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A Retinoid Responsive Cytokine Gene, *MK*, Is Preferentially Expressed in the Proximal Tubules of the Kidney and Human Tumor Cell Lines

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The aim of this study was to survey the expression of an embryonic cytokine gene,* MK, *in the normal organs and neoplastic tissues of adults. Northern analysis showed that* MK *mRNA was exclusively expressed in the kidney among murine organs including thymus, lung, heart, spleen, liver, and kidney.* In situ *hybridization analysis revealed that* MK *expression was localized in the proximal tubules and metaplastic Bowman's epithelium, but not in other nephron segments such as glomeruli, loop of Henle, distal tubules, and collecting ducts. To investigate whether* MK *expression is a marker of tubular cell lineage, several cell lines originating from renal tubules were tested. No expression of* MK *was detected in PtK1 and LLC-PK1 cells derived from marsupial and porcine proximal tubules or in MDBK and MDCK cells from bovine and canine distal/collecting tubules. Unexpectedly, the* MK *gene was expressed in a human renal cell carcinoma line, VMRC-RCW, and the expression was up-regulated in the presence of retinoic acid. To elucidate the involvement of* MK *in the development of tumors, we further examined its expression in a variety of human neoplastic cell lines: YMB-1-C (breast cancer), EBC-1 (lung squamous cell carcinoma), RERF-LC-OK (lung adenocarcinoma), SBC-3 (lung small cell carcinoma), HSC-2 (mouth squamous cell carcinoma), NUGC-2 (gastric cancer), COLO201 (colon cancer), HepG2 (hepatoma), MIA PaCa-2 (pancreatic cancer), MCAS (ovarian cancer), HeLa (cervical cancer), BeWo (chorionic carcinoma), ITO-II (testicular tumor), T24 (urinary bladder tumor), and G-401 (Wilms' tumor). Strong signals were detected in COLO201, HepG2, ITO-II, T24, G-401, and weaker but distinct signals were detected in YMB-1-C, HSC-2, and MCAS cells. The* MK *gene was, therefore, widely expressed in neoplastic cells originating from genital organs, intestinal tract, liver, mammary gland, and urinary tract, and the expression was not restricted to adenocarcinomas, but was also observed in other types of tumor cells. These findings suggest that a retinoic acid responsive gene,* MK, *may play a role in the pathophysiology of renal proximal tubules and tumorigenesis in many types of neoplasms. (Am J Pathol 1993, 142:425–431)

The retinoic acid responsive gene *MK* has been cloned from embryonal carcinoma cells stimulated by retinoid.[1] During the embryogenesis of mice, *MK* is ubiquitously expressed in the mid-gestation period.[2] Thereafter, expression of the the gene localizes rapidly in several sites, including brain, pituitary gland, upper and lower jaw, vertebral column, limbs, stomach, lung, pancreas, small intestine, and metanephros, which finally becomes specific for the kidney after 15 days in the embryonic stage.[2] In adult mice, *MK* is exclusively expressed in the kidney but not in other organs including brain, spleen, testis, muscle, stomach, and small intestine.[1,3] Thus, the designation of *MK* stems from the gene's sequence of expression sites.

Midkine, the product encoded by *MK*, is a 14-kd secreting protein whose amino acid composition is enriched in basic amino acids and cysteine.[4] This

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polypeptide possesses heparin-binding activity, indicating that it is a heparin-binding growth factor.[4] In fact, the polypeptide induces the growth of PC-12, a rat pheochromocytoma cell line, as well as NIH 3T3 cells, and facilitates neurite extension of rat embryonic brain cells *in vitro*.[4,5] These lines of evidence suggest that midkine is a growth and/or differentiation-related cytokine that plays an important role in embryogenesis. However, the fact that the *MK* gene is as well expressed in the adult kidney argues an additional potential role in lifelong kidney function. Still unknown, however, is what part of the nephron expresses this gene and what kinds of pathophysiological functions it carries out in the kidney. In the first step of this study, we surveyed the expression of *MK* in the adult mouse kidney and other organs and cell lines originating from renal tubules by *in situ* hybridization and Northern analysis. During the study, we found that the expression of *MK* was lost in tubular cell lines, but the gene was retranscribed in a line of renal cell carcinoma. We then expanded our survey to various human neoplastic cells. We show here that the expression of the *MK* gene was observed in many types of tumor cells and discuss the basis of *MK* expression in neoplasms as an oncofetal gene or its involvement in tumorigenesis.

Materials and Methods

Organs of adult female ddY mice (10W) purchased from Nippon Clea (Tokyo, Japan) were used for RNA preparations. Renal tubule cell lines and human neoplastic cell lines were provided by the Japanese Cancer Research Resources Bank (JCRB, Tokyo); PtK1 (marsupial proximal tubule), LLC-PK1 (porcine proximal tubule), MDBK (bovine distal/collecting tubule), MDCK (canine distal/collecting tubule), VMRC-RCW (human renal cell carcinoma), YMB-1-C (human breast cancer), EBC-1 (human lung squamous cell carcinoma), RERF-LC-OK (human lung adenocarcinoma), SBC-3 (human lung small cell carcinoma), HSC-2 (human mouth squamous cell carcinoma), NUGC-2 (human gastric cancer), COLO201 (human colon cancer), HepG2 (human hepatoma), MIA PaCa-2 (human pancreatic cancer), MCAS (human ovarian cancer), HeLa (human cervical cancer), BeWo (human chorionic carcinoma), ITO-II (human testicular tumor), T24 (human urinary bladder tumor), and G-401 (human Wilms' tumor). These cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing suggested concentrations of fetal calf serum, with or without 1×10^{-6} mol/L retinoic acid (Sigma Chemical Co., St. Louis, MO), for 48 hours of exposure and used for Northern analysis.

RNA Extraction and Northern Analysis

Total RNA was extracted from mouse organs or frozen cell stocks using a single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction.[6] The RNA samples, 7 to 10 μg/lane, were electrophoresed on a 1.2% agarose gel containing 10% formaldehyde and transferred onto a nitrocellulose membrane (Schleicher & Schuell, FRG). A 0.27-kb *Alu* I fragment of mouse *MK2* complementary DNA was labeled by the random priming method[7] and used as a hybridization probe. Hybridization was performed as described previously.[8] Filters were washed at 50 C in 4 × standard saline citrate containing 0.1% sodium dodecyl sulfate and exposed to x-ray film with intensifying screens.

In Situ *Hybridization*

In situ hybridization was performed using the method described by Nomura.[9] Adult mouse kidneys were fixed in 4% paraformaldehyde in phosphate-buffered saline at 4 C overnight, dehydrated, and embedded in paraffin wax. Sections of 4 μ were mounted on poly-L-lysine-coated glass slides and dried at 37 C. After removal of the wax, sections were postfixed in 4% paraformaldehyde, treated with 0.25% acetic anhydride in 0.1 mol/L triethanolamine, and dehydrated again. To prepare probes, a 0.27-kb *Alu* I fragment of mouse *MK2* was subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA), and [α-^{35}S]UTP-labeled single-stranded sense or antisense RNA probes were synthesized by T7 or T3 RNA polymerase using a Riboprobe system (Promega, Madison, WI) after linearization with *Xba* I or *Xho* I. These probes were added to the hybridization solution (50% formamide, 10 mmol/L Tris-HCl, pH 7.6, 1 mmol/L ethylenediamine tetraacetic acid, 600 mmol/L NaCl, 10 mmol/L dithiothreitol, 1× Denhardt's solution, 0.25% sodium dodecyl sulfate, 10% dextran sulfate, 200 μg/ml *Escherichia coli* transfer RNA) at a final concentration of 1×10^7 or 10^8 cpm/ml. After hybridization at 50 C overnight, a washing procedure including RNAse treatment (25 μg/ml) was performed. Slides containing the dehydrated renal tissue were then dipped in photographic emulsion (Kodak NTB3, Rochester, NY), diluted 1:1 with 2% glycerol in distilled water, dried, and exposed at 4 C for 1 or 3 weeks. After exposure, slides were developed and counterstained with toluidine blue.

Results

Localization of MK *Expression in Renal Tissue*

Kadomatsu and colleagues[1] previously reported that the *MK* gene was exclusively expressed in the kidney, but not in other organs including brain, spleen, testis, and muscle in adult mice. In the first of our experiments, we tested the expression of *MK* in several other organs of adult mice. Total RNAs from thymus, lung, heart, spleen, liver, and kidney were investigated by Northern analysis with an *MK* complementary DNA probe. As a result, an *MK* message of 0.85 kb was detected specifically in the kidney but not in the other examined organs (Figure 1). Next, to determine the precise localization of *MK* transcript in the renal tissue, we studied the gene expression by *in situ* hybridization. Under a low-power field of microscopic examination, *MK* expression was detected in the tubules (Figure 2, A and B) and localized within the renal cortex (Figure 2C), excluding the possibility that the *MK* gene was expressed in the loop of Henle or the collecting tubules. Higher power microscopy revealed that the *MK* mRNA was not detected in the glomeruli or the distal tubules, but was detected in other tubule segments around the glomeruli (Figure 2D). The *MK* gene was transcribed in the invasive proximal tubules called "metaplasia,"[10] a condition in which Bowman's epithelium is replaced by proximal tubules (Figure 2, E and F). The findings illustrated here suggest that the expression site of *MK* is restricted to the proximal tubules, including the S1 portion of convoluted segment of the adult mouse kidney.

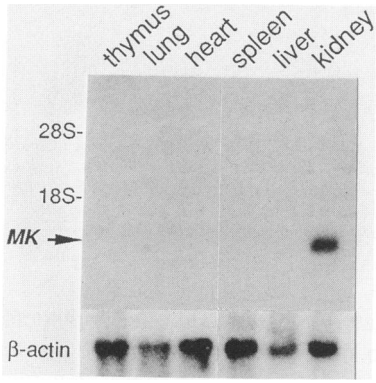

Figure 1. *Total RNAs were extracted from thymus, lung, heart, spleen, liver, and kidney of adult mice, and MK gene expression was examined by Northern blotting. An MK message of 0.85 kb was detected specifically in the kidney, but not in the other examined organs. 28S and 18S markers indicate the position of ribosomal RNAs, whose molecular sizes were identified as 4.7 and 1.9 kb. Expression of the housekeeping gene, β-actin, was tested concurrently to show comparative reactivity.*

Expression of MK *in Renal Tubular Cell Lines*

To investigate whether *MK* expression is a marker of tubular cell lineage, several cell lines originating from renal tubules were tested. PtK1 from marsupial proximal tubules, LLC-PK1 from porcine proximal tubules, MDBK from bovine distal/collecting tubules, MDCK from canine distal/collecting tubules, and VMRC-RCW from human renal cell carcinoma of proximal tubule origin were examined for the expression of the *MK* gene. As shown in Figure 3, the transcript was detected in the VMRC-RCW cells, but not in PtK1, LLC-PK1, MDBK, or MDCK cells. These findings suggest that proximal tubule cells may lose the expression of *MK* when cultured *in vitro*, but the transcription of the gene may be reactivated when they are transformed to neoplastic cells. Even in such a transformed condition, the *MK* gene was responsive to retinoic acid (Figure 3), as has been reported in embryonal cell carcinoma.[1]

Expression of MK *in Human Tumor Cell Lines*

Finally, we investigated whether the expression of *MK* is restricted to renal cell carcinoma or is also observed in other neoplastic cells. Total RNA was extracted from various types of human tumor cells in culture, and the expression of *MK* gene was examined. *MK* mRNA was observed not only in VMRC-RCW, but also in other neoplastic cells (Figure 4). Strong signals were detected in COLO201 (colon cancer), HepG2 (hepatoma), ITO-II (testicular tumor), T24 (urinary bladder tumor), G-401 (Wilms' tumor), and weaker but distinct signals were detected in YMB-1-C (breast cancer), HSC-2 (mouth squamous cell carcinoma), and MCAS (ovarian cancer) cells. The *MK* gene was, therefore, widely expressed in the neoplastic cells originating from genital organs, intestinal tract, liver, mammary gland, and urinary tract, and the expression was not restricted to adenocarcinoma but was also observed in other types of tumor cells. These findings imply that *MK* may play a role in the tumorigenesis of many types of neoplasms.

Discussion

In this report we have revealed that a retinoic acid responsive gene, *MK*, is selectively expressed in the proximal tubules of the mouse kidney. Although the function of *MK* is unknown, some possibilities hinge

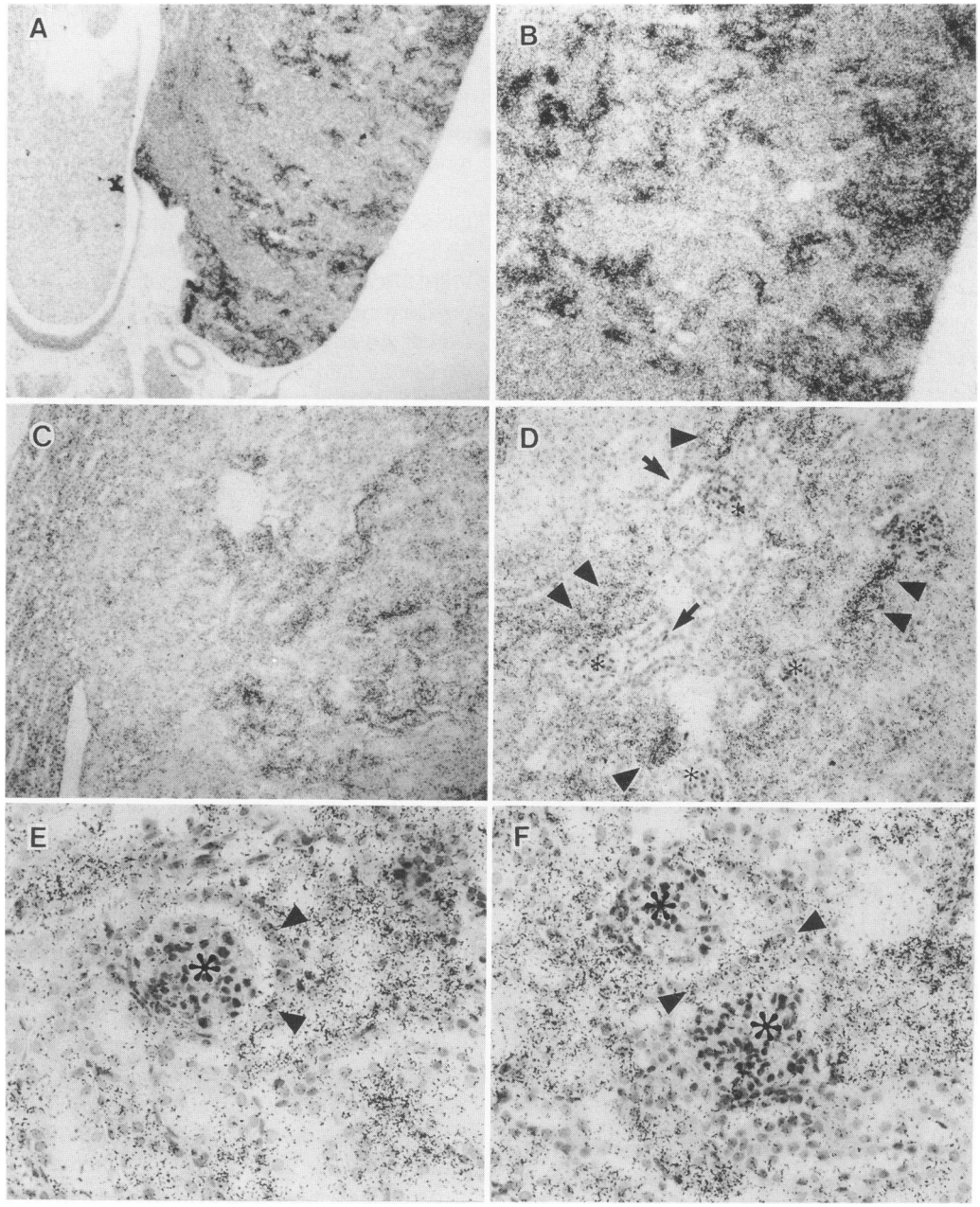

Figure 2. *The expression of MK in the renal tissue of adult mice was examined after in situ hybridization. Paraffin sections of the kidney were hybridized with [α-^{35}S]UTP-labeled sense or antisense RNA probes for mouse MK2. After washing, slides were dipped in emulsion, exposed for 1 or 3 weeks, and developed and counterstained with toluidine blue.* **A, B, C:** *MK expression was detected in the tubules and localized within the renal cortex.* **D:** *MK was not expressed in the glomeruli (*) or distal tubules (arrows), but was in other tubule segments (arrowheads) around the glomeruli.* **E, F:** *The invasive proximal tubules into the Bowman's epithelium also expressed the MK gene (arrowheads). Only the data from the antisense probe are presented.* **A,** *×10;* **B,** *×40;* **C,** *×40;* **D,** *×100;* **E,** *×200;* **F,** *×200.*

on the fact that *MK* polypeptide, midkine, is a member of the group of heparin-binding growth factors. In the physiological state, midkine may regulate normal turnover of tubular epithelial cells and maintain the structure of renal tubules.

Proliferation of renal tubular cells is a common feature in several pathological states of the kidney. For example, in the repair process of tubular injury, compensatory renal growth after nephrectomy, and polycystic kidney disease, enhanced proliferation of tubular cells is often observed. Although some growth factors are thought to contribute to these pathological processes,[11–14] their molecular mechanisms are still unclear. Several lines of evidence have suggested the involvement of retinoic acid in some types of pathological renal growth: 1) cellular retinoic

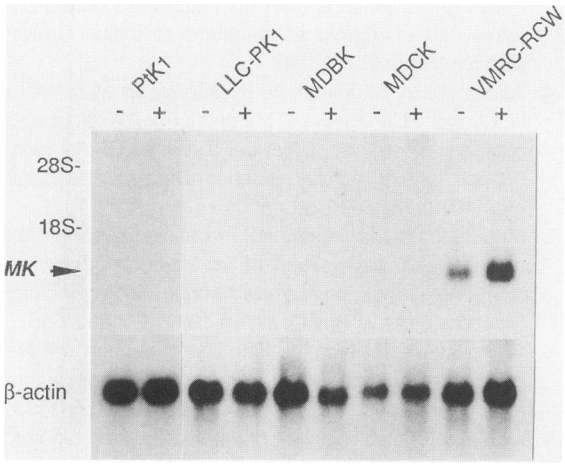

Figure 3. *PtK1 cells derived from marsupial proximal tubules, LLC-PK1 from porcine proximal tubules, MDBK from bovine distal/collecting tubules, MDCK from canine distal/collecting tubules, and VMRC-RCW from human renal cell carcinoma of proximal tubule origin, were cultured with (+) or without (−) 1×10^{-6} mol/L retinoic acid, and Northern analysis was performed. MK gene expression was detected in the VMRC-RCW cells, but not in PtK1, LLC-PK1, MDBK, or MDCK cells. MK expression in VMRC-RCW was enhanced in the presence of retinoic acid. 28S and 18S markers indicate the position of ribosomal RNAs. Expression of β-actin was also tested to show comparative reactivity.*

acid binding protein (CRABP), which is able to bind retinoic acid and mediate its effect in the nucleus, is abundant in the kidney[15]; 2) local expression of CRABP increases after partial nephrectomy, followed by compensatory renal growth[16]; and 3) retinoic acid induces the growth of rabbit kidney epithelial cells *in vitro*.[17] Our observation that retinoic acid induced the gene expression of *MK* in a renal tubule-derived cell, VMRC-RCW, implies that retinoic acid could stimulate the gene transcription of *MK* in the kidney, which may contribute to the pathological basis of renal growth mediated by midkine as a growth factor. The up-regulation of the *MK* gene in the regenerating renal tubules supports this hypothesis (unpublished observations).

We demonstrated that tubular metaplasia in Bowman's capsule was also associated with the expression of the *MK* gene. Such an intraglomerular tubular epithelium is often observed in mice.[10] In other species, however, this phenomenon is rarely observed in normal physiological states but is occasionally detected in some pathological conditions. In humans, tubular metaplasia may be associated with the incidence of hematuria and proteinuria,[18] and its association with glomerulosclerosis is reported in aging rats.[19] Although species difference in the pathophysiology of tubular metaplasia should be addressed further, the difference in responsiveness to retinoic acid or the regulation of this gene might contribute to the frequency of this phenomenon in each species.

In this study, we failed to detect *MK* expression in PtK1 and LLC-PK1 cells, both of which originate from renal proximal tubules. There are some possible explanations for the discrepancy between *MK* expression *in vivo* and *in vitro*. First, established cell lines often lose their original phenotypes because of the loss of cell-to-cell or cell-to-matrix interactions or because of the chromosomal deletion during the multiple passages. The lack of *MK* expression in PtK1 and LLC-PK1 cells may, therefore, be caused by such a cellular alteration *in vitro*. Second, the discrepancy may be derived from the species difference of *MK* expression. Although the *MK* gene is highly homologous between the human and the mouse,[20] the transcriptional regulation of *MK* in the marsupial and porcine kidney might differ from that in the murine kidney. Further studies are necessary to examine these possibilities.

We have shown that the *MK* gene is highly expressed in the human renal carcinoma cell line VMRC-RCW. Because this type of cancer cell originates from the proximal tubules,[21,22] their *MK* expression may be derived from the character of

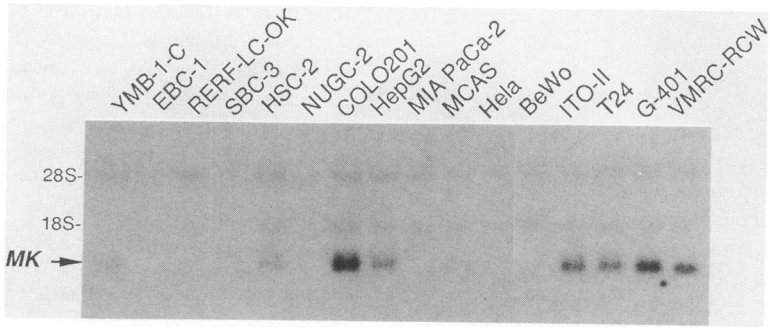

Figure 4. *Total RNAs were extracted from various types of human tumor cells in culture; YMB-1-C (breast cancer), EBC-1 (lung squamous cell carcinoma), RERF-LC-OK (lung adenocarcinoma), SBC-3 (lung small cell carcinoma), HSC-2 (mouth squamous cell carcinoma), NUGC-2 (gastric cancer), COLO201 (colon cancer), HepG2 (hepatoma), MIA PaCa-2 (pancreatic cancer), MCAS (ovarian cancer), HeLa (cervical cancer), BeWo (chorionic carcinoma), ITO-II (testicular tumor), T24 (urinary bladder tumor), G-401 (Wilms' tumor), and VMRC-RCW (renal cell carcinoma), and expression of the MK gene was examined by Northern analysis. Strong signals were detected in COLO201, HepG2, ITO-II, T24, G-401, VMRC-RCW, and weaker but distinct signals were detected in YMB-1-C, HSC-2, and MCAS cells. 28S and 18S markers indicate the position of ribosomal RNAs.*

original cell type. Another study has shown that *MK* expression was observed not only in renal cell carcinoma but also in several other types of human neoplastic cells originating from the genital organs, intestinal tract, liver, mammary gland, and urinary tract. These findings, in addition to the fact that midkine is a member of the group of heparin-binding growth factors, imply that this cytokine might have potential for contributing to tumorigenesis as an autocrine growth factor. Alternatively, various cells may express the *MK* gene as a result of dedifferentiation (a recapture of more primitive developmental stages), because undifferentiated cells commonly express *MK* during the mid-gestational stage of embryogenesis. *MK* may be regarded as one of the oncofetal genes, except for its expression in the normal adult kidney.

In the normal physiological state, renal tubules show little proliferative activity.[13] Because *MK* is expressed in normal tubules, this transcript may have alternative functions in a differentiated state of tubular epithelium. The proximal tubules, compared with other nephron segments, specifically participate in such varied renal functions as transport of electrolytes, reabsorption of glucose and protein/amino acids, and metabolism of vitamin D. The tubular localization of *MK* in the proximal but not distal and collecting ducts indicates that the *MK* gene could be involved in these complex mechanisms specified in the proximal tubules of the normal kidney. Future research focusing on the isolation of a midkine receptor will reveal the pathophysiological functions of this gene.

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