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Epidermal growth factor or transforming growth factor α is required for kidney tubulogenesis in matrigel cultures in serum-free medium

(embryonic growth/extracellular matrix)

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ABSTRACT **The ability of matrigel, a reconstituted basement membrane gel, to induce the differentiation of baby mouse kidney cells has been examined in a hormonally defined serum-free medium. Primary cultures of baby mouse kidney cells were observed to form tubules over a time interval of 1–2 weeks in matrigel. Electron microscopic studies showed that tubules with lumens were present, and the tubule morphology was similar to that of the collecting duct. When using matrigel from which the growth factors had been removed, tubule formation no longer occurred, unless the medium was further supplemented with epidermal growth factor (10 ng/ml). Transforming growth factor α stimulated tubule formation as effectively as epidermal growth factor, whereas transforming growth factor β had an inhibitory effect on tubule formation. These data suggest that both an extracellular matrix and specific growth factors may regulate kidney differentiation during development.**

The epithelial branching morphogenesis that occurs during early mouse kidney development has been attributed to inductive epithelial mesenchymal interactions (1). Inductive signals that are responsible for renal tubulogenesis have not been identified, and thus the molecular mechanisms underlying tubulogenesis are poorly understood. In induced embryonic renal tissue however, basement membranes have been implicated as playing a critical role in the process of morphogenesis (2, 3). Basement membranes are composed of a number of proteins including collagen IV, laminin, heparan sulfate proteoglycan, and nidogen/entactin (4). Laminin in particular has been implicated as playing an important role in cell adhesion, growth, and differentiation (4). In the embryonic mouse kidney the appearance of laminin has been correlated with kidney development *in vivo* and in organ culture (5).

Such inducers of differentiation are very likely present in the extracellular matrix. Purified extracellular matrix has been observed to facilitate animal cell differentiation *in vitro* (6). An abundant source of basement membrane extracellular matrix for *in vitro* studies is the Engelbreth–Holm–Swarm (EHS) sarcoma tumor (4, 7). Matrigel, a reconstituted basement membrane isolated from the EHS tumor (7), has been observed to stimulate testicular cord formation and germ cell development *in vitro* (8). In addition, matrigel promotes tube formation by human umbilical cord endothelial cells (9). Tube formation by human umbilical cord endothelial cells has been shown to be dependent upon laminin (10). Although laminin is the major component in matrigel, the possibility that another inducer is responsible for initiating various cellular events cannot be excluded.

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In this report primary baby mouse kidney cells are cultured within matrigel while in hormonally defined serum-free medium. Tubules formed within matrigel rather than an epithelial monolayer that formed upon a plastic substratum. Not only was tubule growth apparent microscopically but in addition branching morphogenesis occurred. A dependence of tubule formation upon an inducer, either epidermal growth factor (EGF) or transforming growth factor α (TGF-α), was observed.

MATERIALS AND METHODS

Culture Medium. The basal medium consisted of a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (F12) (pH 7.4) containing 15 mM Hepes, sodium bicarbonate (1.1 mg/ml), penicillin (92 units/ml), and streptomycin (200 μg/ml) (DMEM/F12). The hormonally defined medium consisted of DMEM/F12 further supplemented with bovine insulin (5 μg/ml), human transferrin (5 μg/ml), prostaglandin E_1 (25 ng/ml), 5 pM 3,5,3′-triiodothyronine, and 50 nM hydrocortisone (11). Unlike the original formulation, sodium selenite were not added. Water used for medium and growth factor preparation was first purified by reverse osmosis and subsequently by a Milli-Q deionization system.

Primary Baby Mouse Kidney Epithelial Cell Culture. Primary baby mouse kidney tissue was prepared for cell culture as described by Taub and Sato (12). To summarize 10- to 14-day-old baby C57/BL6J mice of either sex were sacrificed by cervical dislocation. The kidneys from the mice were washed with culture medium and minced into 1-mm pieces. The pieces were suspended in a culture tube containing 5 ml DMEM/F12 supplemented with collagenase (1 mg/ml; Worthington 4188) and soybean trypsin inhibitor (1 mg/ml). The kidney tissue was then incubated at 37°C for 15 min with periodic shaking to facilitate the release of nephron fragments. After incubation, the tube was again shaken, and intact tissue was allowed to settle to the bottom by gravity. The supernatant fraction containing free nephron segments was washed by centrifugation. Collagenase treatment of the minced material was repeated two or three times, and tubule segments from the collagenase treatments were pooled. The number of tubule fragments obtained was determined using a hemocytometer.

Matrigel Cultures. Matrigel, a reconstituted basement membrane gel, was prepared from an unfractionated high-salt/urea extract of the EHS tumor as described by Kleinman *et al.* (7). Matrigel was stored at −20°C. Prior to use, the

Abbreviations: EGF, epidermal growth factor; TGF-α and TGF-β, transforming growth factor α and β, respectively.

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matrigel was thawed at room temperature and maintained as a liquid at 4°C until the kidney tissue was ready for cell culture. Dissociated kidney tissue (nephron fragments) were mixed into the liquified matrigel at 1×10^4 nephron fragments per ml of matrigel. Matrigel containing the dissociated kidney tissue was inoculated into 35-mm dishes at 1.0 ml per dish. After an overnight incubation in a humidified 5% CO_2/95% air environment, 2 ml of hormonally defined medium was added to each dish, and the cultures were again maintained at 37°C in a humidified 5% CO_2/95% air atmosphere.

In some studies, ammonium sulfate-treated matrigel was utilized. Low molecular mass proteins [such as TGF-α and transforming growth factor β (TGF-β)] are soluble in 20% saturated ammonium sulfate, unlike the major extracellular matrix proteins laminin, collagen IV, and heparan sulfate proteoglycan. Ice-cold matrigel was precipitated with 20% ammonium sulfate, centrifuged, and resuspended in Tris-buffered saline (TBS = 0.15 M NaCl/0.05 M Tris·HCl, pH 7.4). This process was repeated once to obtain sequential ammonium sulfate-precipitated material. The matrigel was dialyzed for 2 hr against TBS containing 0.5% chloroform, then for 2 hr against TBS alone, and then against medium.

Electron Microscopy. To examine cross sections of tubules in matrigel cultures, glass coverslips (22 × 22 mm) were coated with matrigel for 24 hr prior to initiation of cell cultures. This prevented monolayers formation on the glass coverslip. Baby mouse kidney tissue was dissociated and inoculated into liquid matrigel at 1×10^4 nephron fragments per ml of Matrigel. Matrigel containing this kidney tissue was then spread over the coverslip at 0.5 ml per coverslip. The cultures were incubated for 10 days at 37°C in a humidified 5% CO_2/95% air atmosphere. Hormonally defined medium was added at 2 ml per dish after the first day. The cultures were fixed with 2% (vol/vol) glutaraldehyde in 0.05 M sodium cacodylate (pH 7.4). The cultures were then postfixed first in 0.5% osmium tetroxide/0.8% $K_3Fe(CN)_6$/0.05 M cacodylate (pH 7.4), then in 0.15% tannic acid/0.05 M cacodylate (pH 7.4), and finally in 2% (wt/vol) aqueous uranyl acetate. The cultures were subsequently dehydrated in acetone. Each coverslip was inverted and placed on a 21-mm^3 histomold (Peel-A-Way-Products) containing Epon-araldite (Electron-Microscopy Sciences). Semithin (0.5 μm) and thin (90 nm) sections were cut at 90° relative to the plane of the coverslip and photographed with a JEOL 100CXII electron microscope.

Materials. Hormones, human transferrin, prostaglandin E_1, and other chemicals were purchased from Sigma. TGF-β was purchased from R & D Systems (Minneapolis) and TGF-α was obtained from Peninsula Laboratories.

RESULTS

Baby mouse kidney cells were observed to form tubules (Fig. 1*A*), unlike cells cultured on plastic, which form an epithelial monolayer (Fig. 1*B*). The original inoculum, which consisted of nephron fragments, is illustrated in Fig. 2*A*. After 1 day in matrigel, less organized groups of cells were observed (Fig. 2*B*) and, after 6 days, tubules were observed (Fig. 2*C*).

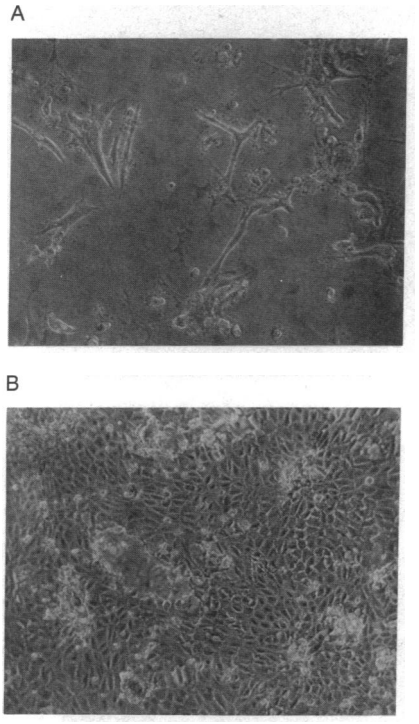

FIG. 1. Appearance of baby mouse kidney cells cultured in matrigel and on plastic. Primary cultures of baby mouse kidney cells were inoculated either into matrigel (*A*) or directly on plastic dishes containing hormonally defined serum-free medium (*B*). After 6 days, photographs were taken of representative microscope fields (×50).

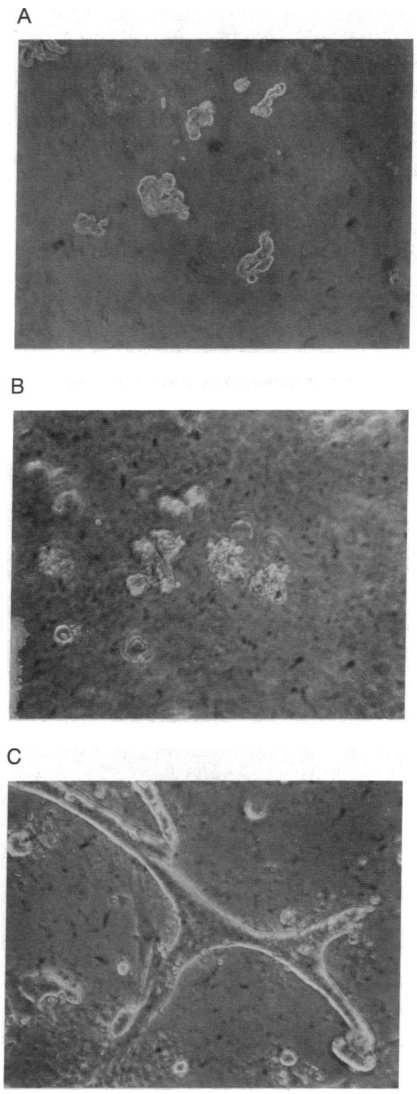

FIG. 2. Baby mouse kidney tubules in matrigel. Primary cultures of baby mouse kidney cells were inoculated into matrigel and cultures. Photographs were taken at the time of inoculation (*A*), after 1 day in culture (*B*), and after 6 days in culture (*C*). (Magnification = ×100.)

To ascertain whether tubule formation was actually occurring *de novo* in culture, particular regions in matrigel cultures were examined over time. Fig. 3*A* shows the beginnings of a branching tubule after a 6-day culture period. After 13 days, the tubule was more than 750 μm long and could not be observed in a single microscope field at ×100 magnification (Fig. 3*B*). Similarly, a region in a 1-day-old matrigel culture was observed to contain groups of cells similar to those illustrated in Fig. 2*B*. After 4 days in culture, an outgrowth was observed to be emerging from one of these original groups of cells (unpublished observation).

Vertical cross-sections of matrigel cultures were examined with the electron microscope. Fig. 4*A* shows a typical tubule with a lumen. The cells within the tubule consisted of polarized epithelial cells with microvilli at the lumenal surface and with centrally localized nuclei. Individual cells were separated by distinct boundaries. These characteristics are typical of the cells in the collecting duct (13). In some cases cellular debris is present in the lumenal space. Fig. 4*B* illustrates at a higher magnification microvilli at the apical surface and junctional complex between two cells.

Matrigel has been found to contain many growth factors (A. Roberts, personal communication). To evaluate the dependence of *in vitro* tubule formation upon growth factors, high molecular mass matrigel components were concentrated with 20% saturated ammonium sulfate. When newborn mouse kidney cultures were initiated in this ammonium sulfate-treated matrigel, tubule formation was dramatically diminished (Fig. 5*A*), unless the culture medium was further supplemented with EGF (10 ng/ml) (Fig. 5*B*). The baby mouse kidney cultures also formed tubules in ammonium sulfate-treated matrigel in serum supplemented medium (Fig. 5*C*).

EGF was observed to increase the frequency of tubule formation in ammonium sulfate-treated matrigel in a dose-dependent manner (Fig. 6). Tubule formation was dramatically stimulated by EGF at concentrations as low as 0.1 ng/ml. Not only did EGF stimulate the frequency of tubules, but also the branching of the tubules in ammonium sulfate-treated matrigel (Fig. 6). However, a significantly higher EGF dosage (10 ng/ml) was required to stimulate the branching of tubules (Fig. 6).

Since TGF-α not only has structural similarity to EGF but also interacts with EGF receptors on animal cells (14), we also tested the effects of TGF-α on tubule formation in ammonium sulfate-treated matrigel. TGF-α-stimulated tubule formation and the stimulatory effect of TGF-α were

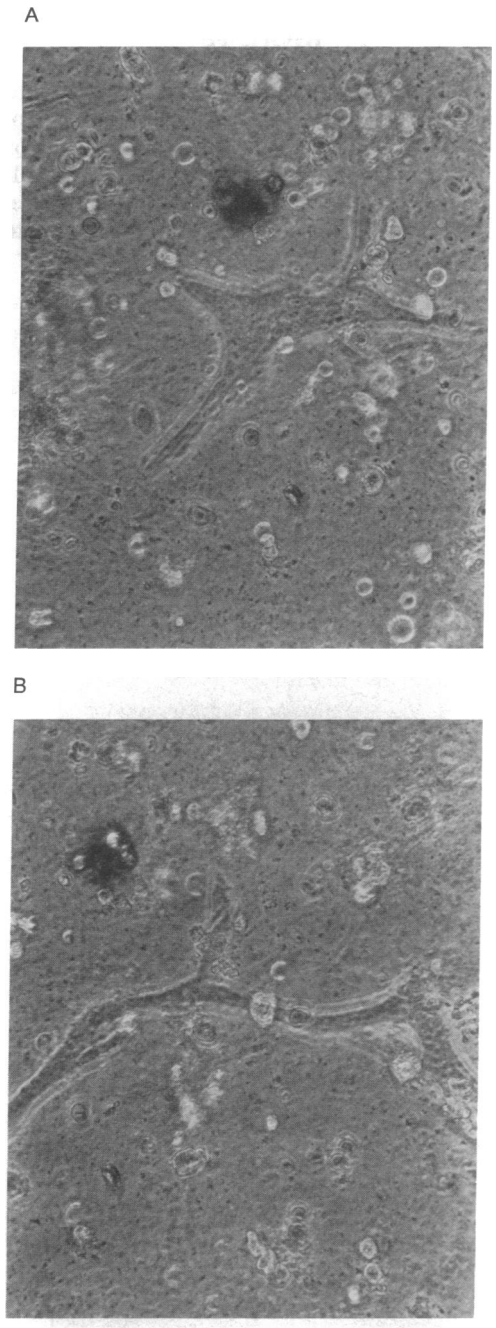

FIG. 3. Baby mouse kidney tubule formation over a 13-day period in matrigel. A specific microscope field was identified by circling the bottom of the culture dishes with a marking pen. Photographs of this region were taken at ×50 magnification on day 6 (*A*) and on day 13 (*B*). The length of the tubule on day 13 was determined using photographs both of the tubule and of a stage micrometer.

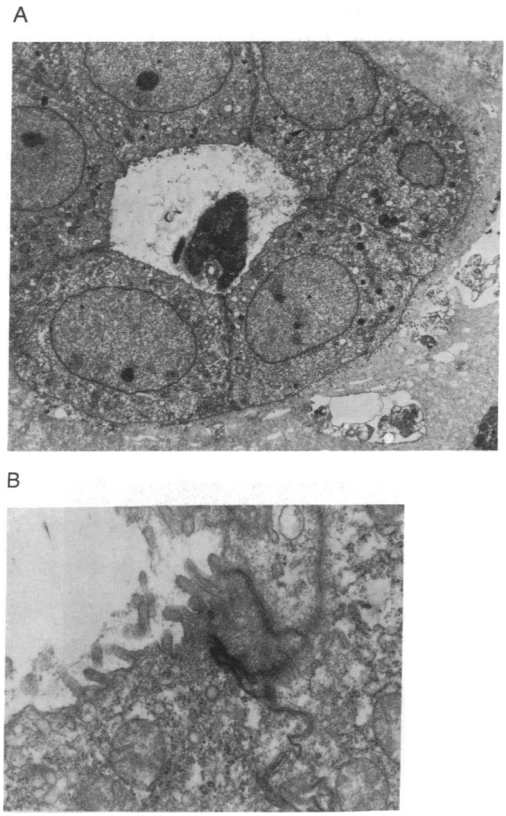

FIG. 4. Electron micrograph of a tubule in matrigel. Baby mouse kidney cells were cultured in matrigel for 6 days and processed for electron microscopy. (*A*) Cross section of tubule. (×1000.) (*B*) Junctional complex within this cross section. (×18,900.)

equivalent to that of EGF (Fig. 7). In contrast, TGF-β had an inhibitory effect on tubule formation in this matrigel (Fig. 7).

DISCUSSION

The extracellular matrix has been proposed to play a critical role in the development of the kidney *in vivo* (3). In this report, the influence of a reconstituted basement membrane gel (matrigel) (7) upon baby mouse kidney cells is examined in a hormonally defined serum-free medium (11). The outgrowth of tubules from dissociated renal tissue in matrigel was observed. Subsequently, growth and branching morphogenesis occurred over a 2-week period. Electron microscopy studies indicated that the tubules consist of polarized epithelial cells interconnected by tight junctional complexes. Lumen formation was observed. When ammonium sulfate-treated matrigel was employed, tubule formation became dependent upon the presence of EGF, TGF-α, or serum. EGF and TGF-α were not similarly required for tubule formation to occur in unextracted matrigel. However, we have observed significant (albeit less dramatic) effects of these factors at 10 ng/ml on tubule formation in unextracted matrigel (unpublished observation), which would be expected if these or other undefined growth factors are present in matrigel at suboptimal concentrations. TGF-β (which is inhibitory to tubulogenesis) has been detected in matrigel at 4 ng/ml (A. Roberts, personal communication). The removal of inhibitory TGF-β from matrigel by ammonium sulfate treatment very likely also results in a stimulation of tubule formation by EGF or serum that is much more dramatic than in untreated matrigel. Presumably, other growth factors are also present in untreated matrigel that are in contrast stimulatory to mouse kidney tubulogenesis in this matrigel. EGF and TGF-α are good candidates as being these factors and inducers of renal development *in vivo*. However, other unidentified inducers may also be present in either serum or matrigel.

Our results indicate that new tubules actually elongate and branch in matrigel. The renal tubule formation that occurs in matrigel may be the consequence of renal growth and differentiation *in vitro* or simply the reaggregation and migration of fully differentiated renal cells. Indeed, the mouse metanephrogenic mesenchyme has been observed to incorporate [^{3}H]thymidine into DNA after induction *in vitro*, while the cells gradually entered the program of epithelial differentia-

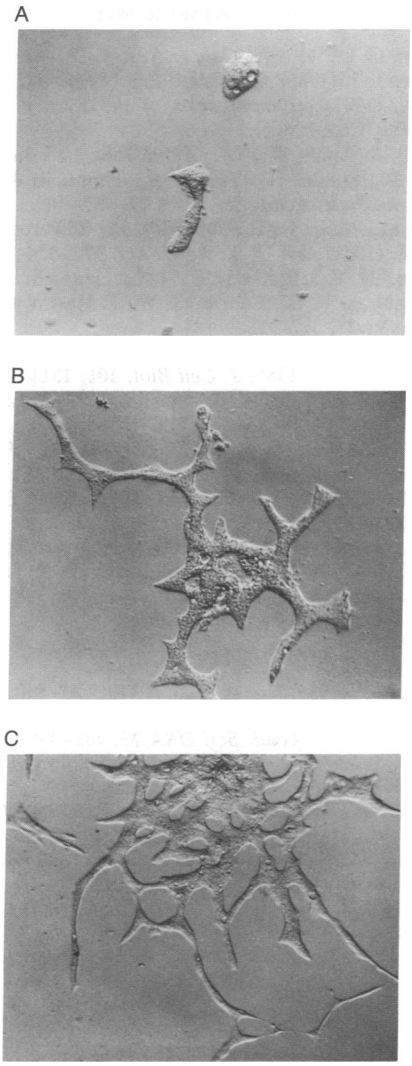

FIG. 5. Photomicrographs of tubules in ammonium sulfate-treated matrigel. Baby mouse kidney cells were cultured for 6 days in ammonium sulfate-treated matrigel in hormonally defined medium with the five supplements with no further supplements (control) (*A*), with EGF (10 ng/ml) (*B*), or with 10% fetal calf serum (*C*). Photographs were taken after 6 days in culture. (×100.)

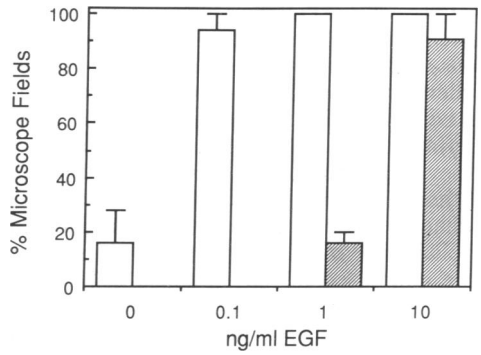

FIG. 6. Dependence of tubule formation upon EGF dosage. Baby mouse kidney cells were cultured for 6 days in ammonium sulfate-treated matrigel in culture dishes containing hormonally defined medium without any further supplements (control), with EGF (0.1 ng/ml), with EGF (1 ng/ml), or with EGF (10 ng/ml). The number of total tubules per microscope field and the number of branched tubules per microscope field was determined in 25 microscope fields per culture (magnification, ×50) in three culture dishes. The percent of microscope fields containing either all types of tubules (open bars) or only branched tubules (hatched bars) was calculated for each dish. The average ± SD was then determined for triplicate dishes.

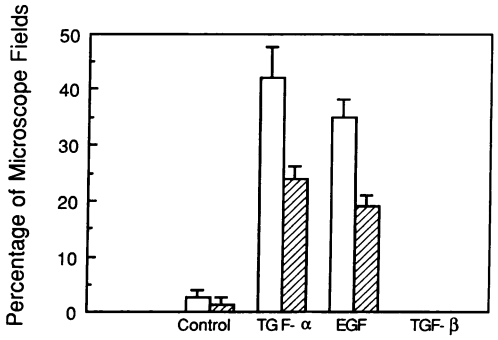

FIG. 7. Dependence of tubule formation upon TGF-α and EGF. Baby mouse kidney cells were cultured for 6 days in ammonium sulfate-treated matrigel within culture dishes containing hormonally defined medium without any further supplements (control), with TGF-α (10 ng/ml), with EGF (10 ng/ml), or with TGF-β (10 ng/ml). Under each condition, the percent of microscope fields containing tubules (open bars) or branched tubules (hatched bars) was measured as described in Fig. 4. Determinations were made in triplicate dishes and are expressed as the average ± SD.

tion (15). However, the cell multiplication and differentiation that occurred was not clearly due to a single inducer.

The mouse kidney matrigel culture system utilized here is ideal for identifying specific inducers, as a defined culture medium is being used. However, the kidney tissue utilized in these experiments was obtained from 1- to 2-week-old mice. Thus, kidney development may not actually be occurring *in vitro*, and the tubulogenesis that is being observed may be an inherent characteristic of completely determined kidney tubule epithelial cells. In this case, the growing nephrons may possibly be composed of only a single cell type. Alternatively, the branching tubules that form in matrigel may originate from stem cells within the baby mouse kidney. In this latter case, the tubules may possibly consist of a gradient of different cell types, as is observed in nephrons *in vivo*.

The development of the metanephros in the embryonic mouse *in vivo* is characterized by the growth and differentiation of the epithelial cells in the ureteric bud on one hand and the surrounding mesenchymal tissue on the other hand (3). The outgrowth that occurs from the ureteric bud results in the formation, initially, of a branching ureter and ultimately in a collecting duct. The mesenchyme, which is localized at the ends of the branches, gradually condenses. Undifferentiated mesenchymal cells ultimately assume a polarized morphology typical of epithelial cells as well as differentiated functions. The baby mouse kidney cells within matrigel similarly underwent branching morphogenesis. Moreover, our electron microscopy studies indicate that the tubular cells in matrigel were similar in structure to collecting duct cells. Mesenchymal condensates were not observed to surround the growing ends of the branching tubules in matrigel, as observed in the organ culture studies of Grobstein (16) and of Ekblom *et al.* (17). This observation can possibly be explained if the second tissue component (mesenchyme) is not present in matrigel. However, the possibility cannot be excluded that cells of mesenchymal origin are similarly undergoing branching morphogenesis in matrigel but in separation from cells originating from the ureteric bud.

In his classic organ culture studies, Grobstein (16) isolated the mesenchyme and ureteric bud from an 11-day embryonic mouse metanephros and placed them in two culture chambers separated by a filter. The results of his transfilter experiments indicated that the mesenchyme and ureteric bud can develop in two separate chambers providing that appropriate cell–cell contacts have been made or that appropriate inductive signals are present or have previously occurred.

After the induction of the embryonic mouse kidney, definite changes in the expression and localization of laminin have been detected (14). Laminin appears in the adherent mesenchymal cells. Laminin, an 850-kDa glycoprotein, consists of three polypeptide chains, designated A, B1, and B2 (4). The appearance of the A chain in particular has been observed to correlate with the development of the polarization of the mesenchymal cells of the metanephros and the onset of tubulogenesis (18). Moreover antisera against specific fragments of laminin containing parts of the A chain have been observed to inhibit the development of polarization of the primitive renal mesenchyme in organ culture (19).

The specific inducer that is responsible for the initiation of the morphogenesis of the embryonic mouse metanephros *in vivo* has not yet been identified. The embryonic mouse metanephros has been observed to differentiate in hormonally defined serum-free medium *in vitro* (19, 20), and a supplement, transferrin, is required for the differentiation of the mouse kidney mesenchyme in serum-free conditions (20). However, the physiologic significance of the transferrin requirement is unclear, and the studies of Grobstein (16) indicate that unidentified inducers are produced by embryonic kidney cells in organ culture. Both EGF and TGF-α are good candidates as being the inducers. Both EGF precursor mRNA and TGF-α mRNA have been observed in the mouse kidney (21, 22). Of particular interest is the finding that TGF-α mRNA is expressed in the kidneys (metanephros) of 9- and 10-day mouse embryos. Thus, TGF-α mRNA appeared immediately prior to the branching morphogenesis of the embryonic metanephros. The reported appearance of TGF-α mRNA at this time is consistent with TGF-α playing a role in early kidney development and with the physiologic significance of our *in vitro* investigations with matrigel.

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