DEFICIENCY IN INTERCELLULAR COMMUNICATION IN TWO ESTABLISHED RENAL EPITHELIAL CELL LINES (LLC-PK₁ AND MDCK)

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SUMMARY
We have studied the cell-to-cell passage of uridine nucleotides in two renal epithelial cell lines (LLC-PK₁ and MDCK) and in porcine aortic endothelial cells (PAE). All three cell types incorporated tritiated uridine. After a 3 h incubation the radioactivity was predominantly in the form of acid-soluble compounds, mainly UTP. Prelabelled LLC-PK₁ or MDCK cells were unable to transfer radioactivity to added adjacent, non-labelled cells, whereas PAE cells readily formed communicating intercellular junctions, as judged by autoradiographic analysis after a 3 h co-culture period. Cell-to-cell communication in either of the renal cell lines was not promoted by treatment with dibutyryl cyclic AMP and methylisobutylxanthine. Radioactivity incorporated into the acid-insoluble pool was not available for intercellular transfer, as assessed in experiments in which cells were prelabelled 24 h before co-culture.

INTRODUCTION
Epithelial cell lines that retain differentiated properties in vitro have recently become available. They are interesting model systems in which to study general physiological problems related to epithelial transport as well as to investigate the mechanisms for the generation of membrane asymmetry. Among the best studied cell lines are MDCK and LLC-PK₁, both of which are apparently immortal.

The MDCK cell line was isolated from normal dog kidney (Gaush, Hard & Smith, 1966). The cells have a distinct epithelial morphology, possess a pseudodiploid karyotype and display several characteristics of normal kidney tubule cells after 60–120 passages in vitro (Cereijido et al. 1978; Saier, 1981). Some of their physiological properties as well as their enzyme content are consistent with the conclusion that MDCK cells originally derived from the distal tubule (Rindler, Chuman, Shaffer & Saier, 1979; Richardson, Scalera & Simmons, 1981). It has been shown that MDCK cells do not form tumours in adult nude mice (Stiles et al. 1976).

LLC-PK₁ cells were isolated from juvenile pig kidney (Hull, Cherry & Weaver, 1976). They form monolayers that exhibit typical epithelial morphology (Mills, Macknight, Dayer & Ausiello, 1979) and display membrane asymmetry of transport functions after more than 200 passages in culture (Mullin & Kleinzeller, 1983;
The presence of a sodium-dependent hexose transport system in LLC-PK₁ cells (Mullin & Kleinzeller, 1984) as well as their enzyme content (Sepúlveda, Burton & Pearson, 1982; Moran, Handler & Turner, 1982) suggest a proximal renal tubule origin for this line. LLC-PK₁ cells have an apparently diploid chromosome number and do not produce tumours in immunosuppressed animals (Hull et al. 1976; Mullin & Kleinzeller, 1984).

Communicating junctions linking neighbouring cells are of common occurrence in animal tissues, both between cells of the same and of different types. They are thought to mediate the spread of electrical current, notably in excitable tissues, and also the intercellular exchange of metabolites of low molecular weight (Löwenstein, 1979, 1981; Pitts & Finbow, 1982; Hooper, 1982). It is almost certain that the gap junction, first characterized by electron microscopy and more recently isolated and purified (Caspar, Goodenough, Makowski & Philips, 1977; Unwin & Zampighi, 1980; Herzberg, Lawrence & Gilula, 1981), is the cellular structure that mediates the functional roles described above. The consequences of intercellular communication in non-excitable tissues are not known, although two major hypotheses have been proposed. These suggest that junctional permeability serves to integrate cellular metabolism within a tissue, thus co-ordinating responses to exogeneous metabolites or hormones, and that the presence of gap junctions is essential to the regulation of cell growth and division (Sheridan, 1976; Löwenstein, 1979).

Electrical coupling between proximal tubule epithelial cells has been demonstrated (Giebisch & Windhager, 1973), and this is correlated with the presence of gap junctions (Silverblatt & Bulger, 1970; Pricam, Humbert, Perrelet & Orci, 1974; Kühn & Reale, 1975). An absence of gap junctions has been noted in distal tubule epithelium (Pricam et al. 1974; Kühn & Reale, 1975), suggesting that in vivo this tissue is unusual in exhibiting little, if any, intercellular communication.

In this paper we have explored the intercellular communication capability of MDCK and LLC-PK₁ cells. The approach used is that developed by Pitts & Simms (1977), in which labelled nucleotide transfer between cells is assessed. We have also investigated the intercellular permeability of subcultured porcine aortic endothelial cells, whose ability to communicate intercellularly has not been tested previously, although primary cultures of bovine aortic endothelial cells have been shown to transfer nucleotides and to be electrically coupled (Larson & Sheridan, 1982). As expected, the endothelial cells were competent in the nucleotide transfer assay. Under the same assay conditions, however, the two epithelial cell lines were transfer-deficient.

**MATERIALS AND METHODS**

**Cell culture**

The renal epithelial cells, LLC-PK₁ and MDCK, were generously given to us by Dr R. N. Hull (Lilley Research Laboratories, Indianapolis, U.S.A.) and Dr N. L. Simmons (Department of Physiology, University of St Andrews, Scotland) respectively. Each was grown in Dulbecco's modification of Eagle's medium containing 10% foetal calf serum, as previously described (Sepúlveda & Pearson, 1982). The LLC-PK₁ cells were used at passages 195–220, and the MDCK cells...
Renal epithelial cell communication

at passages 60–75. Porcine aortic endothelial cells (PAE) were isolated by collagenase treatment of thoracic aortas from piglets and cultured in Dulbecco's medium containing 20% foetal calf serum, as previously described (Pearson, Carleton, Hutchings & Gordon, 1978). PAE cells were used after 3–8 passages.

Labelling of cells with \( {\text{\textsuperscript{3}}H} \)uridine

Cells were plated into 16 mm wells of 24-well plastic culture trays at a density of 30,000 cells per well. Eighteen hours after plating the cells were loaded with \( [5,6-\text{H}] \)uridine (2-5 μCi/ml, sp. act. 45 Ci/mmol) during a 3 h incubation in serum-free culture medium. Loading was terminated by extensive washing with serum-free culture medium and cells were processed at this point or left in unlabelled serum-containing medium for a further 3 h or 24 h before processing, as described by Pitts & Simms (1977). Trichloroacetic acid-soluble and insoluble radioactivity were determined by liquid scintillation spectrometry. The acid-soluble extract was neutralized and, after the addition of unlabelled uridine, UMP, UDP and UTP as markers, resolved by thin-layer chromatography as described by Pearson et al. (1978), \( R_f \) values were: UTP, 0.03; UDP, 0.12; UMP, 0.28; uridine, 0.60. The amount of radioactivity associated with each component was determined after cutting the spots into scintillation vials, eluting with 0.1 M-HCl (1 ml) and adding 3 ml of Packard Pico-Fluor 30 scintillation fluid.

Nucleotide transfer experiments

The method used followed that of Pitts & Simms (1977) with only minor modifications. Donor cells were plated on 13 mm glass coverslips at a density of 5000 cells per coverslip. Eighteen hours after plating cells were preloaded with \( \text{\textsuperscript{3}}H \)uridine as described above. After extensive washing coverslips were either used immediately or left in unlabelled culture medium for a further 24 h. Suspensions of unlabelled recipient cells (20,000–40,000 cells per coverslip) were added above the prelabelled cells and co-cultured for 3 h. Control coverslips, containing only donor cells, were incubated in parallel. The cells were fixed in formal saline, washed in ice-cold trichloroacetic acid (TCA), and processed for autoradiography. After photographic development and fixation, cells were stained with haematoxylin and eosin and mounted for microscopic examination. Silver grains were counted over cells in contact and not in contact with donor cells as described by Hunter & Pitts (1981).

RESULTS

The general strategy developed by Pitts & Simms (1977) to detect the presence of intercellular permeable junctions is based on the ability of animal cells in culture to take up uridine and convert it into its nucleotides. Incorporation also occurs into RNA and, in a minor proportion only, into DNA. Nucleotide transfer occurs across permeable junctions when competent cells are brought into contact in the culture dish; TCA-insoluble material (RNA or DNA) is not exchanged.

We first tested whether the cells used in this study are capable of taking up \( \text{\textsuperscript{3}}H \)uridine. Table 1 shows the radioactivity incorporated by LLC-PK₁, MDCK and PAE cells after a 3 h incubation in the presence of \( \text{\textsuperscript{3}}H \)uridine. Between 66 and 83% of the intracellular radioactivity, depending on the cell type, was in the form of TCA-soluble compounds. Further incubation of the cells for 3 h in non-radioactive serum-containing culture medium caused some loss of total radioactivity and a reduction of the radioactivity in the TCA-soluble extracts in all three cell types, although there was still between one-third and one-half of the TCA-soluble radioactivity found immediately after pre-loading. There was also an increase in radioactivity associated with TCA-insoluble material. Total radioactivity in the cells decreased further over
Table 1. Intracellular radioactivity after 3 h incubations with [3H]uridine

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Time after labelling (h)</th>
<th>Acid-soluble (%)</th>
<th>Acid-insoluble (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLC-PK1</td>
<td>0</td>
<td>66·4</td>
<td>33·6</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>31·5</td>
<td>57·4</td>
<td>88·9</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4·0</td>
<td>63·5</td>
<td>67·5</td>
</tr>
<tr>
<td>MDCK</td>
<td>0</td>
<td>79·8</td>
<td>20·2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>23·3</td>
<td>75·2</td>
<td>98·5</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2·1</td>
<td>70·6</td>
<td>72·7</td>
</tr>
<tr>
<td>PAE</td>
<td>0</td>
<td>82·7</td>
<td>17·3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>28·9</td>
<td>53·8</td>
<td>82·7</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4·8</td>
<td>38·2</td>
<td>43·0</td>
</tr>
</tbody>
</table>

Radioactivity in the TCA-soluble and TCA-insoluble pools is given as a percentage of the total intracellular radioactivity immediately after labelling (0 h). Results are the means from six replicate culture wells.

Table 2. Thin-layer chromatographic analysis of cellular uridine nucleotides after 3 h incubations with [3H]uridine

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Time after labelling (h)</th>
<th>Distribution of radioactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uridine</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>0</td>
<td>0·4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0·8</td>
</tr>
<tr>
<td>MDCK</td>
<td>0</td>
<td>1·3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0·7</td>
</tr>
<tr>
<td>PAE</td>
<td>0</td>
<td>1·2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0·7</td>
</tr>
</tbody>
</table>

Samples of acid-soluble extract from different cells were chromatographed. Radioactivity associated with each compound is given as a percentage of the total radioactivity applied to the thin-layer chromatography plate. Results are the means of three determinations. The $R_F$ value of the unidentified radioactive component was 0·45.

Samples of TCA-soluble cell extract by thin-layer chromatography is shown in Table 2. In all three cell types, immediately after the labelling period most of the label was found associated with UTP together with substantial amounts in UMP and a little in UDP, while non-metabolized uridine formed only a very small proportion of the total intracellular label.

From these measurements we conclude that LLC-PK1, MDCK and PAE cells are able to take up [3H]uridine and to maintain an intracellular pool of TCA-soluble labelled material, predominantly nucleotides, for more than 3 h. They also incorporate label into TCA-insoluble material (probably RNA), which accounts for
essentially all the cellular radioactivity after 24 h. They are therefore suitable cell types in which to test for intercellular junctions using the protocol devised by Pitts & Simms (1977).

Accordingly, cells preloaded with [$^3$H]uridine were used to determine their capacity to transfer nucleotides to unlabelled cells in co-culture. In Fig. 1A–C different groups of closely apposed labelled and unlabelled LLC-PK$_1$ cells can be seen. The

Fig. 1. Autoradiographs of co-cultured LLC-PK$_1$ cells in which potential donor cells were preloaded with [$^3$H]uridine for 3 h. A, B. Examples in which unlabelled cells were added immediately after the preloading period and co-cultured for 3 h. C. An example in which unlabelled cells were added 24 h after the preloading period and co-cultured for 3 h. Autoradiographs were exposed for 22 days. Bars, 50 μm.
Fig. 2. Autoradiographs of co-cultured MDCK cells in which potential donor cells were preloaded with \([^{3}H]\)uridine for 3 h. A, B. Examples in which unlabelled cells were added immediately after the preloading period and co-cultured for 3 h. Autoradiographs were exposed for 14 days. Bars, 50 \(\mu\)m.

grain density above potential recipient cells was not different from that of the background either in controls (c) or when cells were co-cultured immediately after the preloading period (A and B). In autoradiographs of preloaded cells alone, all cells were labelled 3 h or 24 h after the preloading period (not shown).

Fig. 2 shows that a similar result was obtained with MDCK cells. Heavily labelled cells were seen in close contact with cells that show no silver grains above the background level. Identical pictures were obtained (not shown) when cells were cocultured 24 h after the preloading period and again parallel autoradiographs of preloaded cells alone revealed that all such cells were labelled.

The results obtained with the two kidney epithelial cell lines are in contrast to those obtained with PAE cells, which participated efficiently in intercellular uridine nucleotide transfer. Fig. 3A–C, shows heavily labelled donor cells in contact with recipient cells that are also labelled but to a lesser extent. Control autoradiographs of preloaded cells alone helped in the identification of donor cells, which were in general more widely spread than the recipients. Fig. 3D demonstrates that preloaded cells cultured for 24 h in unlabelled medium transfer little radioactivity to potential recipient cells. A quantitative analysis of the autoradiographs is summarized in Table 3. Silver grain counts over recipient cells in contact with donor cells and over isolated cells are given. As discussed by Hunter & Pitts (1981), counts over isolated cells represent
Fig. 3. Autoradiographs of co-cultured PAE cells in which potential donor cells were preloaded with [3H]uridine for 3 h. A, B, C. Examples in which unlabelled cells were added immediately after the preloading period and co-cultured for 3 h. D. An example in which unlabelled cells were added 24 h after the preloading period and co-cultured for 3 h. Autoradiographs were exposed for 22 days. Bars, 50 μm.
Table 3. Transfer of uridine nucleotides between cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Silver grains per cell</th>
<th>C.C.</th>
<th>C.N.C.</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLC-PK₁</td>
<td></td>
<td>7.4 ± 3.2</td>
<td>8.2 ± 2.9</td>
<td>1.31</td>
<td>N.S.</td>
</tr>
<tr>
<td>MDCK</td>
<td></td>
<td>6.3 ± 2.6</td>
<td>8.4 ± 2.0</td>
<td>0.22</td>
<td>N.S.</td>
</tr>
<tr>
<td>PAE</td>
<td></td>
<td>7.3 ± 2.4</td>
<td>4.5 ± 2.5</td>
<td>19.63</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PAE (24 h)</td>
<td></td>
<td>9.9 ± 3.4</td>
<td>8.2 ± 3.2</td>
<td>2.58</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

Counting of silver grains associated with recipient cells in contact (C.C.) or not in contact (C.N.C.) with labelled donor cells. Results shown are for experiments in which labelling was carried out, as described in the text, for 3 h followed by a 3 h period of co-culture. The last row, PAE (24 h), corresponds to an experiment in which donor PAE cells were left for 24 h in unlabelled medium after the labelling period. Autoradiographs were exposed for 14 days. Silver grains were counted over 50-51 cells contacting with donor cells and 50-51 cells not in contact with donor cells. Values shown are the means ± the standard deviation of the two cell populations. Significance of the difference between means is analysed by Student's t-test with 98-100 degrees of freedom. N.S., not significant.

It has been reported previously that cyclic AMP can increase intercellular communication (Hax, van Venrooji & Vossenburg, 1974) or induce junctional coupling and nucleotide transfer in junction-deficient cell types (Wright, Slack, Goldfarb & Subak-Sharpe, 1976; Azarnia, Dahl & Loevenstein, 1981). We therefore attempted to promote uridine nucleotide transfer in MDCK or LLC-PK₁ cells by incubating the cells in the presence of dibutyryl cyclic AMP (2.5X10⁻⁴ M) and methylisobutylxanthine (10⁻⁵ M). The presence of the drugs during the co-culture period or during both preloading and co-culture periods did not alter the inability of MDCK and LLC-PK₁ cells to transfer uridine nucleotides (result not shown).

DISCUSSION

In this paper we have used the assay introduced by Pitts & Simms (1977) to study the cell-to-cell passage of uridine nucleotides in two established renal epithelial cell lines and in porcine aortic endothelial cells. We found no evidence for the formation of nucleotide-permeable junctions between LLC-PK₁ cells or MDCK cells, whereas intercellular nucleotide transfer between PAE cells was readily demonstrated. Transfer of labelled material between PAE cells did not occur when the radioactivity was almost exclusively in the form of TCA-insoluble compounds (probably RNA), showing the usual restriction to low molecular weight substances for this type of transfer.
The lack of observable transfer between LLC-PK₁ cells or MDCK cells was not due to an inability of the renal cells to take up uridine or to convert it to nucleotides: the patterns of distribution of the products of [³H]uridine metabolism and the time courses of the conversion of TCA-soluble to TCA-insoluble material were similar in all three cell types studied. The establishment of communicating junctions in homogeneous co-cultures of competent cells is normally a rapid process starting within minutes of contact, and transfer of label being observed between most adjacent cells within an hour (Pederson, Sheridan & Johnson, 1980). These two renal cell lines, therefore, seem to be deficient in their ability to form such junctions. This finding was unexpected, in view of the almost ubiquitous occurrence of communicating junctions in cultured cells. The few exceptions previously reported include some clones of the L cell line and certain tumorigenic lines (see Loewenstein, 1979). As noted in the Introduction, both LLC-PK₁ and MDCK cells are diploid or near-diploid and non-tumorigenic.

The anatomical structure normally thought to mediate intercellular communication is the gap junction, although, as has been noted by Hooper (1982), other structures such as the tight junction cannot easily be excluded since examples of cell types that form tight junctions but not gap junctions are rare. In this respect it is noteworthy that tight junctions are readily formed between LLC-PK₁ cells or MDCK cells in culture. They have been demonstrated microscopically and their presence confirmed by other techniques—in particular the recognition that both of these cell types form clearly polarized monolayers in vitro with an asymmetric distribution of properties between apical and basolateral membranes (Mills et al. 1979; Meza et al. 1980; Misfeldt & Sanders, 1981; Dragsten, Blumenthal & Handler, 1981; Mullin & Kleinzeller, 1984; Sepúlveda & Pearson, 1984). Thus in LLC-PK₁ cells and in MDCK cells tight junctions do not appear to be able to mediate cellular nucleotide transfer. Moreover, published freeze-fracture electron micrographs of MDCK cells do not reveal the presence of any gap junctions (U, Saier & Ellisman, 1979; Meza et al. 1980), strongly suggesting that the absence of these junctions is indeed the reason for their inability to transfer nucleotides. We have not seen freeze-fracture studies of LLC-PK₁ cells, but published transmission electron micrographs (Mills et al. 1979; Misfeldt & Sanders, 1981) do not show obvious gap junctions.

It must be noted that our finding that MDCK cells are not competent in nucleotide transfer assays is at variance with the report by Hunter & Pitts (1981), who reported that transfer occurred between homogeneous co-cultures of MDCK cells, albeit at a lower level (approx. 4-fold higher grain count above contacting recipient cells than above non-contacting cells) than between several other homogeneous or heterogeneous co-cultures studied at the same time (up to more than 10-fold labelling specificity). It seems unlikely that the small difference in experimental protocol used by Hunter & Pitts (1981), in which donor cells and potential recipient cells were mixed in suspension and plated out together, rather than recipients being added above monolayers of donor cells as in our experiments and those of Pitts & Simms (1977), is responsible for the discrepancy. It therefore seems possible that our strain of MDCK cells is not exactly the same as that used by Hunter & Pitts (1981); strain-
specific differences in other properties of MDCK cells have previously been noticed (Richardson et al. 1981).

Our demonstration that subcultured PAE cells form nucleotide-permeable intercellular junctions confirms and extends the findings of electrical, metabolic and dye coupling in bovine aortic endothelium by Larson & Sheridan (1982), who studied freshly isolated sheets of cells, or cells in primary cultures, but did not examine subcultured cells.

LLC-PK₁ cells have some characteristics that are restricted to proximal tubular epithelium in vivo, the most prominent of these being their Na⁺-dependent hexose transport activity and the presence of high levels of γ-glutamyltransferase (Mullin, Weibel, Diamond & Kleinzeller, 1980; Rabito & Ausiello, 1980; Sepúlveda et al. 1982). This strongly suggests that they originate from proximal tubule epithelium. The cells do not, however, seem to have retained all of the differentiated properties associated with their origin. For example, although they take up amino acids via the basolateral membrane, they lack the typical brush-border transport systems of the proximal tubule (Sepúlveda & Pearson, 1984b). As noted in the Introduction, proximal tubule epithelial cells in vivo are electrically coupled and possess numerous gap junctions (Silverblatt & Bulger, 1970; Giebisch & Windhager, 1973; Pricam et al. 1974; Kühn & Reale, 1975). The lack of nucleotide transfer between LLC-PK₁ cells, correlated with their apparent lack of gap junctions (noted above), implies that this cell line, when cultured routinely, has lost the ability to express another of the differentiated traits found in its tissue of origin.

Amsler & Cook (1982) found that development of Na⁺-dependent hexose transport in LLC-PK₁ cells could be greatly enhanced by dibutyrly cyclic AMP and methylisobutylxanthine, whereas certain other agents such as the tumour promoter 12-O-tetradecanoylphorbol-13-acetate inhibited the development of hexose transport. The same groups of compounds have also been shown, respectively, to enhance and diminish intercellular communication (Hooper & Subak-Sharpe, 1981; Loewenstein, 1981). We therefore used dibutyrly cyclic AMP and methylisobutylxanthine in attempts to stimulate nucleotide transfer between LLC-PK₁ cells, and between MDCK cells, whose ability to express differentiated properties is also enhanced by elevating intracellular cyclic AMP (Lever, 1979). Under the conditions of our experiments neither cell line modified the communication-competent phenotype expressed in the absence of any potential inducers of differentiation.

Whilst the apparent inability of LLC-PK₁ cells to form communicating junctions reflects the loss of a function that is present in the parent tissue, it is not clear that this is the case for MDCK cells. This cell line exhibits characteristics suggesting that it originates from distal tubule epithelium (Rindler et al. 1979; Richardson et al. 1981). This tissue does not appear to be electrically coupled, and additionally possesses few, if any, gap junctions (Pricam et al. 1974; Kühn & Reale, 1975).

Without regard to the relationship between the properties of LLC-PK₁ cells and MDCK cells and their tissues of origin, however, our findings are of interest when considering general hypotheses concerning the physiological roles of junctional permeability. These assume the existence of molecules, necessary for the expression of
Renal epithelial cell communication

Differentiated cellular properties or for the regulation of cell growth, that are transmitted between cells via communicating junctions (Loewenstein, 1979), and thus suggest cells lacking such junctions may not be able to differentiate fully and will be tumorigenic. While these predictions have been borne out in several other experimental systems (Loewenstein, 1981; Hooper & Subak-Sharpe, 1981), both MDCK cells and LLC-PK1 cells retain certain specific differentiated features, exhibit strict growth control in vitro, and are not known to be tumorigenic. Once confirmed, the fact that either of these renal epithelial cell lines lacks communicating junctions, should be taken into account when considering growth control in vitro and the function of cell-to-cell communication in differentiated kidney tubule. It should also be pointed out that our results do not rule out the possibility that growth and differentiation of the stem cells from which kidney tubule cells are derived, is controlled by molecules that pass through communicating junctions.

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Renal epithelial cell communication


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