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27. A Cla I fragment from RSVc-SrcK295R (*12*) was transferred into pLNCX [A. D. Miller and G. J. Rosman, *BioTechniques* 7, 980 (1989); J. Schmidt and J. Brugge, unpublished data]. After transfection of Psi-2 cells, clones of G418-resistant cells were isolated and tested for virus production. The LNCXc-SrcK295R stock gave 2.2 × $10^6$ G418-resistant colonies per milliliter in NIH 3T3 cells.
28. We thank D. Livingston for the ZipTex virus; D. Shalloway for the pcR295 plasmid; J. Schmidt for construction of LNCXc-SrcK295R; B. Meyers and S. Thomas for preparation of virus stock; S. Parsons for the EC10 antibody; J. Bolen for SrcII antiserum and antiserum to Fyn, Lyn, Yes, Hck, Fgr, Blk, and Lck; and S. Thomas, C. Seidel-Dugan, and J. Bolen for critical evaluation of the manuscript. J.E.T. is supported by a fellowship from the NIH and P.S. is a Pew Scholar in the Biomedical Sciences. Supported by the National Cancer Institute (J.S.B.) and the National Institutes of Child Health and Human Development (P.S.).

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# Dependence of Kidney Morphogenesis on the Expression of Nerve Growth Factor Receptor

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**Nerve growth factor receptor (NGFR) serves as the binding site for the neurotrophic growth factors. Although NGFR has been found in several embryonic tissues outside the nervous system, the function of NGFR in embryogenesis of non-neuronal organs remains unknown. NGFR is transiently synthesized by embryonic rat kidney and disappears from nephrons upon their terminal differentiation. Anti-sense oligonucleotide inhibition of NGFR expression inhibits kidney morphogenesis. Therefore, NGFR is required not only for development of the nervous system, but also for differentiation of the kidney tubules.**

A FAMILY OF NEUROTROPHIC GROWTH factors, including nerve growth factor β (βNGF), brain-derived neurotrophic factor, and neurotrophin 3 (hippocampus-derived neurotrophic factor), is likely important for development and maintenance of various sets of neurons (*1*). It is possible that all of these factors bind to the same nerve growth factor receptor (NGFR) (*2*). NGFR is expressed on some neuronal and glial cells (*3*), as well as in several adult and embryonic tissues (*4–8*), including glomeruli of the permanent or metanephric kidney (*4, 8*). The function of NGFR in the development of these non-neuronal organs remains unknown.

We have investigated the expression and effect of NGFR in the developing kidney. The metanephric kidney is formed by the interaction between ureter bud epithelium and the surrounding nephrogenic mesenchyme. The ureter bud induces nephrogenic mesenchyme to form the secretory part of the nephrons (*9, 10*). Reciprocally, nephrogenic mesenchyme causes the ureter bud to branch, thereby forming collecting tubules. The ureter bud can be microsurgically separated from mesenchyme and replaced by several neural or non-neural embryonic tissues that effectively trigger differentiation of kidney tubules (*10, 11*). Thus, the unknown morphogens of kidney tubulogenesis are widespread among embryonic tissues.

By in situ hybridization with a $^{35}$S-labeled rat NGFR riboprobe, NGFR mRNA was first detected in the developing rat kidney at embryonic day 13 in cell condensates that later form nephrons (Fig. 1A). Expression was abundant in the embryonic nephrons (so called S-shaped bodies, Fig. 1, B and C) and ceased a few days after birth upon terminal differentiation of nephrons. A similar pattern of NGFR expression was also revealed by indirect immunoperoxidase staining with monoclonal antibodies (MAbs) to rat NGFR (Fig. 1, D and E), as well as by iodinated-βNGF binding and subsequent autoradiography (Fig. 1, F and G). In the adult kidney, NGFR expression is limited to specific non-epithelial cell types such as mesangial cells of the glomerulus, nerve fibers, and the adventitia of blood vessels (*4*). Expression of NGFR in the nephrons was transient and began during induction of the nephron.

Two classes of NGFR have been character-

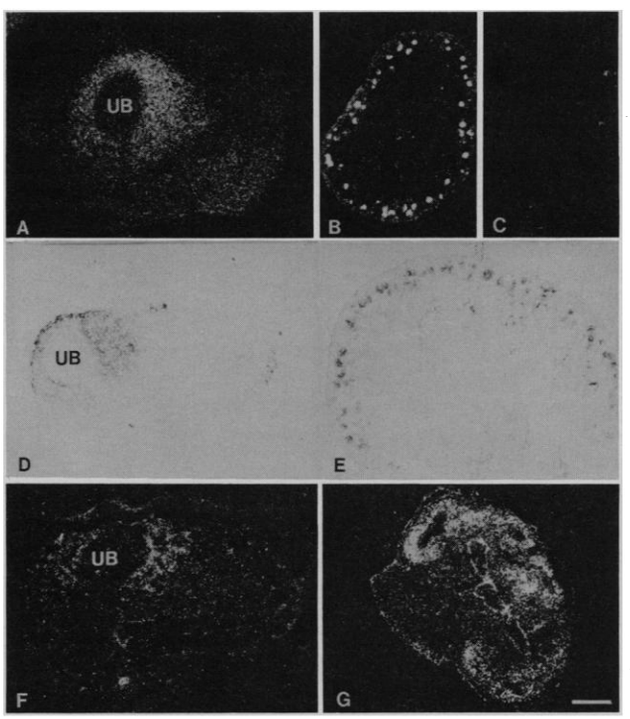


**Fig. 1.** Expression of NGFR in embryonic rat kidneys. (**A** and **B**) In situ hybridization (*20*) with the $^{35}$S-labeled antisense riboprobe to NGFR mRNA. At day 13 (A), nephrogenesis is initiated around the ureter bud (UB) and the mesenchyme condenses to form the first nephrons. The grains are clustered in the condensing mesenchyme. At day 18 (B), nephrogenesis continues only in the outer-most cortex of the embryonic kidney. Each cluster of grains represents an embryonic nephron. (**C**) In situ hybridization with the $^{35}$S-labeled sense riboprobe to NGFR mRNA shows no binding to 18-day embryonic kidney. Immunoperoxidase staining with MAb to NGFR on (**D**) day 13 and (**E**) day 19. Binding of iodinated βNGF (*21*) on (**F**) day 13 and (**G**) day 14. Kidneys incubated in 1000-fold excess cold βNGF did not reveal specific binding. Bar represents 60 μm in (A), (D), and (F); 300 μm in (B) and (C); 700 μm in (E); 120 μm in (H).

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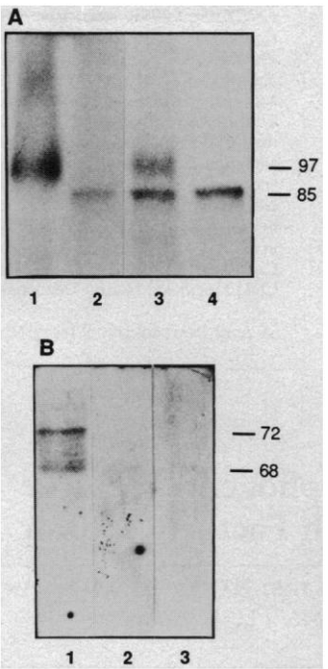


**Fig. 2.** NGFR from embryonic rat kidneys and PC12 cells. (**A**) Affinity cross-linking of NGFR from embryonic kidney and PC12 cells (*15, 22*). Lane 1, whole PC12 cells; lane 2, membranes of PC12 cells; lane 3, whole embryonic kidney cells; lane 4, membranes of embryonic kidney cells. The membranes or cells from 50 14-day embryonic rat kidneys and $5 \times 10^6$ PC12 cells were incubated for 45 min at 37°C with 1 nM iodinated βNGF and placed on ice-cold phosphate-buffered saline. EDC (Pierce) was added at a final concentration of 30 mM (*16, 17*). Excess (1000-fold) βNGF abolished cross-linking of iodinated βNGF to NGFR. (**B**) Iodinated βNGF binding to proteins of embryonic rat kidneys and NR18 cells, which do not express NGFR (*23*), after renaturing the proteins in the gel (*18*). Lane 1, binding to proteins of embryonic rat kidney; lane 2, binding to proteins of embryonic rat kidney in the presence of 1000-fold excess unlabeled βNGF; lane 3, binding to proteins of NR18 cells. Forty 13.5- to 14-day embryonic rat kidneys or $2 \times 10^6$ NR18 cells were lysed in Laemmli buffer, electrophoresed on a 12% polyacrylamide–SDS gel, and subjected to renaturation and diffusion blotting as described (*18, 24*).

ized, low- and high-affinity NGFR (*12, 13*). The product of the *trk* proto-oncogene is involved in high-affinity binding to βNGF, either alone or in complex with the low-affinity NGFR (*14*). The high-affinity receptor mediates biological effects of βNGF in nerve cell cultures (*15*). NGFR from embryonic rat kidney was biochemically characterized by three methods: chemical cross-linking to iodinated βNGF, diffusion protein blotting with subsequent iodinated βNGF binding, and Northern (RNA) blot analysis of NGFR mRNA. The water-soluble cross-linker 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC) has been shown to cross-link iodinated-βNGF to the low-affinity NGFR (*16, 17*) to yield a complex approximately 100 kD in size. Iodinated βNGF was cross-linked by EDC to plasma membrane fractions from 14-day embryonic rat kidneys and PC12 cells, which resulted in 85-kD βNGF-NGFR complexes (Fig. 2A). Similar cross-linking with intact PC12 cells yielded complexes that were somewhat larger (97 kD). Cross-linking by EDC of iodinated βNGF to intact embryonic kidney cells similarly revealed a 97-kD complex. Renaturing proteins in the gel restores the ability of a denatured protein to bind its ligands specifically (*18*); this method was used to verify the molecular size of NGFR in embryonic kidney. Iodinated βNGF specifically binds to proteins of molecular weight 72 kD and 68 kD from membranes of embryonic kidneys (Fig. 2B). Northern blot analysis revealed a single NGFR mRNA species of 3.7 kb from embryonic kidneys and PC12 cells (Fig. 3). These results show the presence of low-affinity NGFR in the embryonic kidney. The difference in mobility of cross-linked complexes from membrane and whole cell preparations corresponds to the size of βNGF. Thus, it is possible that, in whole cells, βNGF is cross-linked in dimeric form, and in membrane preparations, in monomeric form. Alternatively, the mobility differences may reflect differences in glycosylation of NGFR.

The function of NGFR in epithelial differentiation and branching morphogenesis was analyzed in organotypic cultures of embryonic rat kidneys. In these cultures, undifferentiated 13-day embryonic rat kidney undergoes tubule differentiation within 2 days. Phosphothionate deoxyoligonucleotides complementary to the 3′ region of NGFR mRNA were used to inhibit NGFR expression in kidney explants. As shown by immunoperoxidase staining with the use of a MAb to NGFR, phosphothionate anti-sense oligonucleotides (5 μM) inhibit NGFR expression in cultures of 13-day embryonic kidneys (Fig. 4).

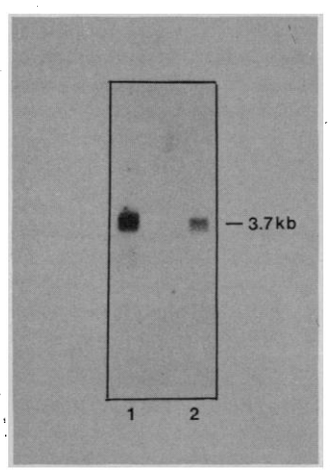


**Fig. 3.** Northern blot analysis of NGFR mRNA from embryonic rat kidneys (lane 1) and PC12 cells (lane 2). Total RNA was isolated from PC12 cells according to miniprep method (*25*), mRNA from 100 14-day embryonic kidneys was prepared with the use of Quick mRNA Preparation Kit (Pharmacia). Total RNA (10 μg) from PC12 cells and polyadenylated mRNA (1.5 μg) from embryonic kidneys were separated on a 1.2% agarose-formaldehyde gel and transferred to nylon membrane (Amersham) as described (*26*).

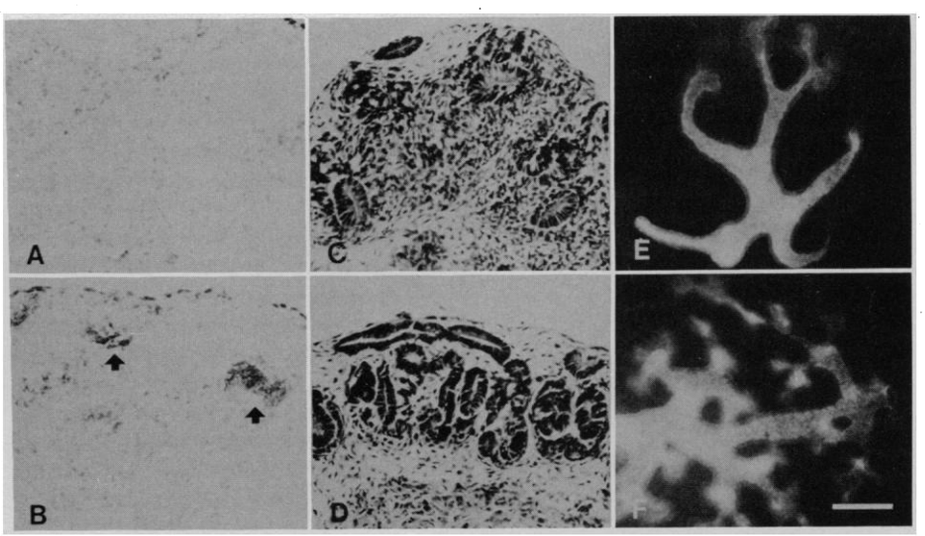


**Fig. 4.** Anti-sense phosphothionate oligonucleotide inhibition of NGFR expression and kidney morphogenesis. Kidneys incubated with (**A, C,** and **E**) anti-sense, and (**B, D,** and **F**) sense phosphothionate oligonucleotides (*27*) for 5 days. (A and B) Biotin-streptavidin peroxidase staining for NGFR demonstrates inhibition of NGFR expression. The arrows mark mesenchymal condensates and early kidney tubules expressing NGFR. (C and D) Kidney explants stained with hematoxylin-eosin. (E and F) Inhibition of the ureter bud branching is visualized in a whole mount immunolabeled for cytokeratin 8. The results shown are representative of nine experiments with 13-day-old kidneys, each with five kidney rudiments in each medium. The oligonucleotide sequences were as follows: anti-sense, 5′-AGTGGACTCGCTGCATAG-3′; sense, 5′-CTATGCAGCGAGTCCACT-3′; and non-sense, 5′-TCTTCTTCAAGCTTTGGC-3′. Bar represents 80 μm in (A) and (B); 120 μm in (C) and (D); and 400 μm in (E) and (F).

Even after 5 days in culture, no tubules were formed and only rudimentary branching of the ureter bud was seen. The corresponding sense and non-sense phosphothionate oligonucleotides did not affect NGFR expression, or growth and differentiation of the kidneys. Removal of the anti-sense oligonucleotides after 2 days in the cultures restored normal nephric differentiation (*19*). Thus, depletion of NGFR specifically perturbed epithelial differentiation of the nephrons. Although the ureter bud did not express NGFR (Fig. 1), its branching was inhibited by anti-sense oligonucleotides, but not by control oligonucleotides. Therefore, inhibition of NGFR expression in nephrogenic tissue also perturbs epithelio-mesenchymal tissue interactions that govern ureter bud branching.

The results show that NGFR expression is required for the formation of epithelial kidney tubules. NGFR is widely distributed in embryonic tissues derived from all germ layers (*7, 8*), so signal transduction by NGFR may be important in differentiation of a variety of organs, as well as of the nervous system.

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# Quantitation of Hepatic Glycogenolysis and Gluconeogenesis in Fasting Humans with $^{13}$C NMR


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**The rate of net hepatic glycogenolysis was assessed in humans by serially measuring hepatic glycogen concentration at 3- to 12-hour intervals during a 68-hour fast with $^{13}$C nuclear magnetic resonance spectroscopy. The net rate of gluconeogenesis was calculated by subtracting the rate of net hepatic glycogenolysis from the rate of glucose production in the whole body measured with tritiated glucose. Gluconeogenesis accounted for 64 ± 5% (mean ± standard error of the mean) of total glucose production during the first 22 hours of fasting. In the subsequent 14-hour and 18-hour periods of the fast, gluconeogenesis accounted for 82 ± 5% and 96 ± 1% of total glucose production, respectively. These data show that gluconeogenesis accounts for a substantial fraction of total glucose production even during the first 22 hours of a fast in humans.**


HEPATIC GLUCONEOGENESIS AND glycogenolysis are essential processes for maintaining plasma glucose during fasting. The relative contributions of these processes to glucose production have been difficult to quantify in humans. The rate of gluconeogenesis has been estimated by measuring the incorporation of isotopically labeled gluconeogenic precursors into plasma glucose or by measuring net splanchnic uptake of gluconeogenic substrates (*1*). Both methods rely on multiple assumptions of uncertain validity (*2*).

The rate of net hepatic glycogenolysis in humans during a 68-hour fast was measured by means of $^{13}$C nuclear magnetic resonance (NMR) spectroscopy. The advantage of $^{13}$C NMR spectroscopy over existing techniques is that hepatic glycogen concentration can be measured noninvasively at multiple time points (*3*). We calculated the rate of net hepatic glycogenolysis by multiplying the


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