observation that no depolarizing activity was measured in media of NRK cells cultured in the presence of COX inhibitors, PGF2α could not be detected in such media with the use of enzyme immunoassay.

These results confirm that COX is involved in the production of the depolarizing factor and support the conclusion that it is PGF2α.

Effect of TransX and PGF2α on the intracellular Ca2+ concentration of quiescent NRK cells. By performing dynamic video imaging measurements of fura-2-loaded, quiescent NRK cells, we investigated whether TransX had effects on intracellular Ca2+ levels similar to those of PGF2α. This prostaglandin induces unsynchronized intracellular Ca2+ oscillations in NRK cells cultured in quiescent monolayers (13).

Figure 4A shows the typical Ca2+ response of an individual NRK cell in a monolayer upon exposure to TransX. In the presence of external Ca2+ (1 mM), TransX induced an oscillatory Ca2+ response in most cells (89.9%), while the initial peak was followed by a plateau phase in the remainder of the cells (n = 168 cells from 4 different monolayers). The frequency of the oscillations was 0.92 ± 0.03 min−1 (n = 151) and ranged between 0.27 and 2.17 min−1. The averaged response of 42 cells from the monolayer shown in Fig. 4A is displayed in Fig. 4B.

Similarly to PGF2α, TransX induces unsynchronized intracellular Ca2+ oscillations in NRK cells, which further supports the finding that specifically this prostaglandin is released by the phenotypically transformed NRK cells.

Contribution of PGF2α to phenotypic transformation of NRK cells. At a high (1 μM) concentration, PGF2α can induce phenotypic transformation of NRK cells (19). We next investigated, in growth stimulation experiments, whether more physiological concentrations of PGF2α can also release NRK cells from growth arrest.

Figure 5, A and B, shows the loss of density arrest of NRK cells induced by TransX and commercially produced PGF2α, both in combination with EGF (5 ng/ml) and insulin (5 μg/ml). The data show that under the conditions tested, TransX and commercially produced PGF2α can induce cellular growth in a concentration-dependent manner to an extent almost similar to that of RA (50 ng/ml). EC50 values for TransX and commercially produced PGF2α were 4.30 ± 1.67 nM (n = 3) and 6.12 ± 0.94 nM (n = 3), respectively, and these data were not significantly different (P > 0.1). Hill coefficients were 1.84 ± 0.18 and 1.94 ± 0.24, respectively, and also were not significantly different (P > 0.1). The parallel action of TransX and PGF2α is in agreement with our finding that TransX contains PGF2α. TransX and PGF2α did not have a growth-stimulatory effect on NRK cells in the absence of EGF (data not shown). These results demonstrate that the PGF2α concentration present in the medium conditioned by phenotypically transformed NRK cells (i.e., −20 nM) is sufficient to induce transformation of nontransformed neighboring cells.

Fig. 4. Effect of TransX on intracellular Ca2+ concentration of quiescent NRK fibroblasts. A: typical Ca2+ response induced by TransX in individual cell selected from a panel of 42 cells within a monolayer. B: averaged response of these 42 cells. The response was measured in 168 cells from 4 different monolayers. Addition of TransX is indicated by bars. An aliquot of TransX was added, causing a final equivalent PGF2α concentration of 100 nM.
Next, we investigated whether the FP receptor, which is the natural receptor for PGF2α (6), is involved in the induction of phenotypic transformation by PGF2α. For that purpose, we stimulated NRK cells with 20 nM PGF2α, but in the additional presence of various concentrations of AL-8810 (Cayman Chemical). This compound has been described as a specific antagonist of the FP receptor partially (12). AL-8810 completely blocked PGF2α-induced intracellular Ca2+ oscillations in NRK cells at concentrations ≥10 μM (n = 3). Figure 5C shows that AL-8810 suppresses PGF2α-induced proliferation of NRK cells. AL-8810 did not completely block phenotypic transformation of NRK cells, because it exerts growth-stimulatory activity by itself, which can be attributed to its weak agonist potency on the FP receptor (12).

The contribution of endogenously released PGF2α to RA-induced phenotypic transformation is shown in Fig. 5D. This image shows that externally added PGF2α (100 nM) amplifies RA-induced proliferation of NRK cells. AL-8810 maximally inhibited PGF2α-induced proliferation of these cells, while this antagonist of the FP receptor partially (~25%) suppressed RA-induced proliferation. Mitogenic activity induced by the combination of RA and externally added PGF2α (100 nM) was blocked by AL-8810 to at least a similar extent as RA-induced proliferation. These results indicate that activation of the FP receptor by endogenously released PGF2α contributes to RA-induced phenotypic transformation. We tested the effect of the mixture of COX inhibitors (indomethacin, flurbiprofen, and ibuprofen) on RA-induced phenotypic transformation of NRK cells and found that this cocktail did not cause a significant reduction of mitogenic activity at ≤10 μM concentrations of each inhibitor (P > 0.1). In contrast, RA-induced phenotypic transformation was significantly reduced by 50% when a nontoxic 50 μM concentration was used (P < 0.01).

Taken together, these data indicate that PGF2α can release NRK cells from density-dependent growth inhibition and contributes to RA-induced phenotypic transformation of NRK cells by a mechanism involving activation of the FP receptor.

DISCUSSION

In the present study, we have shown that phenotypically transformed NRK fibroblasts exhibit a depolarized membrane compared with their nontransformed counterparts as a result of a constitutive activation of Ca2+-dependent Cl− channels. The increase of the intracellular Ca2+ concentration responsible for the opening of these channels is caused by the presence of strongly elevated PGF2α concentrations upon transformation of these cells. This released prostaglandin triggers proliferation of density-arrested NRK cells and enhances RA-induced phenotypic transformation by a mechanism involving activation of the FP receptor.

Phenotypic transformation of NRK cells by RA or TGF-β is accompanied by the loss of density-dependent growth inhibition and the ability to proliferate under anchorage-independent conditions (18, 34). We have shown for the first time that phenotypically transformed NRK fibroblasts share two other typical properties with tumor cells. First, phenotypically transformed NRK cells are depolarized compared with their nontransformed counterparts, which is a characteristic of many tumor cells (21). Second, in parallel with the enhanced COX...
activity observed in various types of both human and animal cancer cells (22), transformed NRK fibroblasts secrete PGF2α in culture medium at concentrations at least 10-fold those released by nontransformed NRK cells. Although tumorigenic transformation results in a reduction or inhibition of gap junctional intercellular communication in many cell types (3, 15, 20), dye-coupling experiments have previously shown that phototypic transformation of NRK fibroblasts is accompanied by an increase in gap junctional intercellular communication (33). In agreement with this finding, preliminary single-electrode patch-clamp experiments revealed that transformed NRK cells are electrically well coupled. Thus transformation of NRK cells does not result in a reduction of gap junctional coupling.

The identification of PGF2α as the biologically active factor in the medium conditioned by transformed NRK cells was based on mass spectrometry and confirmed using an enzyme immunoassay. This discovery was further established by the finding that TransX and commercially produced PGF2α exhibited similar effects on membrane potential, intracellular Ca2+ levels, and cellular growth of NRK cells.

The PGF2α concentration in the medium conditioned by transformed NRK cells determined using enzyme immunoassay was ~20 nM. We previously found that the minimal PGF2α concentration required to constitutively depolarize monolayers of quiescent NRK cells is ~10 nM (13), and therefore the concentration present in the CM of transformed NRK cells was sufficient to explain the depolarizing activity of this medium. Repetitive addition of either the CM of transformed NRK cells or TransX showed no homologous desensitization, in agreement with the finding that PGF2α only partially desensitizes its natural G protein-coupled receptor (10). The binding of PGF2α to this FP receptor previously was shown to result in increased intracellular IP3 and Ca2+ levels (23), and we recently demonstrated that PGF2α can induce intracellular Ca2+ oscillations in NRK cells cultured in quiescent monolayers (13). These oscillations resulted from an intricate interplay between Ca2+ release from internal stores and Ca2+ influx over the plasma membrane and were not synchronized in spite of gap junctional coupling of the NRK cells in such monolayers. In the present study, we have shown that TransX also induces unsynchronized Ca2+ oscillations in quiescent NRK monolayers, which supports the finding that this purified active fraction contains PGF2α.

We have found that PGF2 and PGE2 can mimic the effect of PGF2α on the membrane potential of quiescent NRK monolayers, but only at higher concentrations (data not shown). This finding most likely reflects the ability of these prostaglandins to activate the FP receptor with low affinity (23). Although we cannot exclude the possibility that apart from PGF2α, phenotypically transformed NRK fibroblasts secrete low concentrations of other prostaglandins, including PGE2 and PGD2, these prostaglandins were not found in the mass spectrum of TransX.

The present data indicate that phenotypic transformation of NRK fibroblasts is associated with increased COX activity. In recent years, many studies have been performed to elucidate the role of the COX enzyme and the production of prostaglan- 
dins in relation to cancer (4, 24, 30). Overexpression of COX-2 has been found to play a key role in various stages of tumorigenesis (29), and PGE2 seems to be a major determinant in tumor promotion (27, 39). PGF2α has been identified as an endogenous tumor promoter in the mouse skin model of multistage carcinogenesis (11) and in the kidney (16), and it acts as an endogenous promoter of cell transformation in cultured mouse fibroblasts (37).

The finding that transformed NRK cells secrete PGF2α as a paracrine factor indicates that these transformed cells can induce a transformed phenotype in their nontransformed neighboring cells. The growth-stimulatory action of PGF2α requires binding to its FP receptor, whereas activation of this receptor is also involved in the autocrine enhancement of RA-induced transformation by PGF2α. On the basis of these results, the FP receptor seems to be a potential target in medical therapies, particularly because elevated expression of functional FP receptors has been reported in human endometrial adenocarcinomas (26).

Although the exact mechanism by which endogenous PGF2α contributes to the transformation of NRK cells is still elusive, our data strongly indicate that this prostaglandin plays a crucial role in the transformation process.

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