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Modulation of Cell Differentiation in Perfusion Culture

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Key Words
Kidney · Collecting duct · Development · Differentiation · Gradient · Perfusion culture · Modulation · Sensor · Electrolyte

Abstract
An in vitro model was used to investigate the terminal differentiation mechanisms leading from embryonic to adult renal tissue. For these experiments the capsula fibrosa with adherent embryonic tissue was isolated from neonatal rabbit kidneys. These explants were mounted onto special tissue carriers and cultured in medium containing serum for 24 h. During that time collecting duct (CD) cells grew out and formed a monolayered epithelium covering the whole surface of the explant. The carriers were then transferred to perfusion culture containers to obtain an optimal degree of differentiation. A special type of container allowed us to continuously superfuse the epithelia with individual media on the luminal and basal sides. Using this method it became possible to culture embryonic CD epithelia in a fluid gradient for weeks. The epithelia were superfused with standard Iscove's modified Dulbecco's medium (IMDM) on the basal side, while IMDM containing additional NaCl was used on the luminal side. In controls IMDM was superfused on both the luminal and basal sides. It was found that the degree of differentiation in the CD epithelia is dependent on the influence of fluid gradient exposure. Perfusion culture under isotonic conditions revealed that less than 5% of cells were immunopositive for principal and intercalated cell features, while epithelia cultured in a luminal-basal gradient showed more than 80% positive cells. Immunoreactivity for characteristic markers started to develop after an unexpectedly long latent period of 3–6 days, then increased continuously during the following 5 days and reached a maximum on day 14. After switching back from the gradient to isotonic culture conditions the immunoreactivity for some markers decreased within 5 days, while other characteristic features remained stable. Thus, differentiation was not only under the control of growth factors but was also regulated by the electrolyte environment.

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Out of the Organ into the Test Tube

Cell and tissue cultures are frequently used to study the development and cell biological functions of renal tubular cells [1]. After isolation from the organ, cell suspensions are prepared by enzymatic tissue disintegration. Then the

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cells are usually transferred to a culture dish and cultivated in medium containing serum, where they attach to the bottom of the culture dish and form a confluent monolayer within a few days. Although the cells exhibit an excellent vitality, a loss of characteristic features is observed compared to the situation within the kidney. In the process of dedifferentiation cells lose their characteristic morphological, physiological and biochemical properties. There is evidence that the isolation of the cells from their organ-specific extracellular matrix may contribute to the dedifferentiation process [2]. To date it is not known which further factors cause the change in morphology followed by a loss of functional characteristics.

Perfect Modulation of Differentiation within the Developing Kidney

In the maturing kidney the process of differentiation can be observed as embryonic cells are transformed into functional tissue. Little is known about the factors triggering these maturation steps. Embryonic epithelia within the developing mammalian kidney have their origin at two very different sites.

Nephron epithelia originate from tissue interaction between the embryonic collecting duct (CD) ampulla and the surrounding nephrogenic mesenchyme [3, 4]. In this process mesenchymal cells are converted to nephron epithelial cells. After the inductive stimulus, nephron segmentation, tubular elongation and functional maturation take place. Each of the developing nephron segments is composed of a homogenous cell population with typical morphological and functional features [5].

The functional development of the CD epithelium occurs by a completely different mechanism. The CD epithelium originates from the ureter bud invading the nephrogenic mesenchyme. It acts as an embryonic inducer and, being the head of a hierarchic cascade, it triggers the generation of all of the nephron anlagen [4]. This happens in successive steps, in which the ampullar tip continuously elongates towards the nephrogenic mesenchyme beneath the capsula fibrosa. The elongation zone ends below the neck region of the ampulla. Below the neck, differentiation into a heterogeneously composed epithelium consisting of principal (P) and different kinds of intercalated (IC) cells takes place [6, 7].

Humoral and Environmental Factors Modulate Differentiation

We investigated the humoral and environmental factors affecting the transition from embryonic epithelium to adult renal CD epithelium with its different cell types [8]. The functional differentiation of nephron epithelia on the one hand depends on humoral factors such as vitamin A derivates [9] and growth factors or hormones [10–13], and on the other hand on structural elements such as the extracellular matrix [4]. In addition to these very heterogeneous developmental stimuli it was shown for MDCK cells that also changes in the pH and osmolarity are able to evoke differentiation [14–17].

Very little information is available on the mechanisms by which a heterogeneously composed CD epithelium is derived from embryonic cells [18]. Tissue culture experiments with embryonic CD epithelia revealed that the development of individual CD cell features can be triggered by aldosterone [19], amiloride [20] and the extracellular electrolyte environment [21, 22]. Under static culture conditions in medium containing serum, aldosterone is known to generate a P cell-like epithelium with an amiloride-sensitive Na^+ transport and tight characteristics [23]. In contrast, in perfusion culture and in serum-free medium aldosterone surprisingly not only induces the development of P but also of β-type IC cell features [7, 19, 24].

Prerequisites

Experiments in conventional culture dishes with a static environment do not give satisfactory results because typical features are not found to develop in the CD epithelium [24]. In order to obtain a higher degree of cellular differentiation we improved the culture conditions. To achieve an optimal degree of differentiation under in vitro conditions certain prerequisites are necessary.

Optimal Cell Anchorage. As far as we know, differentiation is influenced not only by a single soluble growth factor, but by a variety of environmental stimuli, which obviously interact in a hierarchic way. At the top is cell anchorage [25]. Without optimal cell anchorage a high degree of differentiation cannot be expected. Suboptimal cell anchorage results in minor cell differentiation. For this reason we culture CD epithelia on the capsula fibrosa as a kidney-specific support without cell dissociation [26].

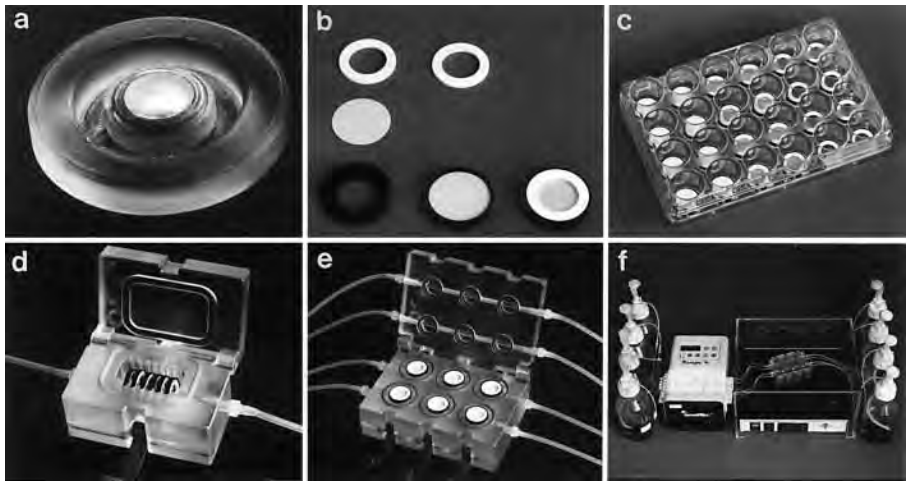


Fig. 1. Perfusion culture of a renal collecting duct (CD) epithelia. **a** The renal explant with the epithelium (E), 6 mm in diameter, is mounted in a special tissue carrier. **b** A different type of carrier can hold individually selected cell supports with a diameter of 13 mm. **c** The tissue carriers can be incubated in a 24-well plate until cells are adherent. **d** A perfusion culture container can hold up to 6 tissue carriers. On the left side fresh culture medium is continuously pumped into the container. Medium with harmful metabolic products flows out on the upper right side. **e** A gradient perfusion container can hold up to 6 carriers and is connected to a luminal and a basal perfusion line. The closed gradient container allows luminal and basal perfusion with two different media. Three tissue carriers are superfused with test medium, while the other 3 carriers are used as controls. **f** A peristaltic pump transports the media and a thermoplate provides constant temperature.

Tissue Carriers. To facilitate handling of the generated tissue different kinds of carriers are used in our experiments. In one type of carrier natural matrices such as the capsula fibrosa explant of neonatal rabbit kidney are placed (fig. 1a). In a second type of carrier individually selected filters, nets or scaffolds, 13 mm in diameter can be used (fig. 1b). For adherence and multiplication of cells the tissue carriers are placed in a 24-well plate (fig. 1c) in a CO_2 incubator. Medium containing serum or growth factors is applied for this limited period of time to stimulate CD cell proliferation.

Perfusion Culture Container. For differentiation the tissue carriers are transferred to different kinds of perfusion culture container (fig. 1d, e). One model allows continuous exchange of medium (fig. 1d), where the tissue is always bathed in fresh medium. While for the multiplication of cells medium containing growth factors is used (fig. 1c), differentiation takes place in a perfusion culture container and in the majority of cases in serum-free medium. By this technique a CD epithelium with P and IC cell features can be generated (fig. 2, 3).

Exchange of Medium. In order to visualize the permanent and complete exchange of medium during perfusion, the container is filled with acidified yellow culture medium. The container is then perfused with alkalized purple culture medium at a rate of 1 ml/h (fig. 4a). After 20 min the whole basal side of the container is covered with purple medium (fig. 4b; arrow). In the time between 30 min (fig. 4c) and 2.5 h (fig. 4g) the level (arrows) of the purple medium continuously increases. After 3.5 h (fig. 4h) the medium in the container is completely exchanged with new medium. Under these conditions the tissue receives constant nutrition, harmful metabolic products do not accumulate and paracrine factors remain at a physiological level. Previous experiments have shown that embryonic CD epithelia can be cultured in a container for weeks and months exhibiting a morphology comparable to the situation within the maturing kidney [6, 7, 20].

Gradient Culture Container. In a second model, the gradient container enables culture under a fluid gradient (fig. 1e), so that a tissue-specific environment for epithelia can be simulated (fig. 1e). The tissue carrier with the developed epithelium separates the container into a luminal and a basal compartment. On both sides of the epithelium either the same medium or media of different compositions can be perfused. A kidney environment can be mimicked by using a hyper- or hypotonic medium on one side, while standard medium is used on the other side. A gradient container can hold 6 epithelia. Three epithelia are used as a control, while the others are used in the experimental series.

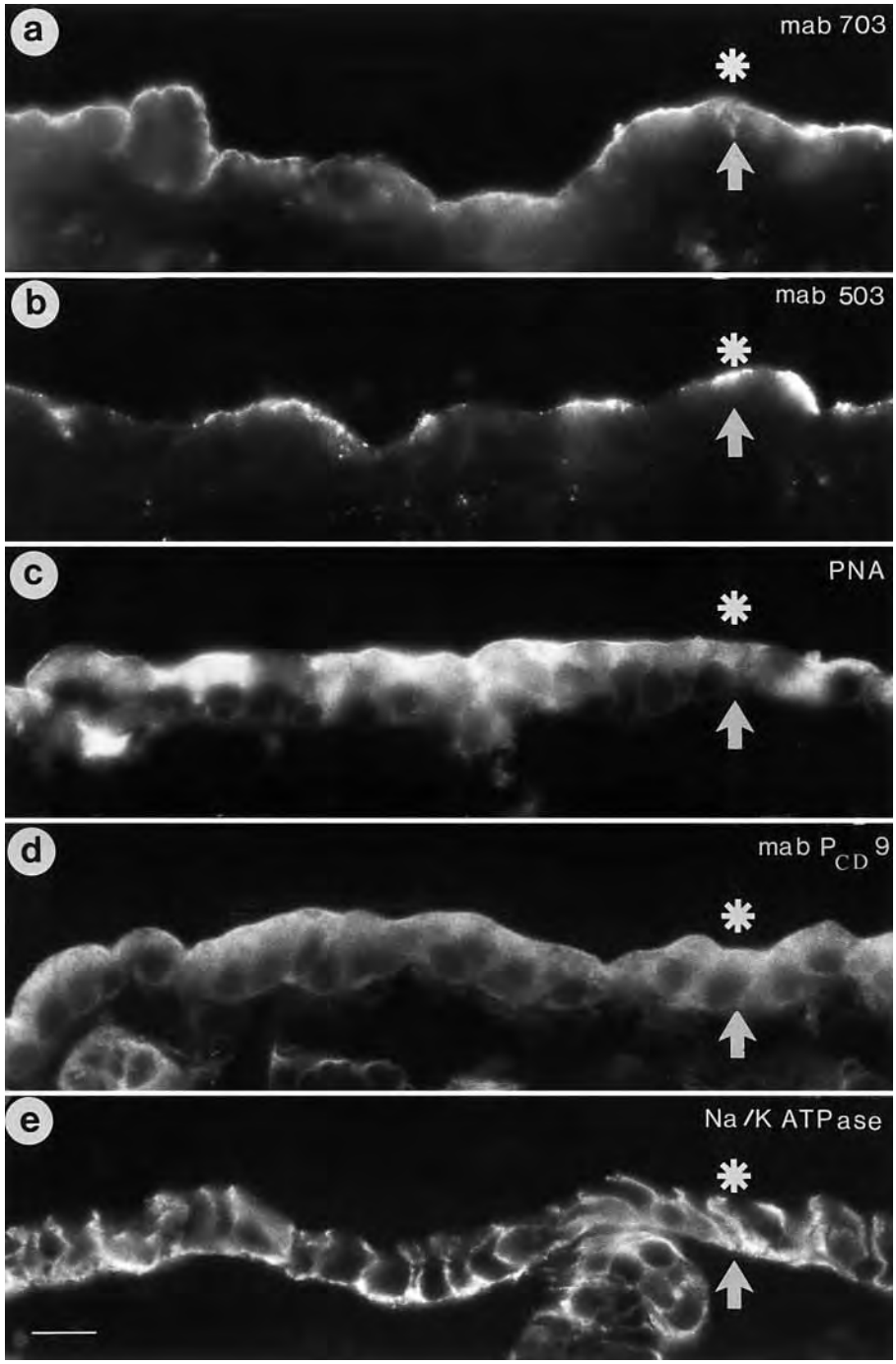


Fig. 2. Immunofluorescence microscopy of CD epithelia kept under gradient culture conditions after 14 days with IMDM on the basal and IMDM including additional NaCl and Na-gluconate on the luminal side. Consecutive sections are used for labeling. Within the epithelium a high percentage of mab 703 (**a**), mab 503 (**b**), PNA (**c**), mab $P_{CD}9$ (**d**) and mab Na/K-ATPase (**e**) was found. This shows the generation of a hybrid cell type with a coexpression of P and IC cell features. Bar = 10 μm. Asterisk = Luminal side; arrow = basal aspect of the epithelium.

Simple Hardware. The culture system can either be used inside a CO_2 incubator or outside on a laboratory table. Only simple equipment is necessary for perfusion culture. A thermoplate (MEDAX, Kiel, Germany) provides a constant temperature of 37°C and a peristaltic pump (IPC N8, ISMATEC, Wertheim, Germany) transports the medium into the container (fig. 1f).

Parameters in the Culture Medium

Medium. Fresh medium is continuously perfused at a rate of 1 ml/h for a 2-week culture period or longer. In the present experiments Iscove's modified Dulbecco's medium (IMDM; order No. 21980-032; Gibco BRL-Life Technologies, Eggenstein, Germany) [27] without serum

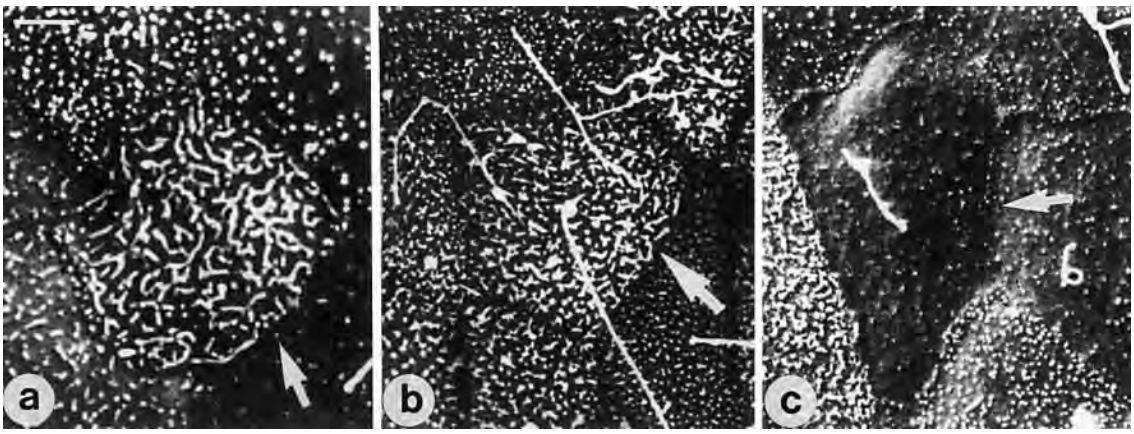


Fig. 3. Scanning electron microscopy of CD epithelia in perfusion culture. **a** On the luminal cell pole microplicae have developed indicating a characteristic of an α-type IC cell. **b** Development of long microvilli and a cilium shows features of immature β-type IC cells. **c** Luminal cell poles with few and short microvilli together with one cilium indicates the development of P cell-like features [24]. **a–c** × 4,500.

is used as the standard medium. Aldosterone (1×10^{-7} mol/l; Sigma-Aldrich-Chemie, Deisenhofen, Germany) and 1% antibiotic-antimycotic solution (Gibco BRL-Life Technologies) are added to all culture media. Furthermore, up to 50 mmol/l HEPES (Gibco BRL-Life Technologies) is used in the medium to maintain a constant pH of 7.4 in perfusion culture under laboratory room atmosphere (0.3% CO_2).

Metabolic Parameters. Metabolic activity of the cells is monitored by analyzing the superfused culture medium. Media parameters such as pH, pCO_2, pO_2, lactate, osmolarity and electrolyte concentrations of Na^+, K^+, Cl^- and Ca^{2+} are determined in undiluted 200-µl samples of the culture medium. The samples are analyzed in a Stat Profile 9 Plus analyzer according to the manufacturer's instructions (Nova Biomedical, Rödermark, Germany). Solutions with defined electrolyte concentrations serve as controls.

Gas Equilibration. Conventional cultures in a CO_2 incubator are usually buffered by a system containing a defined amount of $NaHCO_3$, 95% air and 5% CO_2 to maintain a constant pH of 7.4. If such a medium is used in perfusion culture and outside a CO_2 incubator, pH will shift out of the physiological range into an alkaline pH. For this reason medium used outside a CO_2 incubator has to be stabilized by reducing the $NaHCO_3$ content and by adding HEPES or Buffer All (Sigma-Aldrich-Chemie, Deisenhofen, Germany). To obtain optimal equilibration of pH, O_2 and CO_2 in perfusion cultures the media are pumped through thin, gas-permeable silicone tubes, which allow continuous and optimal exchange of gases. This results in 14.36 ± 5.61 mm Hg CO_2 and 174.12 ± 9.17 mm Hg O_2 (fig. 5). In contrast, in a CO_2 incubator (95% air/5% CO_2) on average 35.10 ± 1.64 mm Hg CO_2 and 143.92 ± 1.22 mm Hg O_2 are measured.

Electrolyte Adaptation. IMDM is used as the standard medium in all the experiments. It contains on average 112 mmol/l Na^+ and 85 mmol/l Cl^- and provides an excellent basis for proliferating cells (table 1) [21]. However, when comparing IMDM with the serum of neonatal rabbits as a model fluid for the interstitial space, a gap in the concentration of electrolytes is observed. The serum contains 137 mmol/l Na^+, while in IMDM only 112 mmol/l Na^+ are measured. Similar differences are found when comparing K^+, Cl^-, and Ca^{2+} concentrations. For gradient culture experiments it is necessary to adapt the medium to mimic the interstitial or the luminal fluid of the epithelia. During the present experiments the culture medium is adapted to a physiological Na^+ concentration in 2 steps: we add 12 mmol/l NaCl (IMDM1) and further 17 mmol/l Na-gluconate (IMDM2) to reach 137 mmol/l Na^+ as observed in the serum of neonatal rabbits (table 1).

Generation of an Embryonic Collecting Duct Epithelium

To obtain a high degree of cellular differentiation under in vitro conditions, we isolate renal CD cells without tissue dissociation and keep them on their specific

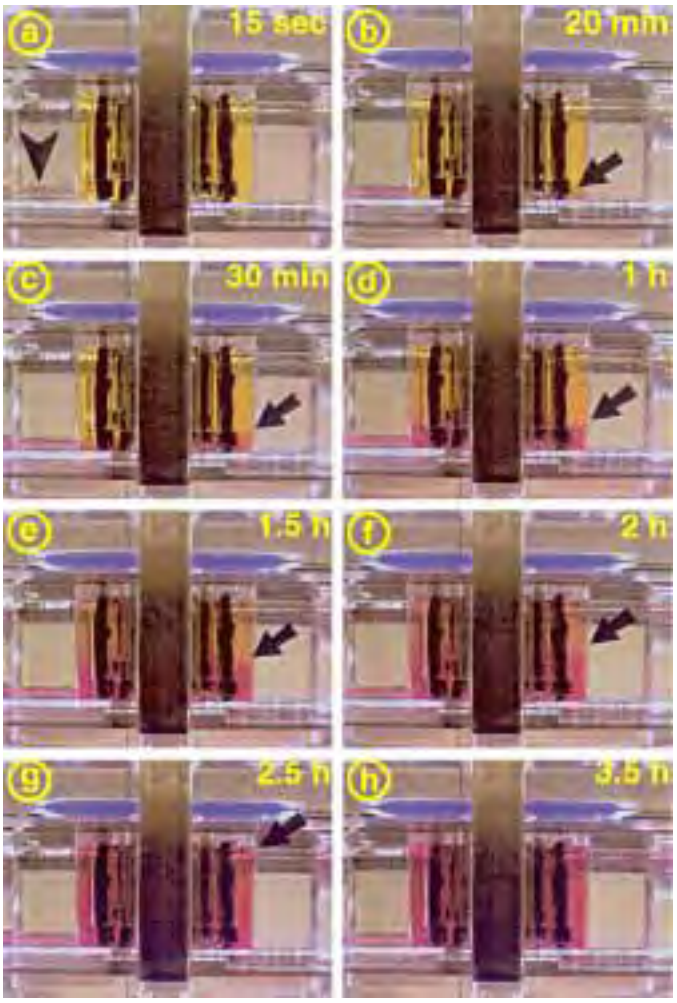


Fig. 4. Exchange of culture medium. In order to visualize the permanent exchange of medium, the container is filled with acidified yellow culture medium. The container is then perfused with alkalized purple culture medium at a rate of 1 ml/h (**a**; arrowhead). After 20 min the whole basal side of the container is covered with purple medium (**b**; arrow). In the time between 30 min and 2.5 h (**c–g**) the level (arrows) of the purple medium continuously increases. After 3.5 h (**h**) the medium in the container is completely exchanged with new medium. Under these conditions tissue receives constant nutrition, harmful metabolic products do not accumulate and paracrine factors remain at a physiological level.

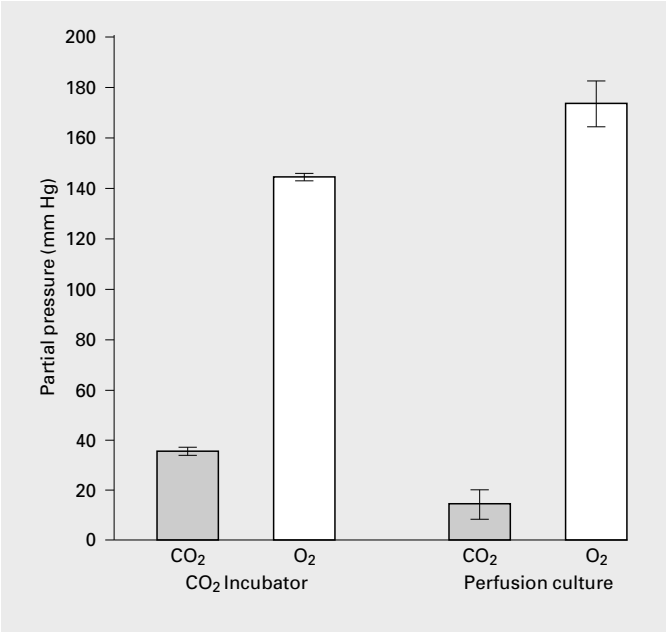


Fig. 5. Comparison of CO_2 and O_2 partial pressure. O_2 and CO_2 content in media during perfusion and under static conditions in an incubator. This results in 14.36 ± 5.61 mm Hg CO_2 and 174.12 ± 9.17 mm Hg O_2 in perfusion culture, while in a CO_2 incubator (% air/5% CO_2) on average 35.10 ± 1.64 mm Hg CO_2 and 143.92 ± 1.22 mm Hg O_2 are measured (n = 9). Under perfusion culture conditions the O_2 partial pressure of the culture medium is considerably higher than in a conventional culture plate.

Table 1. Electrolyte composition (mmol/l) of IMDM, IMDM1 (+ 12 mmol/l NaCl), IMDM2 (+ 12 mmol/l NaCl, 17 mmol/l Na-gluconate) and serum specimens of neonatal rabbit (mean ± SEM)

	IMDM	IMDM1	IMDM2	Serum
Na^+	112.3 ± 1.6	126.4 ± 1.7	136.9 ± 1.0	137 ± 5.7
Cl^-	85.1 ± 1.0	98.7 ± 1.4	98.5 ± 1.2	99 ± 5.8
K^+	4.25 ± 0.1	4.25 ± 0.04	4.25 ± 0.04	6.04 ± 1.7
Ca^{2+}	1.15 ± 0.27	1.15 ± 0.1	1.15 ± 0.03	1.66 ± 0.46
n	40	31	32	17

extracellular matrix. Cortical explants from the kidneys of newborn rabbits are isolated microsurgically according to the methods described earlier by stripping off the capsula fibrosa of neonatal rabbit kidneys [26]. The explants consist of a piece of capsula fibrosa with adherent CD ampullae, S-shaped bodies and nephrogenic blastema.

Proliferation of CD Cells under Static Culture Conditions with Serum Supplementation. The renal explants are mounted in sterile tissue carriers (fig. 1a; Minucells and Minutissue, Bad Abbach, Germany), which are placed in 24-well culture dishes (fig. 1c; Greiner, Nürtingen, Germany). Culture for the first 24 h is carried out in a tissue incubator (Heraeus, Hanau, Germany) at 37°C in a

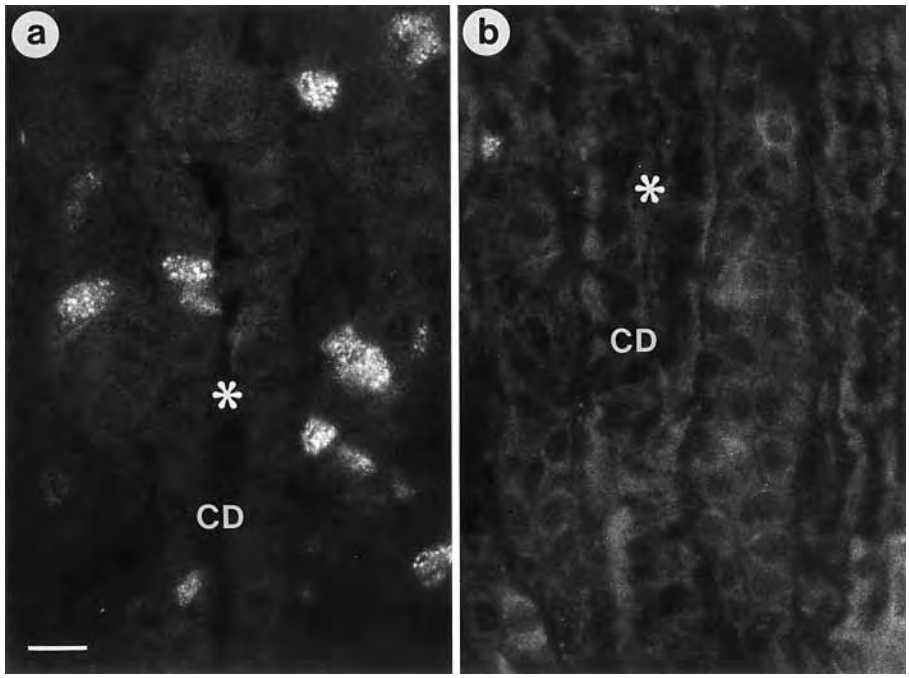


Fig. 6. Immunofluorescence microscopy of mab Ki 67 binding in neonatal and adult rabbit kidney. In the developing kidney a large number of cell divisions are visible (**a**), while in the adult kidney labeled cells are extremely rare (**b**). Bar = 10 μm. Asterisk = Luminal side of the epithelium.

humidified atmosphere containing 5% CO_2/95% air. During culture in IMDM (Gibco BRL-Life Technologies, Eggenstein, Germany) containing 10% fetal bovine serum (Boehringer, Mannheim, Germany) an outgrowth of cells from the CD ampullae is observed. Within 24 h of the initiation of culture the entire surface of the explant, 6 mm in diameter, is covered by a polarized CD epithelium.

Perfusion Culture with Embryonic Collecting Duct Epithelia

Differentiation in Perfusion Culture. The degree of cellular differentiation in cultured epithelia depends not only on the organ-specific extracellular matrix, but also on a continuous supply of nutrients and growth factors, the elimination of metabolic products and the prevention of an accumulation of synthesized paracrine factors [25]. Because all these demands cannot all be met in the static environment of a culture dish 24 h after the initiation of the cultures the tissue carriers (fig. 1a) are transferred to gradient containers (fig. 1e). The tissue carriers with the epithelium separate the container into a luminal and a basal compartment. Different media (table 1) are perfused at the luminal and basal sides so that the epithelium is exposed to a fluid gradient for the whole culture period.

Markers to Register Modulation of Differentiation

To register the primary appearance of individual CD features we use a set of cell type-specific markers. Mab 703 recognizes a 70-kD protein on P cells, while mab 503 detects a 31-kD protein on IC cells identified on adult renal CD cultures [28]. Both of the antibodies were kindly provided by Dr. M. Tauc, Department of Cellular and Molecular Physiology, University of Nice, France. Mab $P_{CD}9$ was generated in our laboratory and binds to a 32- to 39-kD protein on all of the CD cells of the neonatal kidney [29]. PNA-rhodamine conjugate (Vector, Burlingame, Vt., USA) is used to detect β-type IC cell features [17]. A monoclonal antibody against Na/K-ATPase, developed by D.M. Fambrough, was obtained from the Development Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, under contract NO1-HD-7-3263 from the NICHD. Proliferating cells are detected by mab Ki 67, a generous gift from Dr. J. Gerdes, Research Centre Borstel, Germany [30].

To calculate the degree of immunopositive cells in the cultured epithelia a double-labeling procedure is applied. The epithelia are first labeled with the nuclear marker propidium iodide (4 μg/ml; Sigma-Aldrich-Chemie, Deisenhofen, Germany) followed by incubation with a cellu-

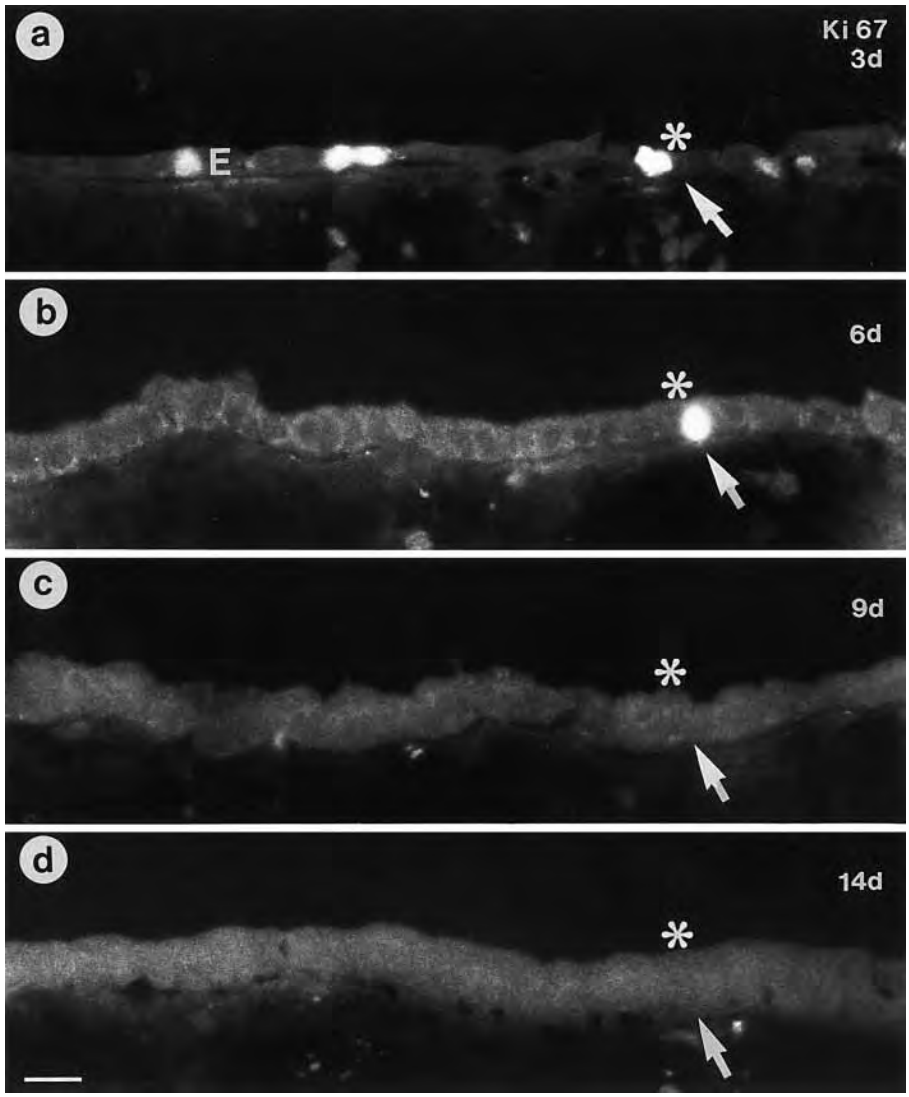
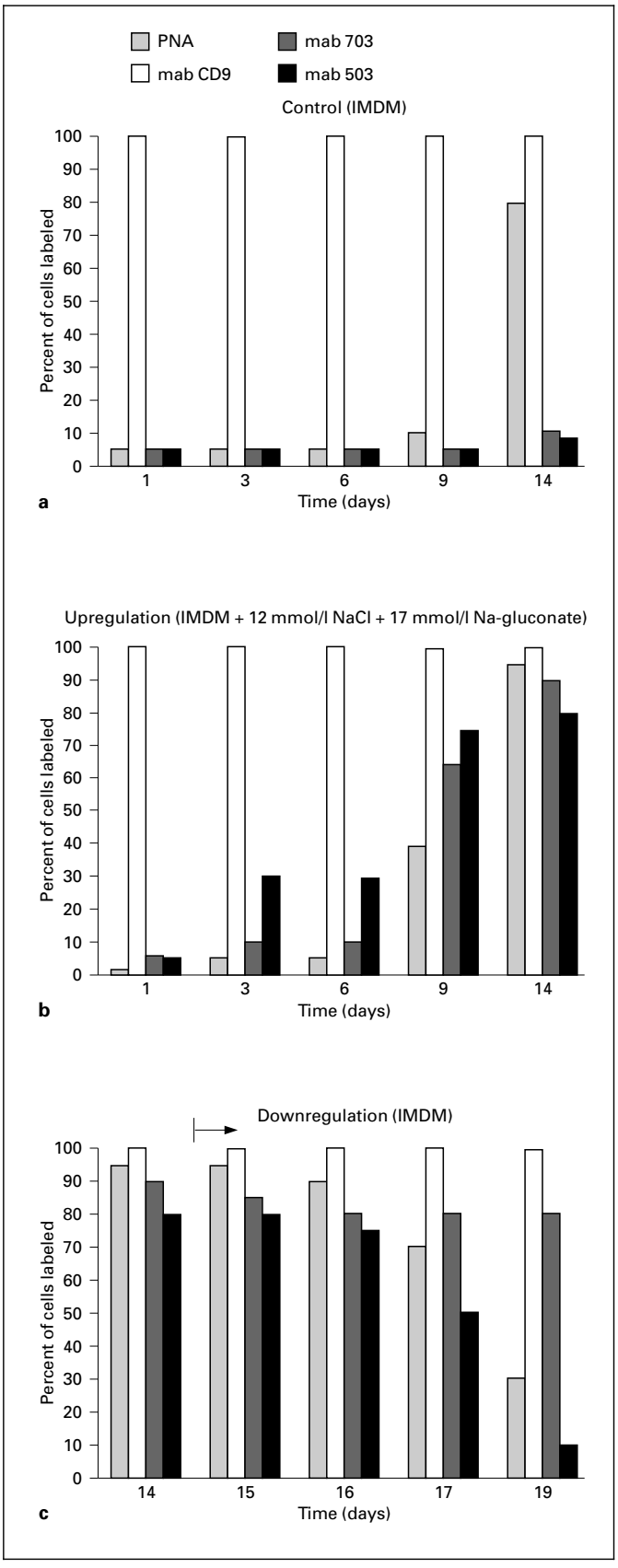


Fig. 7. Immunofluorescence microscopy of mab Ki 67 labeling in cultured CD epithelia. Downregulation of mitotic activity can be registered in the cultured CD epithelium. **a** After day 3 multiple mab Ki 67-positive cells are observed. **b** Then at day 6 the amount of proliferating cells drastically decreases, so that at day 9 (**c**) and 14 (**d**) no more cell divisions are visible. Bar = 10 µm. Asterisk = Luminal side; arrow = basal aspect of the epithelium.

lar marker such as mab 703, 503, $P_{CD}9$ or PNA. Using this method the amount of labeled and unlabeled cells within the epithelium can easily be determined.

Mitotic Activity Is Downregulated while CD Features Start to Appear

Mitotic activity can be demonstrated by the proliferation marker Ki 67 [30]. In the neonatal, still developing kidney a high labeling rate is found (fig. 6a), while in the adult kidney practically no labeling can be detected (fig. 6b). The result shows that most of the cells in the adult kidney do not proliferate but remain in the functional interphase. Since mitosis and differentiation are not parallel but successive steps, under in vitro conditions we have to apply two different experimental steps. In the first step we multiply the cells in medium containing fetal bovine serum. Then after 24 h we switch to serum-free medium to maintain the cells in the interphase as long as possible. Downregulation of mitotic activity can be registered in the cultured CD epithelium. On day 3 multiple dividing, mab Ki 67-positive cells are observed (fig. 7a). Then on day 6 (fig. 7b) the amount of proliferating cells drastically decreases, so that by days 9 and 14 (fig. 7c, d) no cell divisions are visible any more. The result shows that the later described upregulation of individual cell features in the CD epithelium does not occur in a phase of high but in a phase of low mitotic activity.



What Happens when an Embryonic Epithelium Is Exposed to a Gradient for the First Time?

All adult renal epithelia form a functional barrier between a luminal and a basal fluid compartment. The two compartments are different in their fluid composition. In contrast, embryonic epithelia first develop in an environment of equally composed luminal and basal fluids as long as no connection of the CD with functional nephrons is established. During maturation the epithelial cells polarize and form a functional tissue with an individual barrier function. Therefore we analyzed whether differences in protein expression exist, when embryonic epithelia are cultured with the same media at the luminal and basal side or when they are exposed to a gradient [21, 22].

Perfusion Culture in Gradient Containers with Isotonic Media: Luminal-IMDM/Basal-IMDM

The primary appearance of mab 703, mab 503, PNA and mab $P_{CD}9$ binding in the embryonic epithelia is monitored from the first until the 14th day of perfusion culture in a gradient container (fig. 8a). In the first series of experiments the embryonic CD epithelia are superfused in a gradient container with standard IMDM on both the luminal and basal side of the tissue (table 1). On day 1 less

Fig. 8. a Development of CD epithelia features under isotonic culture conditions after 1–14 days in a gradient container with standard IMDM on the luminal and basal sides. At day 1 mab 703, mab 503 and PNA binding is detected only on less than 5% of cells, while all of the cells are labeled with $P_{CD}9$. After day 14 less than 5% of the cells show labeling with mab 703 and mab 503, while more than 80% of cells react with PNA and 100% with mab $P_{CD}9$, respectively. **b** Development of mab 703 and 503 antigen features in embryonic collecting duct epithelia cultured for 14 days in a gradient container. At the basal side of the epithelium standard IMDM was used, while on the luminal side IMDM including additional 12 mmol/l NaCl and 17 mmol/l Na-gluconate was superfused. The antigen detected by mab 503 is expressed in high amounts already on culture day 3 and is further upregulated until day 14. The increase in PNA labeling is paralleled by an increase in mab 703 binding. **c** Downregulation of mab 503 and PNA binding features. On day 14 the medium was changed back. Until day 19 again standard IMDM was applied on both sides of the tissue. Mab 503 and PNA binding are downregulated within 4 days, while mab 703 and $P_{CD}9$ expression remain stable.

than 5% of cells are positive for mab 703, mab 503 and PNA, while mab $P_{CD}9$ labels all of the cells within the CD epithelium (fig. 8a). On day 14 less than 5% of the cells show reaction with mab 703 and mab 503. In contrast, more than 80% of cells show PNA binding and all of the cells show immunohistochemical labeling with mab $P_{CD}9$. The development of PNA binding is exclusively triggered by aldosterone [19]. Thus, the development of mab 703 and mab 503 binding is not stimulated under these culture conditions.

Perfusion Culture in a Gradient Container with Different Media: Luminal-IMDM with Additional Na^+/Basal-IMDM

In this series of experiments the embryonic CD epithelia are superfused in a gradient container with IMDM2 on the luminal and IMDM on the basal side mimicking a fluid gradient (table 1).

Mab 703 binding is found on less than 5% of the cells within the cultured epithelium on day 1 (fig. 8b). Until days 3 and 6 only 10% of the cells are positive. Then on day 9 more than 70% and on day 14 more than 90% of the cells become positive for mab 703. As compared to cultures under isotonic conditions (fig. 8a) IMDM2 on the luminal side induces the primary appearance of mab 703 binding.

Mab 503 is found on less than 5% of the cells on day 1 (fig. 8b). On days 3 and 6, 30% immunopositive cells are found. A further increase in immunoreactivity is registered during days 9 and 14 so that up to 70 and 80%, respectively, of the cells are positive for the antibody.

PNA binding is localized on less than 1% of the cells on day 1 (fig. 8b). On days 3 and 6 less than 5% of positive cells are detected. In contrast, 40% positive cells are found on day 9. Nearly all of the cells are positive on day 14. As compared to cultures with standard IMDM, IMDM2 does not affect the expression of PNA-binding molecules.

Mab $P_{CD}9$ labels all of the maturing CD cells in the neonatal rabbit kidney [29]. From culture day 1 (fig. 8b) until day 14 mab $P_{CD}9$ labels all of the CD cells. In contrast to mab 703 and mab 503 the change of the luminal culture medium from IMDM to IMDM containing additional Na^+ does not affect the expression of the antigen.

Development of CD Cell Features Takes Place after an Unexpectedly Long Latent Period

Summarizing the time course of development of mab 703, 503 or PNA binding, the embryonic CD epithelia show no or only minor immunoreactivity after days 1, 3 and 6 (fig. 8b). In contrast, after days 9 and 14 numerous intensively labeled cells are found. It is obvious that the development of mature CD cell features starts after an unexpectedly long latent period of 6 days. As shown earlier the long latent period is paralleled by a decrease in mitotic activity in the cultured epithelium (fig. 7). Thus, the upregulation of individual cell features takes place when mitotic activity is lost and the interphase is reached. In this context it is important to note that the differentiation of the cultured CD epithelia is not terminated and that hybrid types consisting of common P and IC cell features are generated (fig. 2).

Electrolyte Environment Maintains Individual Cell Features

The next experimental series shows that the electrolyte environment is not only able to evoke the upregulation of mature CD features but also controls their maintenance. The downregulation of individual CD characteristics can be prevented simply by the administration of NaCl. The embryonic CD epithelia are cultured in a gradient container. For 14 days IMDM containing additional Na^+ is superfused on the luminal, while IMDM is used on the basal side (table 1). At day 14 IMDM containing additional Na^+ is replaced with standard IMDM for another 5 days (fig. 8c). In the first phase of culture an upregulation of mature CD features is monitored (fig. 8b). In the second culture phase only part of these characteristics remain constant, while others are gradually lost (fig. 8c).

The primary appearance of mab 703 and mab 503 binding is induced by the extracellular electrolyte environment (fig. 8b). In contrast, if the high Na^+ load is reduced after the 14th day mab 503 and PNA binding on the cells is found to be diminished within the next 5 days (fig. 8c). Most astonishingly, mab 703 binding remains unchanged until the 19th day despite the lack of Na^+ load for 5 days. Thus, preservation of mab 503 binding is controlled by the extracellular electrolyte environment, while the maintenance of mab 703 binding is not affected. Mab $P_{CD}9$ antigen expression is not controlled by the extracellular environment, either in this up- or its downregulation.

Table 2. Triggering of differentiation by an electrolyte sensing mechanism

Site	Neonatal rabbit, embryonic CD epithelium [26]
Prerequisites	Gradient perfusion culture [20]
Stimulation	12 mmol/l NaCl, LiCl, or KCl and 12 mmol/l NaCl/17 mmol/l Na-gluconate [21]
Site of reception	Luminal and basal side of the epithelium [21, 22]
Specifity	Evocation of differentiation, individual modulation depending on NaCl, LiCl and KCl [21]
Initiation	Latent period of 6 days, primary appearance and upregulation of individual features [22]
Time of development	10–14 days [22]
Modulation	P and IC cell features within one cell, generation of a hybrid cell type [7]
Lack of stimulation	Downregulation of individual features, lack of maintenance of characteristics [22]
Action	Unknown molecular mechanism

Luminal versus Basal Medium Electrolyte Load on Embryonic CD Epithelia

The present experiments show that medium changes on the luminal side of the embryonic CD epithelium are able to modulate cell features. Since the epithelium is polarized, it is important to know if changes in electrolyte composition on the basal side evoke the same alterations in protein expression as observed on the luminal aspect. Culture experiments with IMDM containing additional Na^+ on the basal side [21] show principally the same alterations in protein expression as observed with luminal application [22].

Not only NaCl but also Other Electrolytes Evoke Differentiation

It is unknown if the primary development of CD cell features in the embryonic epithelia is caused by increasing concentrations of NaCl alone or whether KCl or LiCl also have the same effect as observed in other publications [31–33]. Superfusion of embryonic epithelia with IMDM causes less than 10% mab 703, less than 5% mab 503, and 100% mab $P_{CD}9$-positive cells. 80% of the cells show PNA binding. However, as shown in an earlier publication [21] epithelia superfused with IMDM containing additional 12 mmol/l LiCl show 40% mab 703, 40% mab 503 and 100% mab $P_{CD}9$-positive cells. 80% of the cells reveal PNA binding. Replacement of LiCl against KCl leads to 90% mab 703 and 90% mab 503-positive cells. The amount of PNA-binding cells is found to be decreased to 30%. In consequence, the experiments show that not only NaCl but also LiCl and KCl are able to influence primary protein expression in embryonic CD epithelia. Therefore, different electrolytes do not evoke the same differentiation profile, but show individual patterns.

Generation of a Hybrid Cell Type

In experiments with embryonic CD epithelia and IMDM2 up to 90% mab 703 (fig. 2a) and mab 503 (fig. 2b) positive cells can be detected. In addition, more than 90% PNA (fig. 2c) and 100% mab $P_{CD}9$ (fig. 2d) positive cells arise. Most interestingly, all of the cells within the epithelium show an intensive labeling or Na/K ATPase (fig. 2e). Thus, the majority of cells within the cultured CD epithelia are able to develop both IC cell characteristics as well as P cell features simultaneously. This can only be explained by the fact that hybrid cell characteristics are generated under in vitro conditions [6, 7]. Regarding CD development within the kidney it is indicated that a common precursor cell type for P and IC cells exists in the embryonic ampulla.

The Sensor

We show that the embryonic CD epithelium is sensitive to the electrolyte environment (fig. 8). Consequently, an electrolyte sensor for differentiation must be localized in the epithelium (table 2). Up to now we do not have information about the subcellular localization and its cell biological mechanism.

The sensor is accessible from both sides of the epithelium, since electrolyte load on the luminal and basal sides causes nearly equal changes in the differentiation pattern, which is an indicator for an intracellular localization [21, 22]. Concerning specificity we know that the sensor is not specific for an individual but for different electrolytes [21]. Not only NaCl, but also LiCl and KCl are able to evoke alterations in the differentiation pattern. Most interesting is that NaCl, LiCl and KCl do not evoke the same differentiation profile, but show very different patterns. Thus, the sensor is not only able to evoke but can also modulate differentiation. Furthermore, the sensor does not only control the upregulation (fig. 8b) of individual features but is also able to maintain cellular character-

istics. When the Na^+ load is replaced with standard medium part of the features are preserved, while others are downregulated (fig. 8c). An electrolyte sensor has the advantage that it can regulate differentiation according to the needs in the close vicinity of the cells and in a very sensitive manner. By this mechanism adaptation processes caused by a compensatory upregulation of functional features become interpretable [34–40].

Many of the related questions are not answered. Earlier investigations showed that Na^+ load induces chaperones [41] or switch on an untypical protein synthesis [42]. In contrast, our experiments show that P and IC cell features can be regulated under the control of electrolytes. It is unknown how sensitive the sensor is to changes in the electrolyte environment. Such changes are combined with alterations in osmolarity. Consequently, it has to be elaborated if changing osmolarity activates the same sensor that was observed in earlier experiments [43, 44].

Experiences

For the present experiments it was necessary to improve the culture conditions. This could be achieved with the help of a suitable matrix, tissue carriers, specific culture containers and permanent superfusion of medium. By this technique it is possible to bring the cells from a dormant to an activated state in which the cells are sensitive to differentiation signals.

Today hundreds of experiments with embryonic CD epithelia in perfusion culture are being performed. These experiments allow us to gain a completely new insight into the terminal differentiation process of renal CD epithelia. The experiments show without exception that only epithelia under improved culture conditions develop a degree of differentiation suitable for further investigation. From our point of view today, only a small fraction of the possible differentiation features can be activated and modulated under in vitro conditions. For this reason we believe that we are only at the beginning of generating improved tissues.

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