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Polypeptide growth factors in metanephric growth and segmental nephron differentiation

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Abstract. Although the developing nephron expresses receptors for various polypeptide growth factors, the specific roles of such factors in renal organogenesis are unknown. Therefore, the effects of epidermal growth factor (EGF) (8.2×10^{-11} M–1.6×10^{-8} M), multiplication stimulating activity (MSA) (6.6×10^{-10} M–1.3×10^{-8} M) and transforming growth factor beta (TGF-β) (1×10^{-12} M–1×10^{-9} M) on organotypic renal growth and segmental nephron differentiation were studied in a serum-free hormone-supplemented, murine metanephric organ culture system. Following culture in control or growth-factor-supplemented medium, explant growth was assessed, and explant growth and differentiation were determined morphometrically in four defined nephron segments which were identified morphologically or immunohistologically with segment-specific antibodies and/or lectins: glomeruli, proximal tubules, thick ascending limb-early distal tubules, and collecting tubules. Results showed that EGF increased overall renal growth and specific differentiation of distal elements, but retarded differentiation of glomeruli and proximal tubules. EGF also induced hyperplastic cystic malformation in proximal tubules. MSA stimulated explant growth and promoted segmental differentiation of all tubular segments. TGF-β globally retarded in vitro nephrogenesis. Such data demonstrate that polypeptide growth factors have multiple and often disparate effects on overall renal growth in relation to differentiation of discrete nephron segments and provide insight into the factors which may regulate normal and abnormal renal embryogenesis.

Key words: Polypeptide growth factor – Epidermal growth factor – Metanephric growth – Transforming growth factor beta – Nephron differentiation – Organogenesis

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Introduction

Renal organogenesis is a complex process during which growth and differentiation of a variety of nephronic structures occurs within a specific patterned framework of three-dimensional organ formation. Although the factors that regulate metanephric growth and differentiation are largely unknown, recent data suggest that polypeptide growth factors, in concert with cell adhesion molecules, specific angiogenesis factors, and the extracellular matrix have an important role in renal organogenesis [1–3]. We therefore sought to determine the specific roles of epidermal growth factor (EGF), multiplication stimulating activity (MSA; or rat insulin-like growth factor II) and transforming growth factor beta (TGF-β) on renal epithelial growth and segmental nephron differentiation in our previously described murine metanephric organ culture system. In this model system, advanced organotypic tubular and glomerular epithelial differentiation proceed in the absence of vascularization, perfusion, or urine production [4–7]. The murine metanephric organ culture system thus experimentally separates the processes of three-dimensional organ growth and renal epithelial differentiation from filtration and flow-related phenomenon, endothelial or mesangial cell interactions, or the effects of uncharacterized growth factors or other substrates present in serum or urine. The current study demonstrates that EGF, MSA, and TGF-β have multiple and often disparate effects on overall renal growth in relation to differentiation of discrete nephron segments.

Materials and methods

Metanephric organ culture. The basic methodology of serum-free organ culture of embryonic mouse metanephros has been described in detail [4–7]. Intact metanephric tissue from Swiss Webster albino mice embryos (13 ± 0.4 days of gestation) was cultured in chemically defined medium for 120 h in a Trowell-type organ culture assembly at 36 ± 0.5° C and 95% humidity in a mixed air-5% CO_2 environment. Basal medium consisted of equal volumes of Dulbecco's modified Eagle's medium and buffered Ham's F-12 medium supplemented with

Table 1. Explant growth in metanephric culture[a]

	DNA (μg/explant)	Protein (μg/explant)	Viability (% trypan exclusion)
Control	5.10±0.18	53.33±2.02	96±2
Control + EGF (8.2×10^{-11} M)	5.24±0.31	55.47±1.86	96±3
+ EGF (8.2×10^{-10} M)	5.70±0.26*	58.66±1.53*	96±2
+ EGF (8.2×10^{-9} M)	5.13±0.12	51.66±2.08	91±4
+ EGF (1.6×10^{-8} M)	4.36±0.15*	48.33±2.52	87±5
Control + MSA (6.6×10^{-10} M)	5.61±0.34*	56.12±1.61	95±3
+ MSA (6.6×10^{-9} M)	5.83±0.30*	59.05±2.15*	90±4
+ MSA (1.3×10^{-8} M)	5.13±0.35	53.08±2.89	81±6*
Control + TGF-β (1×10^{-12} M)	4.97±0.45	49.62±2.74	95±3
+ TGF-β (1×10^{-11} M)	4.46±0.42*	45.41±2.19*	93±2
+ TGF-β (1×10^{-10} M)	4.16±0.38*	41.66±2.08*	90±1
+ TGF-β(1×10^{-9} M)	3.93±0.42*	40.33±3.79*	76±4*

[a] All values are mean ± SD
* P <0.05 vs control
EGF, Epidermal growth factor; MSA, multiplication stimulating activity; TGF-β, transforming growth factor beta

selenium (6.8×10^{-9} M), triiodothyronine (2×10^{-9} M), insulin (8.3×10^{-7} M), transferrin (6.2×10^{-8} M), and prostaglandin E_1 (7.1×10^{-8} M). Polypeptide growth factors, added individually to create the various treatment regimens of the current experiment, included EGF (8.2×10^{-11} M–1.6×10^{-8} M), MSA (6.6×10^{-10} M–1.3×10^{-8} M), and TGF-β (1×10^{-12} M–1×10^{-9} M).

In all experiments culture medium was replaced with fresh medium every 24 h. All basal culture medium was obtained from Flow Laboratories (McLean, Va.) and all hormones were obtained as lyophilized tissue culture reagents from Sigma (St. Louis, Mo.). MSA (source: buffalo rat liver cells) was obtained from Sigma, and EGF (source: mouse submaxillary gland) and TGF-β (source: human platelets) were obtained from Collaborative Research (Bedford, Mass.).

Determination of growth. Growth of metanephric explants was assessed for control and each growth factor treatment group following 120 h of incubation utilizing previously described micro-methods of explant DNA and protein content determination [4–8]. Explant viability was determined utilizing previously described methods of cellular trypan blue exclusion and neutral red uptake [4–7, 9]. Twelve explants from each treatment group were utilized for determination of DNA or protein content, and 6–8 explants for each treatment group were used for determination of viability.

Histology, immunohistology, and determination of segmental nephron growth and differentiation. At each day of incubation, control and growth-factor-treated explants were assessed histologically. Tissue was fixed in 2% formaldehyde-2.5% glutaraldehyde in phosphate buffer (pH 7.4) for 2 h at 4°C. Tissue was then washed, dehydrated through graded acetone, and infiltrated and embedded with Immunobed plastic embedding medium (Polysciences, Warrington, Pa.). Sections were cut at 3 μm on an ultramicrotome, mounted on glass slides, and stained with hematoxylin.

Morphometric analysis was combined with light microscopy and immunohistologic techniques to quantitate segmental nephron growth and differentiation in control and growth-factor-treated explants. The final concentrations of polypeptide growth factors utilized in these studies were EGF 8.2×10^{-10} M, MSA 6.6×10^{-9} M, and TGF-β 1×10^{-11} M. These concentrations were derived from organ culture dose/response studies which defined the optimal concentrations of each growth factor which maximized biological effect while minimizing epithelial toxicity ([10]; and Table 1). Segmental growth and differentiation were assessed quantitatively by determining the number of epithelial glomeruli, proximal tubules, thick ascending limb-early distal tubules, and collecting tubules formed under each set of treatment conditions. Glomeruli were identified histologically as previously described [6, 7], and the different tubular segments were identified immunohistologically by specific staining with segment-specific antibodies and lectins as previously described [11–14]. Proximal tubules were identified by staining with affinity-purified antibody to the brush border enzyme, gamma-glutamyl transpeptidase, and the lectin *Lotus tetragonolobus*. Thick ascending limb-early distal tubular segments were identified by staining with affinity-purified antibody to Tamm-Horsfall glycoprotein, and collecting tubules were identified by staining with the lectin *Dolichos biflorus*. The number of epithelial glomeruli or specific immunostained segments per explant was determined by established light microscopic morphometric techniques using a standard test grid as previously described [6, 7, 15]. More specifically, intact explants were serially sectioned at 3 μm, and specific structures counted in sections at 15 μm intervals utilizing a micrometer grid. A total of 6–8 explants for each treatment group was utilized for quantitation of epithelial glomeruli and each segment, with 10–12 histologic or immunohistologic sections assessed per explant.

Antibodies to gamma-glutamyl transpeptidase and Tamm-Horsfall glycoprotein were provided by Drs. N. P. Curthoys (Colorado State University) and John R. Hoyer (University of Pennsylvania School of Medicine), respectively, and prepared by their previously described methods [16, 17]. Lectins were obtained from Sigma, and all other antisera were DAKO immunochemicals (Accurate Chemical & Scientific Corp., Westbury, NY). The specific immunostaining procedure utilized was our previously described post-embedding technique specifically developed for immunolocalization of basement membrane and brush border antigens in plastic sections of fetal murine tissue [12–14, 16].

Statistical methods. Determinations of protein content, DNA content, and viability per explant generated parametric data for each treatment group. Therefore, group data were expressed as mean ± standard deviation, and significance of differences of the means between treatment groups determined by Student's t-test. However, the data from morphometric determination of epithelial glomeruli and specific segment formation generated a non-parametric distribution. Therefore, these data were expressed as mean value with ranges, and the significance of differences between treatment groups determined by the Wilcoxon rank sum test [18]. Following statistical analysis, growth and differentiation data generated from each treatment group were expressed as a percentage of the control to facilitate graphic presentation.

Results

Growth

The effects of EGF, MSA, and TGF-β on explant growth are summarized in Table 1. Treatment of explants with either EGF (8.2×10^{-10} M) or MSA (6.6×10^{-9} M) produced a statistically significant increase in explant DNA and protein content when compared to controls following 120 h of incubation. In contrast, treatment with TGF-β (1×10^{-11} M) caused a statistically significant decrease in DNA and protein content relative to controls. At these concentrations, none of the growth factors tested had any significant effect on cellular viability of explants.

Differentiation

The effects of EGF (8.2×10^{-10} M), MSA (6.6×10^{-9} M), and TGF-β (1×10^{-11} M) on explant differentiation are summarized in Fig. 1 A–E and Fig. 2. At the time of explantation into organ culture (13±0.4 days of gestation)

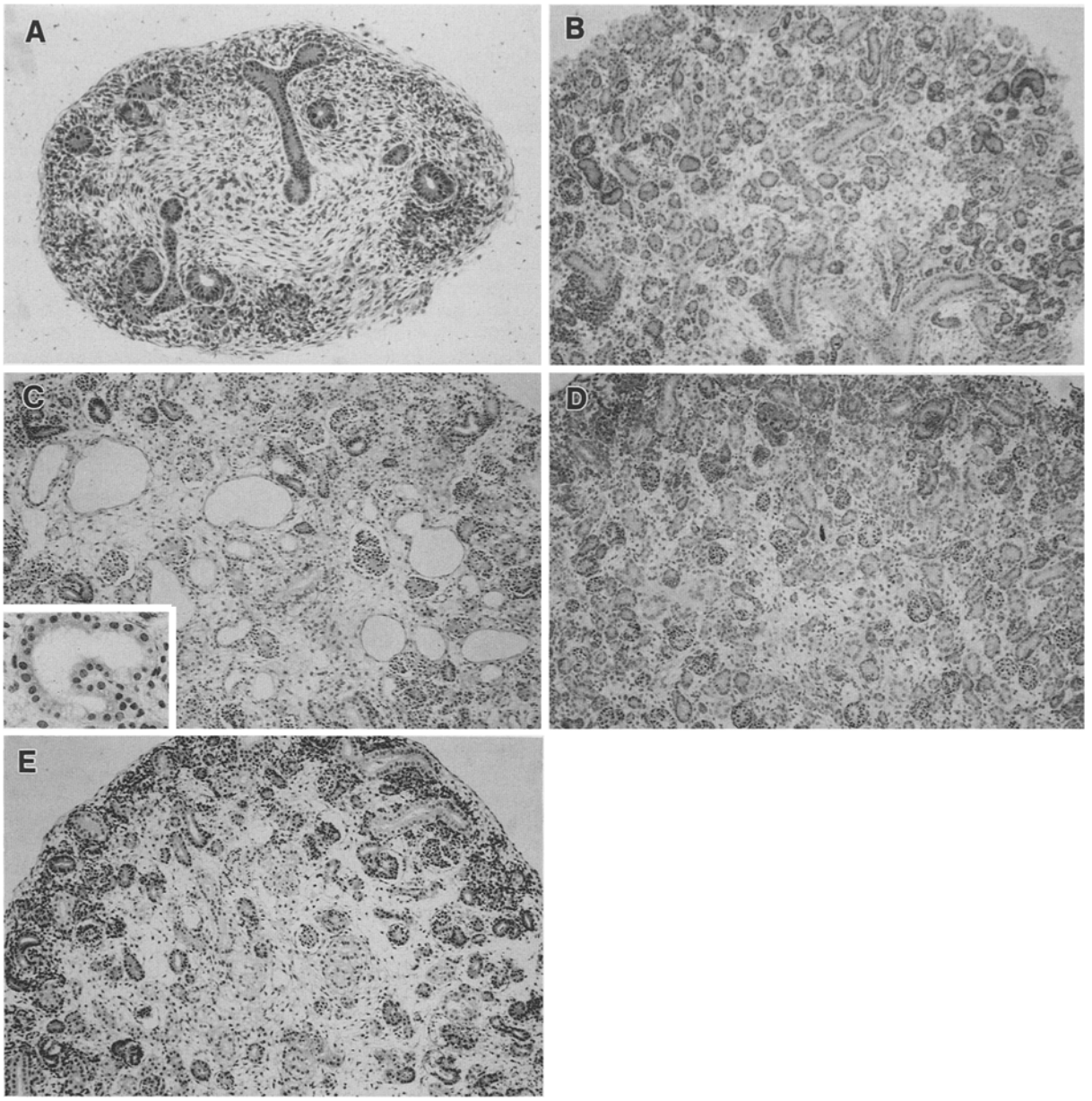

Fig. 1 A–E. Morphology of explant growth in metanephric organ culture. **A** Pre-culture murine metanephric explant at 13 ± 0.4 days gestation. Cellular aggregates of metanephric blastema and immature tubular structures surround distal ramifications of the ureteric bud. **B** Metanephric explant following 120 h of culture under control conditions. Noted is the reniform organization with active tubulogenesis and glomerulogenesis surrounding branches of the ureteric bud. **C** Metanephric explant following 120 h of culture with epidermal growth factor (EGF) (8.2×10^{-10} M). Numerous cystic lesions, localized to proximal tubules by immunostaining with anti-gammaglutamyl transpeptidase (not shown, see text), are seen throughout the explant against a background of normal in vitro nephrogenesis. *Inset:* Tubular cyst demonstrates epithelial hyperplasia and micropolyp formation. **D** Metanephric explant following 120 h of culture with multiplication stimulating activity (MSA) (6.6×10^{-9} M). Overall nephrogenesis is similar to control, but there is an increased density of tubular structures surrounding branches of the ureteric bud. By immunohistology, increase in all tubular populations studied is noted. **E** Metanephric explant following 120 h of culture with transforming growth factor beta (TGF-β) (1×10^{-11} M). Noted is the decrease in overall glomerulogenesis and tubulogenesis as compared with control. Hematoxylin; original magnifications: **A** $\times 50$; **B–E** $\times 25$; **C** (inset) = $\times 198$

embryonic murine metanephros was at an early stage of tubular development. Explants consisted of dense aggregates of metanephric blastema and primitive tubular structures surrounding the actively inducing ampullae of distal ramifications of the ureteric bud (Fig. 1A). No tubular structures beyond early S-shaped tubules were present, and no pre-glomerular differentiation of the lower limb of S-shaped tubules was noted. Following 120 h of culture, explants of all treatment groups demonstrated some degree of epithelial glomerular and segment specific tubular cytodifferentiation (Figs. 1B–E, 2). As previously described, control explants demonstrated advanced glomerular visceral epithelial and proximal tubular differentiation following 120 h of culture ([4–7]; Fig. 1B). EGF treatment of explants resulted in statistically significant increased growth and differentiation of thick ascending limb-early

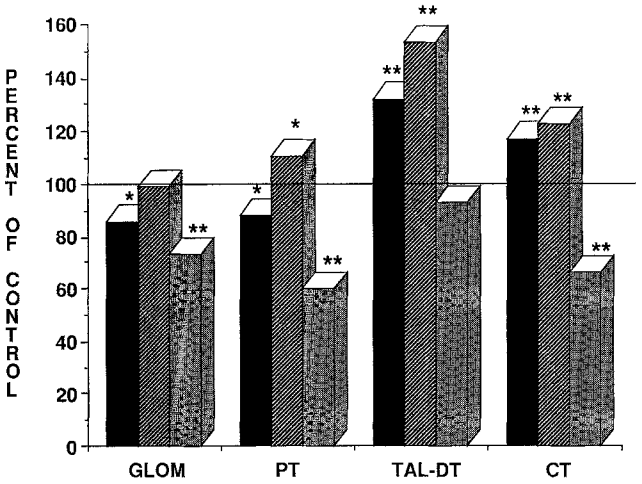

Fig. 2. Segmental nephron growth and differentiation in metanephric organ culture. The effects of EGF (■) (8.2×10^{-10} M), MSA (▨) (6.6×10^{-9} M), TGF-β (▒) (1×10^{-11} M) on growth and differentiation of glomeruli (*GLOM*), proximal tubules (*PT*), thick ascending limb-early distal tubules (*TAL-DT*), and collecting tubules (*CT*) are displayed in relation to control metanephric explant differentiation. Data used to construct histogram were mean number of glomeruli (assessed histologically) or tubular segments (assessed immunohistologically) demonstrated in serial 3-μm tissue sections (see Materials and methods) as follows:

	Mean (range) per section			
	GLOM	PT	TAL-DT	CT
Control	28 (24–33)	58 (47–73)	11 (6–15)	38 (28–47)
EGF	23* (19–26)	49* (39–61)	15** (11–23)	45** (38–49)
MSA	27 (21–36)	68* (58–79)	17** (13–23)	47** (40–54)
TGF-β	20** (16–24)	35** (29–41)	10 (7–15)	(18–33)

In both the graph and numerical data: * $P < 0.05$ vs control; ** $P < 0.01$ vs control

distal tubule and collecting tubule segments, but statistically significant decreases in differentiation of epithelial glomeruli and proximal tubules (Fig. 2). In addition EGF produced striking morphologic abnormalities in developing metanephric explants (Fig. 1C). EGF-treated explants developed cystic lesions which were specifically localized to proximal tubules immunohistologically by exclusive staining with anti-gamma-glutamyl transpeptidase and which were lined with a hyperplastic tubular epithelium which in some areas formed intraluminal polyps (Fig. 1C, insert). EGF induced proximal tubular cysts amid a background of normal organ culture differentiation without evidence of tubular damage or epithelial cytotoxicity.

MSA also had variable effects on nephron segmental differentiation in vitro (Figs. 1D, 2). While having no effect on glomerular differentiation, MSA promoted statistically significant differentiation of all tubular segments. This was most pronounced in thick ascending limb-early distal tubules and collecting tubules and less pronounced in proximal tubules. MSA treatment produced no morphologic abnormalities in explants treated for 120 h (Fig. 1D). Similarly, TGF-β produced no morphologic abnormalities in developing explants (Fig. 1E). However, TGF-β treatment resulted in a statistically significant decrease in segmental nephron growth and differentiation of glomeruli, proximal tubules, and collecting tubules without affecting thick ascending limb-early distal tubular differentiation (Fig. 2).

Discussion

The current study defines the differential effects of EGF, MSA, and TGF-β on metanephric growth and development in murine serum-free, hormonally supplemented organ culture. In addition to establishing optimal culture conditions for renal organogenesis in the system, the results provide insight into the control of normal and abnormal nephron growth and segmental differentiation by specific growth factors. A limitation of the methodology utilized to quantitate segmental nephron growth and differentiation in the current study is that it does not distinguish between increased numbers of differentiated structures (i.e., new nephron formation or acquisition of lectin specificity) and increased growth of segments which are already differentiated (i.e., increased tubular length in a lectin-positive segment). Because of this limitation, absolute numbers of specific structures per kidney cannot be derived from the presented data, and quantitative data from each treatment group are meaningful only in relation to simultaneously cultured controls (Fig. 2).

The intact organ metanephric culture system is particularly suited for the study of renal embryogenesis in that it permits morphological as well as biochemical assessment of the effects of experimental manipulation on renal development [7, 12, 14, 16, 19, 20]. In the controlled experimental organ culture environment, metanephric explants undergo growth and advanced organotypic differentiation in which the developing metanephric blastema maintains its normal three-dimensional relationship to inducing ureteric bud. Explants provide their own extracellular matrix in culture [21], and thus study of growth and differentiation factors are not complicated by additional requirements for cell attachment factors or variable matrix substrates [22, 23]. Further, the maintenance of normal spatial relationships between epithelial anlagen during experimental nephrogenesis permits organotypic intercellular contact, adhesion, and movement. Such a system minimizes disruption of cellular morphogenetic interactions which may be critical during three-dimensional organ growth and development [1, 2, 24, 25].

In metanephric culture, EGF increased overall renal growth and specific differentiation of distal nephron segments, but decreased growth and differentiation of glomeruli and proximal tubules (Table 1, Fig. 2). Although EGF has been demonstrated to affect the development of many organs [26], and EGF production has been localized to distal nephron segments [27, 28], this is the first demonstration of significant EGF-induced effects on embryonic renal growth and nephron differentiation. The current data

support the earlier suggestion that EGF may stimulate undifferentiated renal mesenchyme [3] and are consistent with a recent report that EGF treatment of fetal human kidneys in vitro stimulates overall DNA and protein synthesis without promoting maturation of proximal tubular enzymatic systems [29]. The differential effect of EGF on the growth and differentiation of thick ascending limb-early distal tubules when compared to proximal tubular and glomerular differentiation is of interest given the heterogeneous distribution of EGF receptors along the developing nephron [30]. These findings suggest that EGF receptor localization and activity may be developmentally regulated in the kidney, as has been suggested in other organ systems.

In addition to its overall effects on renal growth and specific segmental nephron differentiation, EGF also induced hyperplastic renal cyst formation in developing proximal tubules (Fig. 1 C). EGF has previously been reported to be highly mitogenic for proximal tubular cells in primary culture [31] and the polyploid cystic tubular lesions produced by EGF are similar to those seen in a variety of human cystic diseases [32]. Such findings, in concert with recent preliminary observations that EGF is present in high concentrations in renal cyst fluid from a number of human and experimental renal cystic diseases [33], raise the possibility that EGF or EGF-like substances may have a role in segmental tubular cystic malformation.

In metanephric organ culture, MSA promoted renal embryonic growth and differentiation of all tubular segments studied (Table 1, Fig. 2). Levels of MSA (or its human counterpart IGF-II) are high during early gestation and rapidly decline during late gestation and following birth [34, 35]. This, in addition to evidence that IGF-II is developmentally regulated in different organs, has led to the suggestion that this polypeptide growth factor is important in normal fetal embryogenesis [35, 36]. Although IGF-IImRNA has been localized to fetal undifferentiated renal mesenchyme and metanephric blastema [34], the current study is the first to document a potential role for this insulin-like growth factor in mammalian renal growth and segmental tubular differentiation. The relationship of the current findings to data documenting high fetal levels of IGF-II gene expression in Wilms' tumors [37, 38] is unclear, but suggests that aberrant regulation of normal fetal renal growth factors may have pathogenic import in certain renal tumors.

TGF-β retarded overall growth and segmental differentiation of all segments except thick ascending limb-early distal tubules in metanephric organ culture (Table 1, Fig. 2). These inhibitory growth effects are similar to those seen following TGF-β treatment of proximal tubular epithelial cells in primary culture [39]. Although mitogenic for certain cell types, TGF-β is a potent inhibitor of the proliferation of many cells in vitro, particularly epithelial cells [40]. Since TGF-β gene expression has been demonstrated developmentally in the mouse fetal kidney [41], it is possible that it functions physiologically as a negative autocrine or paracrine growth factor during nephrogenesis. It is also possible that TGF-β, in the presence of EGF or other growth factors, may promote renal growth or differentiation, as has been reported in other systems [42]. However, in preliminary studies, we have been unable to demonstrate any effect of TGF-β on either EGF- or MSA-induced stimulation of growth and segmental nephron differentiation in metanephric organ culture. Finally, TGF-β may have an important role in renal embryogenesis through biological effects which are unrelated to proliferation or segmental nephron differentiation, since it enhances the formation and inhibits the degradation of extracellular matrix [42].

We conclude that EGF, MSA, and TGF-β have multiple and often disparate effects on overall renal growth in relation to differentiation of discrete nephron segments in the developing mammalian kidney. Such factors may have important roles in normal and abnormal renal organogenesis.

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