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TGFβ2, LIF and FGF2 cooperate to induce nephrogenesis

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SUMMARY

The metanephric kidney develops from interactions between the epithelial ureteric bud and adjacent metanephric mesenchyme, which is induced by the bud to form the epithelia of the nephron. We have found that leukemia inhibitory factor (LIF) and transforming growth factor β2 (TGFβ2) are secreted by inductive rat bud cells and cooperate to enhance and accelerate renal tubule formation in uninduced rat metanephric mesenchymal explants. LIF alone or TGFβ2 with fibroblast growth factor 2 induced numerous tubules in isolated mesenchymes over an 8 day period, while (in combination) all three caused abundant tubule formation in 72 hours. Furthermore, neutralization of Wnt ligands with antagonist-secreted Frizzled-related protein 1 abrogated these responses and combinatorial cytokine/growth factor stimulation of explants augmented nuclear activation of Tcf1/Lef1, suggesting that LIF and TGFβ2/FGF2 cooperate to regulate nephrogenesis through a common Wnt-dependent mechanism.

Key words: Induction, Kidney, Tubulogenesis, Wnt signaling, Stat, TGFβ2, LIF, FGF2, Rat

INTRODUCTION

From the classical studies of biologists such as Lewis, Spemann and Mangold, it has long been recognized that tissue differentiation is mediated by factors secreted from one cell population that cause commitment of another population to a specific cell fate – the so-called process of induction. Despite its acknowledged importance, there remains a void in our understanding of the complete nature and complement of paracrine factors responsible for inductive signaling in any given tissue. The metanephric kidney is no different in this regard. An inductive process that directs development of the epithelia in the nephron from the intermediate mesoderm of the nephrogenic cord, i.e. the metanephric mesenchyme (MM), has been demonstrated both in vivo (Gruenwald, 1943) and with heterotypic tissue inductors in vitro (Grobstein, 1953). These studies implicated the ureteric bud, an outgrowth of the mesonephric duct, as the source of signaling and suggested the existence of a secreted soluble factor(s) as inductor of nephron formation. Evidence of such a soluble activity was provided using tissue homogenates (Perantoni et al., 1991a; Perantoni et al., 1991b), and fibroblast growth factor 2 (FGF2) was subsequently purified and shown to mediate the early inductive events in metanephric development, i.e. condensation or compaction of MM (Perantoni et al., 1995). More recently, this factor was also characterized as a weak inducer of the signature event of nephrogenesis, namely, the epithelial conversion of MM (Karavanov et al., 1998).

Epithelial conversion and tubule development in MM typically occur within 72 hours of exposure to inductive signaling in the metanephros in situ and in tissue recombinations in vitro. However, the response of explanted MM to FGF2 is delayed (10-14 days) and incomplete (primitive tubules lacking glomeruli), suggesting the involvement of additional factors, and efforts to implicate numerous cytokines by trial and error have been unsuccessful (Weller et al., 1991). As an alternative approach in delineating potential complementary metanephric morphogens, cell lines that secrete inductive activity for MM were derived from rat ureteric buds (Perantoni et al., 1985; Karavanova et al., 1996; Barasch et al., 1997). Using this system, leukemia inhibitory factor (LIF) was purified and found, in combination with FGF2, to cause epithelial conversion and tubule formation in MM with 7 day treatments (Barasch et al., 1999). As morphogenesis is delayed in explants even with LIF/FGF2 cooperation, other factors must regulate this process. We report that transforming growth factor β2 (TGFβ2) is secreted by inductive rat ureteric bud cells along with LIF, that TGFβ2 can induce the epithelialization of MM independently of exogenous LIF, and that TGFβ family members function synergistically with LIF and FGF2 to cause tubulogenesis in 72 hours, as in vivo.

MATERIALS AND METHODS

Tissues and culture conditions

The ureteric bud and surrounding metanephric mesenchyme were isolated and/or cultured as previously described (Karavanova et al., 1996). Briefly, metanephric rudiments were surgically removed from

13 dpc rat embryos and trypsinized for separation. MMs were placed on polycarbonate filters (0.1 μm pore size) coated with Type IV collagen. Filters were floated on a basal medium (Karavanova et al., 1996), which always included transforming growth factor α (TGFα; 10 ng/ml), as described. TGFα is incapable of supporting survival or growth of MM cells. Cultures were re-fed every 2-3 days.

Serum-free conditioned medium from RUB1 cells was collected every 2-3 days to amass a total volume of approximately 3 l, centrifuged to remove cellular debris, concentrated sevenfold with a Filtron mini-ultrasette (8 kDa cut-off), and further reduced in a Filtron 10 kDa Macrosep unit to a final 50× concentration. The RUB1 cell line was derived from 14 dpc rat ureteric bud cells and has previously been demonstrated to release inductive factors for MM into a defined culture medium (Karavanova et al., 1996).

Secreted frizzled-related protein 1 (sFRP1) was purified from cell culture medium conditioned by MDCK cells transfected with an sFRP1 expression vector as previously described (Finch et al., 1997).

Factor purification

Concentrated conditioned medium was adjusted to pH 4.5 with acetic acid, and acid-soluble proteins fractionated either on an anion-exchange HPLC column (Waters Protein-Pak DEAE 15HR) with a linear gradient of 0.5 M NaCl in 75 mM Tris, pH 9.3, for LIF purification or on a cation-exchange HPLC column (Waters Protein-Pak CM 8HR) with 0.5 M NaCl in 50 mM acetate, pH 5.0 for TGFβ2 purification. Biologically active fractions were concentrated by microfiltration using a 10 kDa Filtron Macrosep unit, dialyzed with 0.1% trifluoroacetic acid/10% acetonitrile, and eluted with a linear gradient of 70% acetonitrile (0.4 ml/minute). Fractions were lyophilized and reconstituted with 50 mM acetate, pH 5.0 (70 μl). Fractions (10 μl) were tested for biological activity by incubating two freshly isolated 13 dpc MMs on Type IV collagen-coated filters that were floated on basal medium (1.5 ml) with TGFα (10 ng/ml).

RT-PCR conditions

RNAs were purified using a Qiagen RNeasy kit according to manufacturer's instructions. RT-PCR was performed with Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech) also according to the manufacturer. Reaction mixtures containing 20 ng DNase-treated total RNA were run for 35 PCR cycles (30 seconds at 94°C, 30 seconds at 50-60°C (depending upon primer optimal annealing temperatures) and 30 seconds at 72°C).

Primers were as follows: LIF (438 bp), 5′-ACGGCAACCTCATG-AACCAGATCAAGAGTC-3′ and 5′-AGCTGGCAGCCCAACTTC-TTCCTTTGG-3′; TGFβ1 (442 bp), 5′-ATCGACATGGAGCTGG-TGAA-3′ and 5′-GGTAACGCCAGGAATTGTTG-3′; TGFβ2 (250 bp short form and 335 bp long form), 5′-CAGATCCTGAGCAA-GCTGAA-3′ and 5′-GTAGGGTCTGTAGAAAGTGG-3′; TGFβ3 (305 bp), 5′-TTCGACATGATCCAGGGGCT-3′ and 5′-CGCACAG-TGTCAGTGACATC-3′; activin β_A (573 bp), 5′-CTTTGCCGAGT-CAGGCACAG-3′ and 5′-ACCTTGCCATCACACTCCAA-3′; activin β_B (215 bp), 5′-CAACATCACGCACGCTGTCC-3′ and 5′-GAGA-CGAAGAAGTACAGGCG-3′; and Gdf11 (200 bp), 5′-TTCGCCA-GCCACAAAGCAAC-3′ and 5′-GGCAGCAGCGGGACTCACTC-3′. No-RT controls were always included to rule out possible DNA contamination. All RT products were sequenced to confirm identity.

Tissue embedding and In situ hybridization

Tissue explants and resected rat metanephroi (16 and 19 dpc) were fixed and processed as previously described (Karavanova et al., 1996). For explants, tissues were paraformaldehyde-fixed (4% in PBS) directly on their filters and embedded perpendicular to the block's cutting surface. All explants were serially sectioned (50 slides/block). Every fifth section was stained by Hematoxylin and Eosin. In situ hybridization analyses were performed using [^{35}S]-labeled riboprobes (Wilkinson and Green, 1990).

Immunoblotting and immunohistochemistry

Aliquots of dialyzed conditioned media (50 μg protein) were diluted with 2× Tricine sample buffer with or without dithiothreitol. Proteins were resolved in 10-20% gradient Tricine gels (Novex), fixed in transfer buffer and transferred to Immobilon-P filters at 15 volts overnight at 4°C. Filters were blocked with 5% powdered milk in Tween 20/Tris-buffered saline (TTBS) for 1 hour, incubated with primary antibodies (1:1000 dilution) in TTBS overnight and washed three times in TTBS for 15 minutes each. Filters were then incubated with species-appropriate HRP-labeled secondary antibody (1:10,000) for 1 hour and washed three times with TTBS. Proteins were visualized using an ECL Western Blotting System (Amersham Pharmacia Biotech) and X-Omat AR film (Kodak) according to manufacturer's instructions.

Paraformaldehyde-fixed/paraffin-embedded tissues were de-paraffinized, heated in sodium citrate for antigen exposure and incubated with a dilution series of primary antibodies. Antigen was visualized with a Vectastain ABC kit (Vector Labs) according to manufacturer's instructions. For whole-mount preparations, explants were fixed in 100% methanol (−20°C) for 10 minutes, followed by 50% methanol (4°C) in PBS for 5 minutes, and then stored in PBS. For immunohistochemistry, tissues were incubated for 5 hours in 10% normal sheep serum in Tris-buffered saline (TBS, pH 7.4) at room temperature and then with primary antibody (1:100) in 1% sheep serum in TBS overnight at 4°C. Tissues were then washed twice with 1% sheep serum in TBS over an 8 hour period and incubated with a FITC-labeled species-appropriate secondary antibody (1:100) at 4°C overnight. Tissues were finally washed in TBS twice for 1 hour each at room temperature and mounted in Prolong Antifade (Molecular Probes) for visualization.

Nuclear extract preparation

Each nuclear extract was prepared as described previously (Dignam et al., 1983) from 36 explanted MMs. Filters with explants were combined and vortexed in 1 ml of ice-cold PBS. Cells were centrifuged (3000 rpm), resuspended in 300 μl of cold buffer A (10 mM Hepes, pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT) with protease inhibitors Complete™ (Boehringer Mannheim)) and incubated on ice for 10 minutes. Nuclei were collected by centrifugation (1 minute/7000 rpm at 4°C), resuspended in 50 μl of cold buffer B (20 mM Hepes, pH 7.4, 1.5 mM $MgCl_2$, 400 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 20% glycerol with protease inhibitors Complete™), incubated on ice for 30 minutes and centrifuged (30 minutes/14,000 rpm). Protein concentrations were determined using the BioRad Protein Assay Dye Reagent. Extracts were stored at −70°C.

Electrophoretic mobility shift assay (EMSA)

DNA-binding reactions were performed for 30 minutes at room temperature in a volume of 25 μl, containing 0.5 μg of nuclear protein extract in 5 μl of buffer B, 7.5 μg of acetylated bovine serum albumin (Promega), 0.5 μg of sonicated salmon sperm DNA (Stratagene) and 0.5 ng of [^{32}P]-labeled probe (1×10^5 cpm) with or without an 80-fold excess of unlabeled competitor DNA. Stat3 consensus and mutant oligonucleotides used as labeled probes or unlabeled competitors were from Santa-Cruz Biotechnology. For optimal Tcf1/Lef1-binding sites, we used double-stranded oligonucleotides (Korinek et al., 1997) that were purified in 10% polyacrylamide gels. In supershift EMSA, nuclear extracts were preincubated with 1-2 μg of antibody for 30 minutes at room temperature prior to addition of labeled probe. To obtain a supershift with anti-β-catenin antibody, EMSA conditions as previously described (Korinek et al., 1997) were used. DNA-binding reaction mixes were supplemented with 5 μl of glycerol (Sigma) and separated at 4°C in 4% native Tris-glycine polyacrylamide gels (1.5 hours, 10 V/cm), which were dried and exposed to Kodak X-Omat AR film.

RESULTS

Purification of inductive factors from medium conditioned by a cell line of rat ureteric bud (RUB1)

Two separate fractions that cooperate with FGF2 to induce tubule formation in isolated MMs were originally identified by reversed-phase HPLC (RP-HPLC) with a C8 column from serum-free RUB1 cell-conditioned medium. For individual characterization of these two factors, conditioned medium was ultrafiltered and acidified (pH 5.0). Soluble proteins were resolved by anion-exchange (Waters Protein-Pak DEAE 15 HR column), eluting a condensation-inducing activity for isolated 13-dpc MMs with 65 mM NaCl and 50 mM Tris, pH 9.3. Active fractions were pooled, concentrated in Filtron Macrosep microfiltration units and dialyzed with 10% acetonitrile in 0.1% trifluoroacetic acid. This material was injected onto a Delta-Pak 5 μm C18 column to improve the chromatographic resolution of proteins beyond that of the C8 column. Fractions (1 ml/minute flowrate) eluting at 78-82 minutes (50% acetonitrile) yielded a single, somewhat diffuse 45 kDa band of biologically active protein (Fig. 1A). These fractions contained growth-promoting and condensing activities for uninduced 13 dpc rat MMs in serum-free medium. (In the absence of inductive signaling, MMs rapidly become apoptotic (Koseki, 1993).) Other fractions showed no independent biological activity. N-terminal amino acid sequencing of protein in an active fraction yielded a peptide fragment SPLPITXVNA, which conformed to LIF, a member of the gp130 receptor-binding family that includes oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and interleukin 6 (IL6). LIF interacts with a preferred gp190 low-affinity receptor (LIFR), which associates with gp130 to activate a JAK/Stat signaling pathway (Heim, 1999). By this mechanism, LIF promotes cell survival and differentiation in other tissues (Koblar et al., 1998).

Previously LIF was shown to induce tubule formation in combination with weak inductor FGF2 (Barasch et al., 1999). In the current studies, recombinant LIF protein with TGFα caused morphogenesis independent of FGF2 treatment and at substantially lower concentrations (20 ng/ml versus 100 ng/ml) in serum-free explant cultures. LIF-treated MMs enlarged, condensed, and developed numerous epithelial tubules and avascular glomeruli by 8 days in culture (Fig. 1B,C). Membrane-localized immunohistochemical staining (Fig. 1C) for E-cadherin further established the epithelial nature of these structures (Vestweber et al., 1985). LIF concentrations ranging from 1-100 ng/ml were effective, and at 20 ng/ml, tubulogenesis was consistent and extensive (24/24 explants after 8 days in three independent experiments). Higher concentrations did not accelerate or inhibit the appearance of tubules. OSM and CNTF could effectively replace LIF in inducing tubules at equimolar concentrations. OSM or CNTF did not cooperate with LIF to accelerate tubule formation; however, LIF treatment with FGF2 (50 ng/ml) did induce abundant tubules in some cultures (two out of eight) by 6 days (data not shown).

As multiple gp130-binding cytokines may be secreted by bud cells, we have evaluated the effect of neutralizing antibody for LIF on differentiation of MMs induced by RUB1-conditioned medium in a previously characterized model (Karavanova et al., 1996). With non-immune goat IgG (10 μg/ml), explants formed the typical widespread tubular network with conditioned medium after 72 hours (Fig. 1D) and

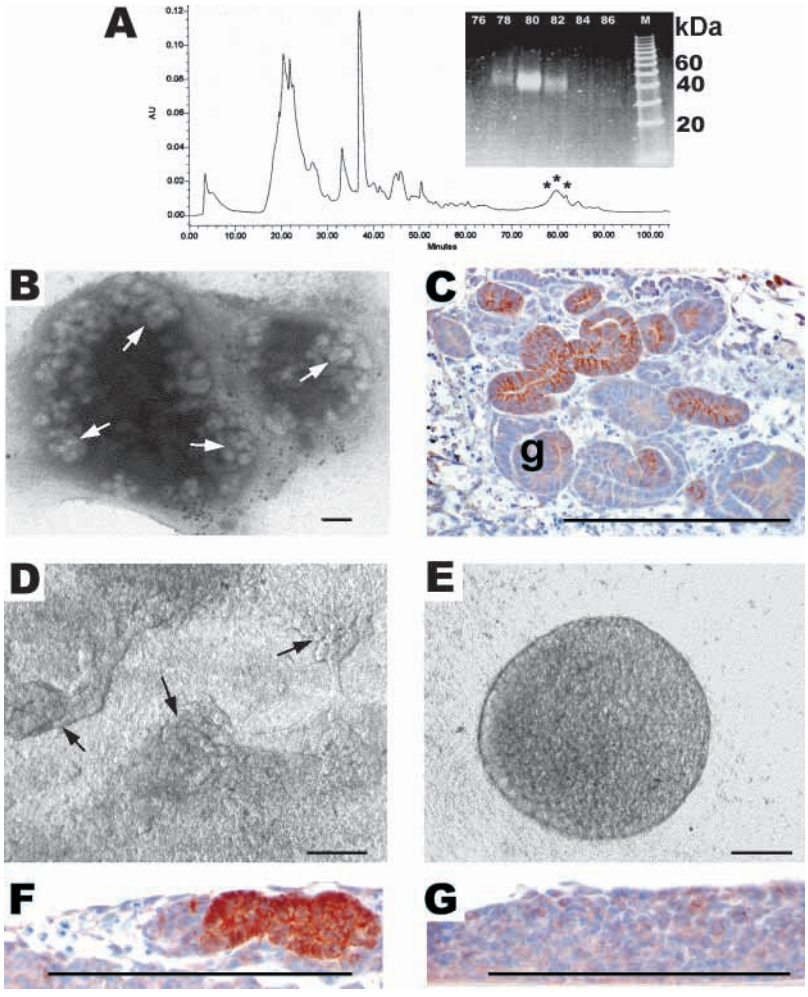

Fig. 1. Purification and characterization of leukemia inhibitory factor from RUB1-conditioned medium. (A) Reversed-phase HPLC separation of biologically active protein. Inset shows Sypro orange stained purified protein at 45 kD. (B) Induction of tubule formation (arrows) in 13 dpc rat MM after 8 days with recombinant LIF (20 ng/ml) plus TGFα (10 ng/ml). (C) Section of LIF-induced tubulogenesis in MMs, showing membrane-associated E-cadherin immunostaining and glomerular-like structure (g). (D,F) MM explants treated with RUB1-conditioned medium (7.5 μl), FGF2 (50ng/ml) and non-immune IgG (10 μg/ml) form tubular networks (arrows). (E,G) MM explants treated with conditioned medium (7.5 μl), FGF2 (50 ng/ml), and anti-LIF neutralizing antibody (10 μg/ml) remain condensed. Tubule formation is demonstrated by membrane localization of E-cadherin in control cultures and is not observed in cultures treated with LIF-neutralizing IgG. Scale bars: 0.10 mm.

cross-sections of these cultures reveal epithelial development as demonstrated by membrane localization of E-cadherin in a tubular structure (Fig. 1F). In the presence of LIF-neutralizing IgGs (10 μg/ml) over the same period (Fig. 1E), explants remained blastemal in appearance and failed to form epithelia, as indicated by the absence of membrane-associated E-cadherin (Fig. 1G). As this antibody does not crossreact with OSM, CNTF, IL6 or colony stimulating factor in ELISAs, LIF is believed to be the predominant gp130-binding cytokine in our conditioned medium.

TGFβ family members also mediate tubule formation

The activity in the second RP-HPLC fraction from the C8 column was purified on the basis of its ability to complement LIF and FGF2 in inducing tubulogenesis over a 72-hour period. Soluble proteins from RUB1 cell-conditioned medium, which was titrated to pH 5.0 with acetic acid, were resolved by cation-exchange (Waters Protein-Pak CM 8 HR column with 0.19-0.24 M NaCl and 50 mM acetate, pH 5.0) and RP-HPLC with a C18 column. Proteins eluting at 70-76 minutes (35% acetonitrile) cooperated with LIF and FGF2 to accelerate tubulogenesis in 13 dpc MMs (Fig. 2A). In silver-stained gels, proteins were resolved at 12-14 kDa under reducing conditions, which corresponds with the monomeric form of several TGFβ family members. As TGFβ2 null homozygotes manifest renal abnormalities (Sanford et al., 1997), this family member was tested first. Western analysis revealed two immunoreactive bands at 12-14 kDa with a rabbit polyclonal antibody specific for TGFβ2 (Fig. 2A inset) but not with antibodies for TGFβ1 or TGFβ3 (not shown).

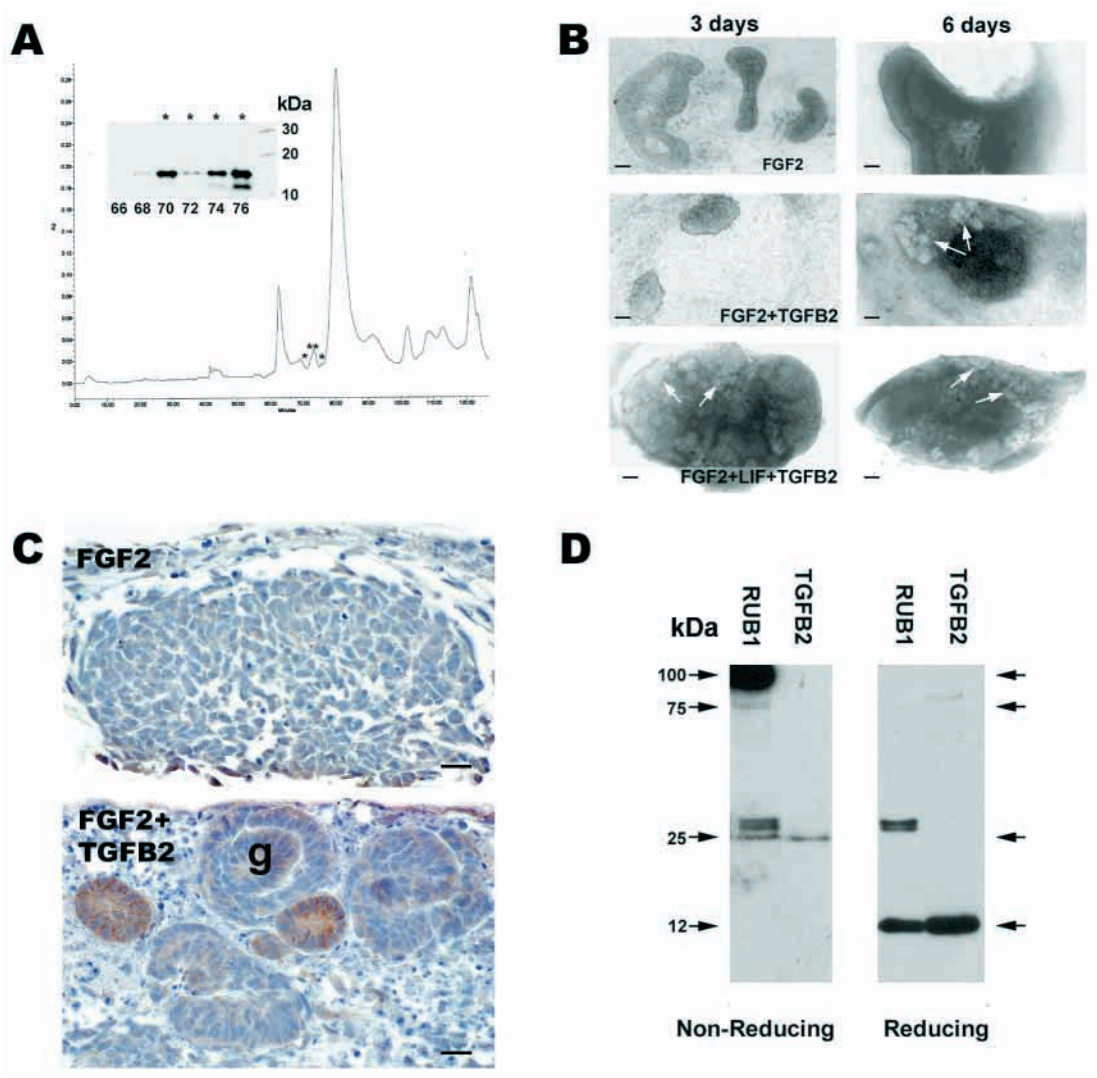

Fig. 2. Purification and characterization of transforming growth factor-β2 from RUB1 conditioned medium. (A) Reversed-phase HPLC purification of biologically active protein of 12-14 kDa (inset), which reacts with antibody specific for TGFβ2. (B) Explants of MM treated with recombinant cytokines/growth factors (FGF2, 50 ng/ml; TGFβ2, 0.67 ng/ml; LIF, 20 ng/ml; TGFα, 10 ng/ml). FGF2-treated explants have no tubules, while TGFβ2/FGF2 and LIF/TGFβ2/FGF2 induced tubules (arrows) at 6 days and 3 days, respectively. Scale bars: 0.10 mm. (C) Section of FGF2-treated (top) or TGFβ2- and FGF2-treated (bottom) explant immunostained for E-cadherin. Membrane-stained epithelial structures and glomerular-like elements (g) arise in tissues treated with TGFβ2. Scale bars: 0.01 mm. (D) Immunoblot for active TGFβ2 in conditioned medium from RUB1 cells. Blot reveals 25 kDa (biologically active) and 100 kDa (inactive) bands under non-reducing conditions and a 12 kDa band under reducing conditions. Lane with TGFB2 contains 5 ng recombinant TGFβ2.

To verify the activity of TGFβ2, recombinant protein (0.01-100 ng/ml) was applied to isolated MMs with LIF, FGF2 and TGFα. Epithelial tubules could be induced in 72 hours with the addition of TGFβ2 concentrations of 0.05-1 ng/ml (Fig. 2B), but above 10 ng/ml, it completely blocked tubulogenesis. TGFβ2 by itself could not support the survival of MM. With the addition of FGF2, it caused tubulogenesis in a few cultures by 6 days (Fig. 2B) but induced tubules in most explants (23/24 from three independent experiments) by 8 days, which is similar to the response of explanted MM to LIF. Treated explants exhibited both avascular glomeruli and typical primitive comma- and S-shaped epithelial structures (Fig. 2C). Immunhistochemical staining for E-cadherin revealed strong expression at the epithelial cell membranes in tubules and weak expression in glomerular-like structures (Fig. 2C), as observed for 19-dpc kidney using this antibody (not shown). Over the same timecourse, FGF2 alone (50 ng/ml) was insufficient to induce epithelial structures either grossly or in any of the serial sections taken from embedded explants (Fig. 2B). Furthermore, E-cadherin expression was not detected in FGF2-treated explants by immunohistochemistry (Fig. 2C). Recombinant TGFβ1, TGFβ3 or activin β_A could replace TGFβ2 as inductive signaling factors, although activin β_A required higher concentrations (10-100 ng/ml).

TGFβ family members are secreted in both biologically active (e.g. 25 kDa homodimer) and inactive (e.g. 100 kDa latent complex) forms (Bonewald et al., 1991). To determine if bud cells release active cytokine, untreated 100×-concentrated RUB1-conditioned medium was analyzed by gel electrophoresis under reducing and non-reducing conditions with immunoblotting. Under non-reducing conditions, immunoreactive bands at 25 and 100 kDa were observed, indicating that both active and latent forms of TGFβ2 are secreted by RUB1 cells (Fig. 2D) and suggesting that bud cells are capable of activating the latent form (Pedrozo et al., 1999). These bands disappeared under reducing conditions, and a new band at 12 kDa, corresponding to monomeric processed TGFβ2, appeared. We were unable to detect either active or inactive form directly in isolated buds or MMs (140 each), presumably owing to limitations of sensitivity.

Neutralizing antibody specific for TGFβ2 and/or a soluble neutralizing form of the TGFβ Type III receptor failed to block differentiation in MM explants induced with FGF2 plus conditioned medium, suggesting that other TGFβ family members may be present in this preparation. As Type III receptors bind TGFβ1-β3, multiple family members were considered using RT-PCR. Expression of the genes for TGFβ1-3, activins β_A and β_B, and growth/differentiation factor 11 (Gdf11) has been previously described in developing kidney tissues (Pelton et al., 1991; Ritvos et al., 1995; McPherron et al., 1999). TGFβ1 expression has been reported in metanephric rudiments by RT-PCR (Rogers et al., 1993). With our conditions, it was detected in 19 dpc kidneys (FK) but not in isolated 13 dpc ureteric buds (UB) or RUB1 cells (data not shown). For TGFβ2, expression was noted in 13 dpc buds and RUB1 cells, but not in 12 dpc MM, which surrounds the mesonephric duct prior to bud outgrowth and induction (Fig. 3A). This indicates that TGFβ2 is upregulated early in nephrogenesis and is consistent with the ability of this factor to autoregulate in some cell types (O'Reilly et al., 1992). Sequences specific for the genes for TGFβ3, activin β_A, activin β_B, and Gdf11 were also amplified by RT-PCR from bud isolates, and amplification was noted in RUB1 cell RNA for TGFβ3, activin β_B and Gdf11 (Fig. 3B), demonstrating that tissue inductors express multiple TGFβ family members.

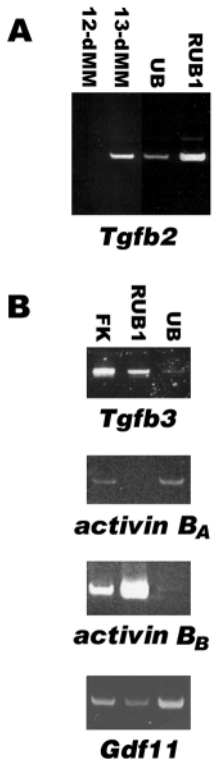

Fig. 3. The genes for multiple TGFβ family members are expressed in inductive bud tissues (*Tgfb2*, TGFβ2; *Tgfb3*, TGFβ3). (A) RT-PCR of DNase-treated total RNA (20 ng) from isolated ureteric buds (UB), rat ureteric bud cell line (RUB1), or isolates of 12 or 13 dpc metanephric mesenchymes, MM. (B) RNA from 19-dpc rat fetal kidney (FK) served as a positive control. No-RT reactions were included to detect possible contamination. All amplified products were confirmed by dideoxy sequencing.

Inductive factors cause expression of epithelial markers in MM

To characterize the extent of differentiation in MMs, the expression of several renal stage-specific genes was evaluated in cultures treated with LIF, FGF2/TGFβ2 (Fig. 4) or LIF/FGF2/TGFβ2 (not shown) by in situ hybridization (ISH) and immunohistochemistry (IHC). Markers were chosen for both early (condensation, i.e. transcription factors Wt1 and Pax2, and secreted patterning molecule Wnt4) and late events (epithelial conversion and/or glomerulogenesis, i.e. Wt1, homeobox gene *Lim1*, secreted frizzled-related protein 2 (sFRP2) and E-cadherin (as shown above in Figs 1C and 2C)) in morphogenesis. For Wt1 (Kreidberg et al., 1993), Wnt4 (Stark et al., 1994), Lim1 (Shawlot and Behringer, 1995) and Pax2 (Dressler et al., 1993), severe renal abnormalities have been reported in gene targeting studies, demonstrating the critical nature of these proteins in normal tubular development. All markers were expressed in cultured explants regardless of treatment and all patterns of expression reflected those reported in vivo. *Wt1* expression was pronounced in condensates and associated with epithelial aspects of glomerular-like structures (Fig. 4A), while *Wnt4* was strongly expressed in condensates but not mature epithelia (Fig. 4). As autocrine *Wnt4* expression in MMs is sufficient for tubule induction (Kispert et al., 1998), it is possible that the cytokines function in part through upregulation of *Wnt4* transcription. Pax2 antibody provided strong nuclear staining of tubular epithelia and induced nephrogenic mesenchyme while more mature glomerular

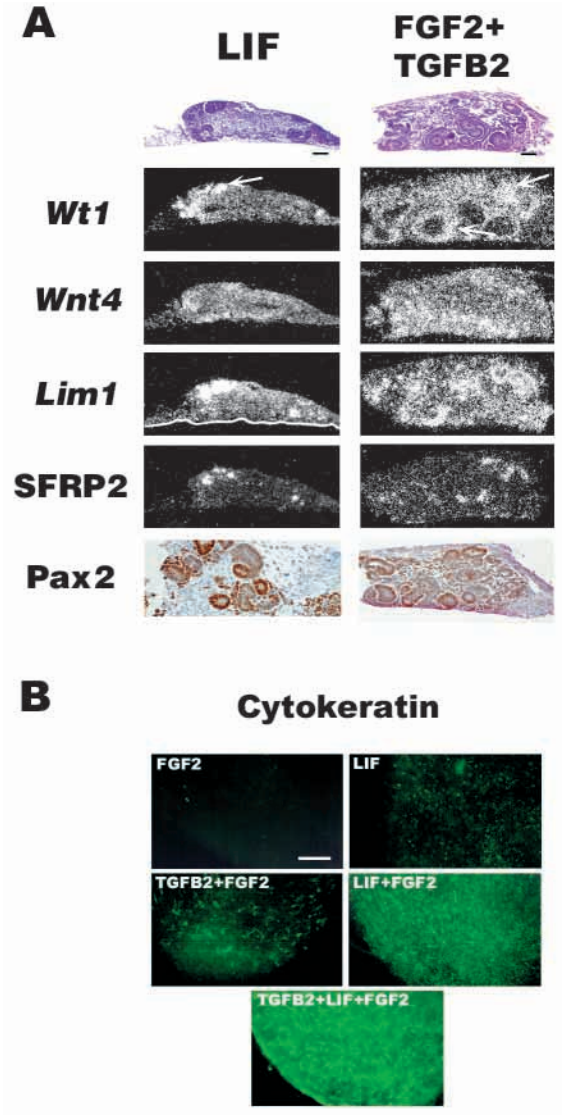

Fig. 4. Expression of epithelial markers in cytokine/growth factor-induced metanephric mesenchymes. (A) explants treated for 8 days with LIF (20 ng/ml) and TGFα (10 ng/ml) or TGFβ2 (0.67 ng/ml), FGF2 (50 ng/ml) and TGFα (10 ng/ml) were probed by in situ hybridization or immunohistochemistry for markers of condensation (*Wt1* and *Wnt4*) and epithelial conversion (*Wt1*, *Lim1*, the gene for sFRP2, or Pax2 protein). Expression patterns resemble those described in vivo for metanephric development. Glomerulogenesis is shown by focal expression of *Wt1* in and around glomerular-like epithelial structures (arrows). (B) FITC-immunofluorescent staining for cytokeratins in 6-day explants treated with various cytokines/growth factors. Image intensity correlates positively with the ability of the inductive factor to stimulate tubulogenesis as shown in Fig. 2B. Scale bars: 0.10 mm.

structures stained weakly. In all explants, *lim1* was highly expressed in foci of newly formed epithelia (Fig. 4A). The gene for sFRP2, which encodes a secreted protein with homology to frizzled family proteins (Wnt receptors) and is expressed as a result of Wnt4-mediated signaling (Lescher et al., 1998), is detected in vivo only in primitive nephrogenic epithelia. In treated explants, expression of this gene occurred in an intense focal pattern associated solely with newly formed epithelia (Fig. 4A). The findings show that LIF and TGFβ2 induce patterns of gene expression in cultured MM that correspond to the structure- and stage-specific localization of these markers in vivo and suggest that Wnt4 is involved in the differentiation mediated by these inductive cytokines.

We also evaluated cytokine/growth factor-induced MM explants for the epithelial marker cytokeratin, which appears in renal vesicles and subsequently in all epithelial segments of the developing metanephros but not in blastemal cells (Oosterwijk et al., 1990). MM explants were cultured for 6 days in various combinations of factors and then evaluated for expression using a pan-cytokeratin antibody and FITC-labeled secondary antibody. While explants treated with FGF2 contained few cells that expressed cytokeratins, the combination of TGFβ2/LIF/FGF2 caused an extensive diffuse expression throughout a comparably sized tissue mass. Both LIF and FGF2/TGFβ2 treatments induced expression in many cells within the explants (Fig. 4B), and in the case of LIF, this was significantly enhanced in combination with FGF2. These patterns closely correlate with the morphogenetic results of cytokine/growth factor treatment as presented in Fig. 2B.

The distribution of inductive ligands and their receptors is appropriate for paracrine signaling

To determine the distribution and relationship of LIF and its gp190 receptor in the metanephros, 16 and 19 dpc rat rudiments were evaluated by ISH or RT-PCR and IHC. By RT-PCR (Fig. 5), a single intense 439 bp band with an appropriate sequence for *Lif* was amplified from RNAs of RUB1 cells (R), isolated 13 dpc ureteric buds (B) and 19 dpc fetal kidneys (FK). Weak amplification was noted from 13 dpc MMs, which may be attributed to contamination by bud cells or low constitutive levels of *Lif* mRNA in these tissues. IHC with anti-LIF antibody, however, revealed staining for LIF in both ductular (branched bud) and tubular structures in FK (Fig. 5B), suggesting it may be induced in MMs.

LIFR, on the other hand, is expressed solely in structures of MM lineage. By IHC (Fig. 5C) using a polyclonal antibody for LIFR, moderate staining was seen in condensed mesenchyme surrounding the branched bud and intense staining was observed in tubular structures. These studies were confirmed by ISH. In 16 dpc metanephroi, LIFR was expressed in mesenchyme (Fig. 5D,F) and tubules. All segments of the bud (ub) were not labeled. LIFR expression was more prominent in maturing epithelial segments of 19 dpc kidneys (Fig. 5E,G) but absent from glomeruli (g) and bud-derived ducts (ub). Our studies reveal that the ureteric bud is the principal source of LIF in the metanephros prior to induction, while the surrounding mesenchymal cells and newly formed tubules elaborate LIF receptors.

Expression of TGFβ2 has been described (Pelton et al., 1991) as prominent staining in and around the bud and structures of newly formed epithelia such as the S-shaped bodies, findings that we have confirmed (not shown). Prior expression studies of receptors for TGFβ family members have also extensively documented receptor components in the developing metanephros both for TGFβs and activins (Mariano et al., 1998; Ritvos et al., 1995). TGFβ Type I and Type II receptors have been described in developing tubules, and these decrease with maturation (Mariano et al., 1998). Furthermore, both activin RII and RIIB have been detected in developing

metanephroi (Ritvos et al., 1995), and we have amplified receptor sequences by RT-PCR from preparations of isolated buds and MMs (data not shown).

Exogenous LIF and TGFβ2 induce cell commitment and endogenous cytokine expression in the first 24 hours

Induction or a commitment to differentiate is maximal and complete in MM after a 24 hour interaction with a heterotypic tissue inductor (Saxén and Lehtonen, 1978). To determine if inductive cytokines/growth factors function with similar kinetics, explants were treated for 24 hours with LIF (20 ng/ml), or TGFβ2 (0.67 ng/ml) in combination with FGF2 (50 ng/ml), washed and then maintained in basal medium containing FGF2. Under these conditions, explanted MMs developed tubules by 6 days, regardless of treatment (Fig. 6A), while FGF2 alone was incapable of inducing tubular morphogenesis, suggesting that continuous paracrine signaling by these factors is not required to sustain induction.

One possible explanation for the kinetics of induction is that LIF or TGFβ2 are causing a shift from paracrine-to-autocrine signaling by upregulating the expression of *Lif* or the gene for TGFβ2 in responding MM. To test this hypothesis, LIF- or TGFβ2-treated explants were evaluated by RT-PCR and compared with preparations of 13 dpc MM (Fig. 6B). Explants exposed to LIF for 1 day (L1) or 3 days (L3) showed upregulation of *Lif* and expression of the gene for TGFβ2 (Fig. 6B) relative to MM. Conversely, explants treated with TGFβ2/FGF2 for 1 day (T1) or 3 days (T3) showed increased *Lif* expression and sustained or enhanced expression of the gene for TGFβ2. Thus, both exogenous LIF and TGFβ2 may facilitate a paracrine-to-autocrine shift for expression of *Lif* and the gene for TGFβ2 in induced MMs.

Cytokine/growth factor-mediated responses to induction are Wnt dependent

The identification of FGF2, LIF and TGFβ2 in induction provides substantial clues as to the possible regulatory

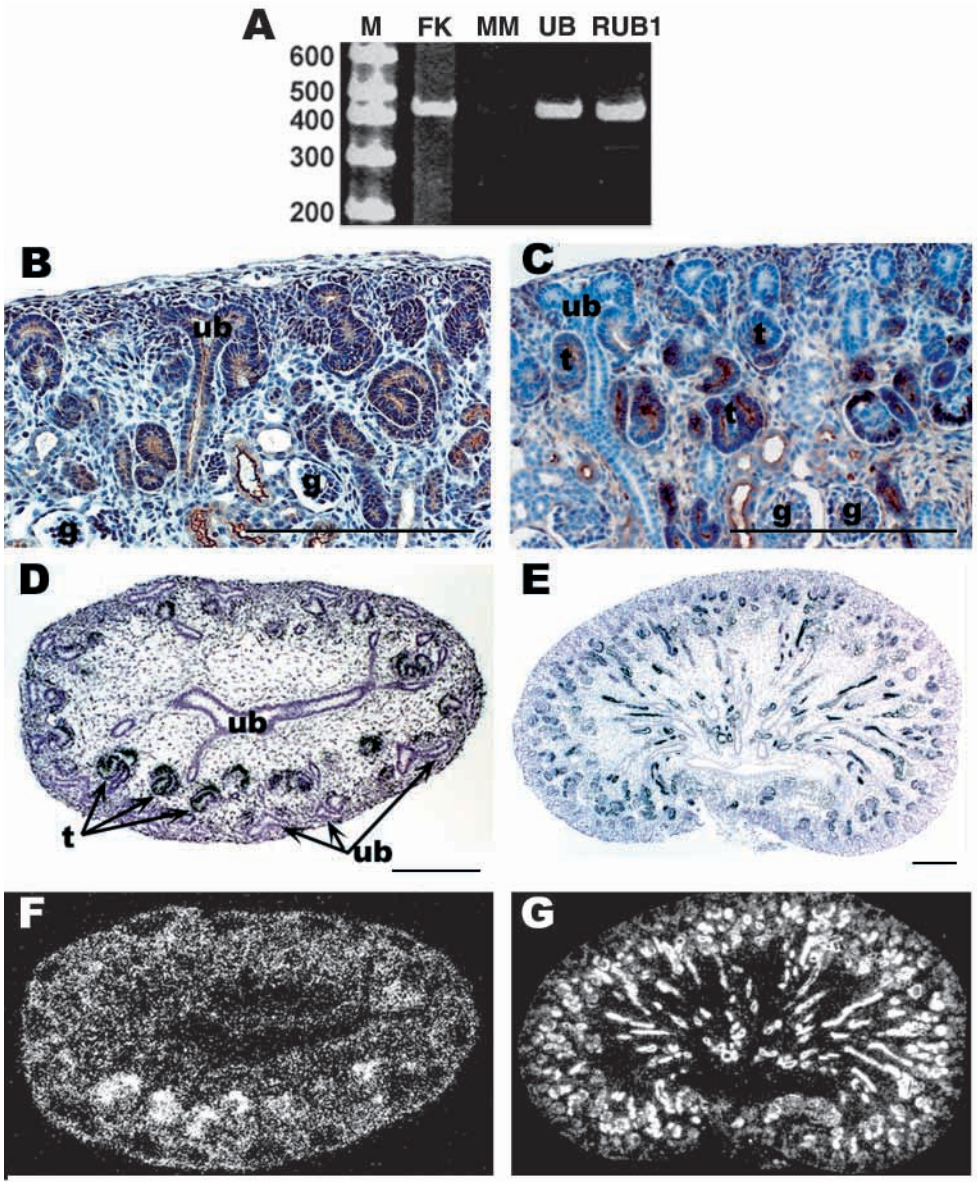

Fig. 5. Expression of LIF and LIFR in the developing metanephros. (A) RT-PCR expression of LIF in metanephric tissue isolates: 19 dpc rat fetal kidney (FK), 13 dpc metanephric mesenchyme (MM), 13 dpc rat ureteric buds (UB) and rat bud cell line (RUB1). (B) Immunohistochemistry for LIF in FK (ub, ureteric bud). (C) IHC for LIFR in FK (g, glomerulus). (D,F) Brightfield/darkfield images for expression of LIFR by in situ hybridization in 16 dpc rat kidneys (t, tubules). (E,F) Brightfield/darkfield images for LIFR in FK. LIFR is localized to tissues originating from MM, including epithelia of the developing nephron. Scale bars: 0.1 mm.

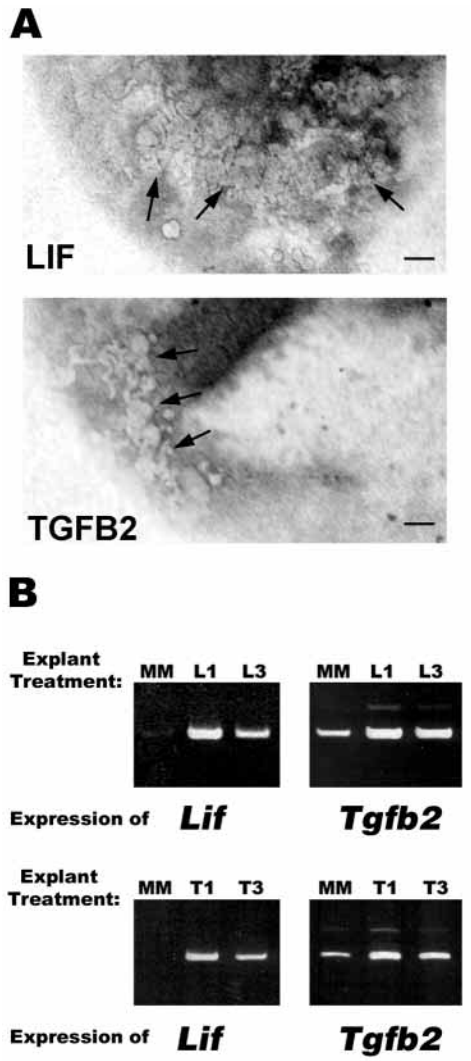

Fig. 6. Kinetics for cytokine induction in metanephric mesenchyme. (A) LIF (20 ng/ml)/FGF2 (50 ng/ml)/TGFα (10 ng/ml) or TGFβ2 (0.67 ng/ml)/FGF2 (50 ng/ml)/TGFα (10 ng/ml) treatment for 24 hours is sufficient to commit MM to tubule formation (arrows). Explant cultures were maintained with FGF2 (50 ng/ml) alone. Scale bar: 0.10 mm. (B) RT-PCR for expression of *Lif* or the gene for TGFβ2 (*Tgfb2*) after treatment of MM explants with LIF for one (L1) or three (L3) days or TGFβ2/FGF2 for one (T1) or three (T3) days compared with untreated isolates of MM.

signaling pathways for the morphogenesis of MM. Furthermore, as autocrine expression of Wnt4 in MM is required to induce tubule formation (Kispert et al., 1998), and as we have shown that *Wnt4* is strongly expressed in cytokine/growth factor-induced MM, it is possible that Wnt signaling provides a common downstream mechanism for the differentiation of MM. In order to test this hypothesis, uninduced MMs were incubated with recombinant sFRP1, a secreted frizzled-related protein that antagonizes Wnt signaling (Bafico et al., 1999; Yoshino et al., 2001). LIF-treated explants showed abundant tubules by 8 days as expected (Fig. 7A); however, sFRP1-treated cultures failed to progress. Small condensates formed but did not epithelialize. Instead, many cells fluoresced with TO-PRO-1 staining (Molecular Probes), showing their nonviability. In explants induced with TGFβ2/FGF2, tubule formation was blocked with sFRP1 treatment, but explants increased in size and remained condensed. These results are consistent with the hypothesis that Wnt4 may be acting downstream of LIF/TGFβ2/FGF2 induction, but do not exclude the possibility that these inductive signals may also be interacting synergistically with downstream components in the Wnt pathway. To address this issue and confirm involvement of Wnt signaling, we have evaluated the effects of FGF2, LIF and TGFβ2 on the ability of nuclear lysates from treated MM to bind to a Tcf1/Lef1-specific oligonucleotide probe in electrophoretic mobility shift assays (EMSAs). ISH studies of Tcf1 and Lef1 expression have localized these transcription factors to the mesenchyme adjacent to the tips of the ureteric bud and to primitive epithelia (Oosterwegel et al., 1993). In our EMSA studies, there was a clear shift in the mobility of a Tcf probe when incubated with lysates from LIF-, FGF2- and/or TGFβ2-treated MMs (Fig. 7B). One predominant band was detected with induction at 24 and 72 hours, and its intensity increased with combinations of inductive factors. The band was competed with an 80-fold excess of unlabeled wild-type oligonucleotide but not with a mutant form, and pretreatment of nuclear extracts with antibody to TCF1 abolished band formation. Furthermore, it was partially supershifted with anti-β-catenin antibody (see arrows in Fig. 7B). Demonstration of this supershift required the modification of incubation conditions for EMSA, which resulted in the appearance of two competed bands and two supershifted bands with antibody. Another candidate interacting component in this system is Smad4, a common downstream signaling cofactor of diverse TGFβ family receptors. Smad4 has recently been shown to mediate Tcf family activation through an association with Tcf and β-catenin molecules in transcriptional complex formation (Nishita et al., 2000). To examine its possible involvement, nuclear lysates from LIF/FGF2- or TGFβ2/FGF2-treated explants were preincubated with an anti-Smad4 antibody or a pan-Smad antibody that does not crossreact with Smad4. The anti-Smad4 antibody, but not the pan-Smad, shifted the mobility of the Tcf probe, suggesting the presence of Smad4 protein in the complex. While it is not possible to determine the functional significance of this interaction in our explant culture system, these data are consistent with an inductive mechanism involving a Smad4-dependent synergistic transcriptional activation of Wnt signaling.

LIF, as described, functions primarily through the JAK/Stat signaling pathway by phosphorylation and nuclear localization of Stat3. Although Stat3 signaling is described as rapid and transient in branching renal cells (Boccaccio et al., 1998), the constitutive expression of LIF in induced explants, as indicated in RT-PCR studies (Fig. 6B), suggests that Stat3-mediated signaling may be constant in these cultures. We have therefore evaluated explanted MMs treated with FGF2, LIF or TGFβ2 for 24 and 72 hours by EMSA (Fig. 7C). In these experiments, labeled DNA probes with a Stat3-binding motif formed complexes with nuclear proteins derived from most explant preparations. DNA-protein complexes were competed with an 80-fold excess of unlabeled Stat3-binding oligonucleotide but not with equal levels of an unlabeled mutant or unrelated oligonucleotide. Cytokine/growth factor induction caused oligonucleotide mobility shifts and specific increases in the

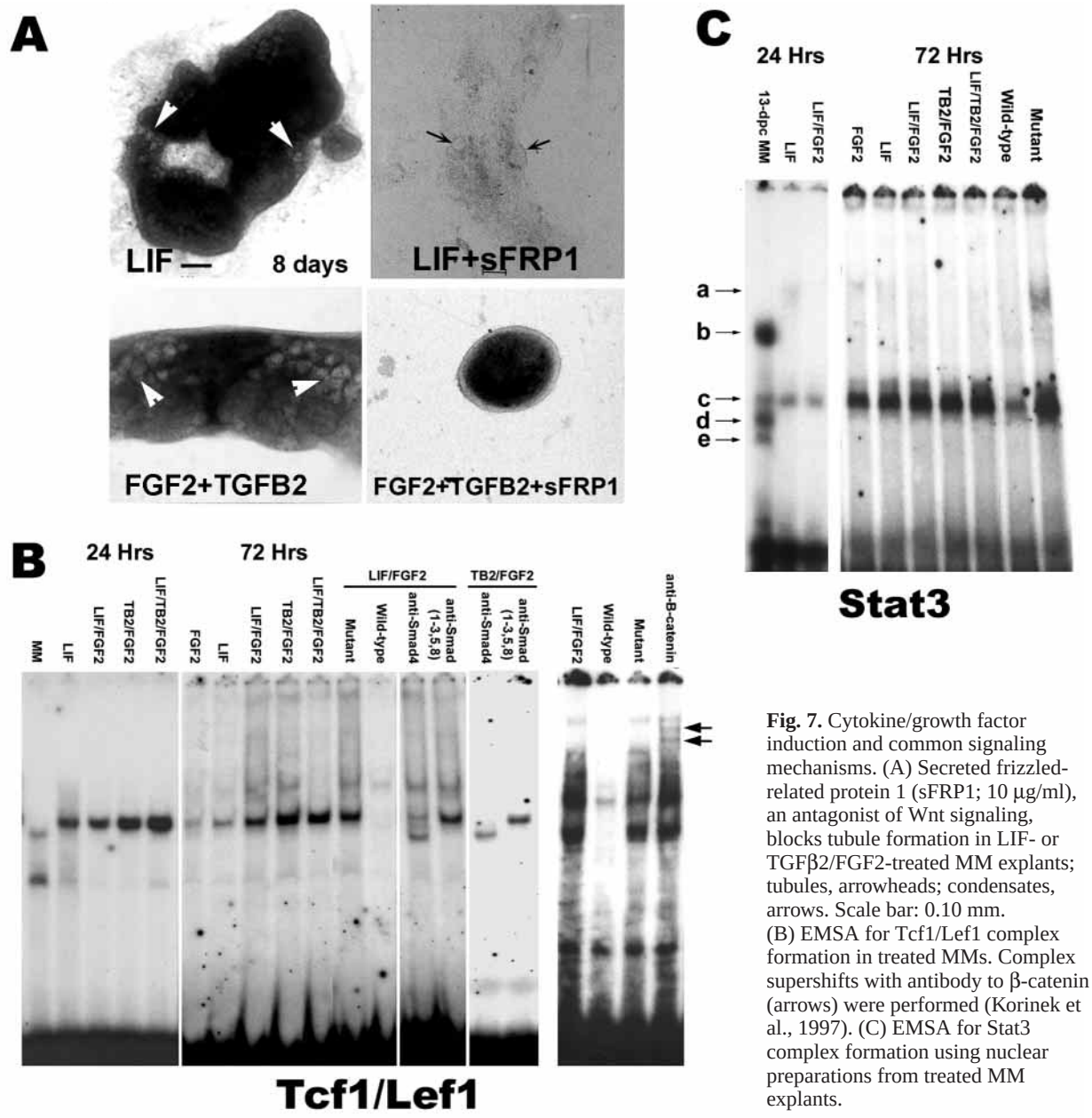

Fig. 7. Cytokine/growth factor induction and common signaling mechanisms. (A) Secreted frizzled-related protein 1 (sFRP1; 10 μg/ml), an antagonist of Wnt signaling, blocks tubule formation in LIF- or TGFβ2/FGF2-treated MM explants; tubules, arrowheads; condensates, arrows. Scale bar: 0.10 mm. (B) EMSA for Tcf1/Lef1 complex formation in treated MMs. Complex supershifts with antibody to β-catenin (arrows) were performed (Korinek et al., 1997). (C) EMSA for Stat3 complex formation using nuclear preparations from treated MM explants.

DNA-protein complexes formed at 24 and 72 hour treatments as compared with isolated MMs. After 24 hours, nuclear lysates from all treatments (only LIF and LIF/FGF2 shown) exhibited relatively low levels of protein binding (complex 'c'), but at 72 hours, formation of Stat3 complexes was enhanced with all treatments (Fig. 7C). LIF stimulated complex formation, which was further increased in combination with FGF2. Even TGFβ2 with FGF2 enhanced binding to the Stat3 probe. These findings suggest that induction with TGFβ2/FGF2 and/or LIF alters Stat3-binding complexes from MMs in a sustainable quantitative manner that reflects the ability of these particular cytokines to cooperate in inducing tubulogenesis. While the functional involvement of Stat3 signaling in renal tubulogenesis has not been established, this secondary messenger is clearly activated by our inductive cytokines/growth factors.

In an effort to characterize FGF2 signaling, we also applied EMSA to studies of AP-1 activation. While binding to an AP-1-specific oligonucleotide occurred and was increased over time for all preparations (24 versus 72 hours), levels of binding were similar among the various treatments (data not shown). No binding was detectable though with nuclear lysates from uninduced MMs.

DISCUSSION

The ureteric bud and its originating epithelium, the mesonephric duct, elaborate a diverse array of signaling molecules responsible for maintenance, growth and differentiation of mesenchymal populations in the urogenital ridge. The current studies describe three distinct families of

cytokines that cooperate to induce differentiation of MMs with comparable kinetics and morphogenetic outcome as reported using an established inductive model (Karavanova et al., 1996). These studies also demonstrate for the first time that LIF without FGF2 or FGF2 with a TGFβ family member can independently induce epithelial conversion and tubule formation in MM, and that these cytokines cooperate to accelerate differentiation.

In these studies, LIF functioned as a maintenance, growth and differentiation-inducing factor, and, in combination with FGF2, which behaves as a weak inductor of tubule formation (Karavanov et al., 1998), LIF may accelerate the appearance of tubules. Furthermore, LIF was initially expressed by the ureteric bud and subsequently upregulated in LIF-treated MM, while LIF receptors were detected by ISH and IHC only in cells originating from MM. TGFα is routinely included in our culture medium, and we have reported that its addition has no effect on MM explant survival, growth or differentiation (Karavanova et al., 1996). However, it has been shown (Barasch et al., 1999) and we have also found (unpublished observation) that LIF requires the presence of TGFα to support and sustain MMs. Signaling through the EGF receptor blocks Fas-induced apoptosis in HEK293 cells, which are derived from MM, and may have a similar effect in treated explants (Gibson et al., 1999). Nevertheless, it appears that TGFα does not function as a morphogen. However, FGF2 clearly contributes to the differentiation process, independently causing tubule formation (Karavanov et al., 1998) and activation of Stat3 (current study; Megeney et al., 1996) and, with LIF, enhancing Tcf/Lef1 DNA-binding activity. Previous studies documented the inductive effects of LIF primarily with concurrent FGF2 treatment (Barasch et al., 1999), making it necessary to distinguish LIF activity from that of the established morphogen FGF2. LIF has been shown in a variety of culture systems, e.g. oligodendrocytes (Mayer et al., 1994), pituitary corticotroph cells (Stefana et al., 1996) and adipocytes (Aubert et al., 1999) to induce differentiation in the absence of FGF supplementation. In the current studies, LIF promoted growth and tubular development in MM at levels (20 ng/ml) that did not inhibit nephron formation in intact rudiments (A. O. P., unpublished observation; Bard and Ross, 1991). However, DNA-binding activities for Stat3 and Tcf1/Lef1 as well as the rate of tubule formation were increased in FGF2/LIF-treated explants versus LIF or FGF2 alone, suggesting that combinations of factors enhance inductive signaling. LIF and FGF2 function synergistically to induce proliferation and vasculogenesis in endothelial cells (Gendron et al., 1996) and proliferation in myoblast cells (Austin et al., 1992). These findings may reflect the ability of each factor to stimulate similar signaling pathways. In fact, FGF2 and LIF have both been shown independently to activate Stat3, ERK1 and ERK2 in myoblasts (Megeney et al., 1996).

In addition to its apparent paracrine effects, LIF may also promote endogenous LIF expression in induced mesenchymal cells. There is considerable evidence for the existence of autocrine signaling by LIF in a variety of tissues, including renal mesangial cells (Hartner et al., 1994). It has also been shown that other gp130-binding cytokines can upregulate the expression of LIF (Knight et al., 1999). The current studies, however, provide the first indication that LIF may regulate its own expression in responding MMs, as exogenous LIF treatment of MM explants appeared to stimulate endogenous *Lif* expression. This apparent paracrine-to-autocrine shift in cytokine expression may provide an explanation for the established kinetics of metanephric cell commitment to tubule formation.

TGFβ molecules have been implicated in the regulation of cell proliferation and tissue differentiation, including metanephric development, by gene targeting studies, e.g. TGFβ2 (lung, limb, craniofacial, heart, and urogenital defects; Sanford et al., 1997); activin β_B (eyelid and urogenital defects; Vassalli et al., 1994); and Gdf11 (skeletal, palate and renal defects; McPherron et al., 1999). Null homozygotes for TGFβ2 are especially interesting because they manifest a progressive deterioration of the metanephros as a result of tubular degeneration late in organogenesis (Sanford et al., 1997). Development, however, appears grossly normal during the initial rounds of morphogenesis, indicating that it is not solely responsible for nephrogenesis. Its absence, though, produces an abnormal renal phenotype, unlike the loss of LIF in null homozygotes (Stewart et al., 1992). For other tested TGFβ family members, activin β_A, while possibly expressed in isolated ureteric buds, is not detected in cells from the immortalized rat bud cell line. However, it was expressed in LIF- or TGFβ2-treated MMs (A. O. P., unpublished observation). Conversely, activin β_B is highly expressed in the bud cell line but only weakly demonstrable in bud isolates and not at all in preparations from MM, suggesting that the activins are differentially regulated in the metanephros. Although activin β_B null homozygotes do manifest abnormalities in the urogenital tract, only reproductive tissues have thus far been implicated. Recent reports also indicate that growth/differentiation factor 11/Bmp11 may play a crucial role in kidney development (McPherron et al., 1999). Thus multiple TGFβ/activin family members may cooperate to invoke TGFβ signaling. It is worth noting that only factors eliciting Smad2/3 signaling are effective. Efforts to induce tubules with Bmp7, which signals through Smads1/4, have been unsuccessful (Dudley et al., 1999).

TGFβ family members have been reported to limit tubulogenesis in intact metanephroi (Rogers et al., 1993). This may be due either to the inhibitory effect of TGFβ on ureteric bud growth and branching or on the higher concentrations (25 ng/ml versus 0.67 ng/ml) of TGFβ used in those studies. We previously reported that TGFβ1 directly blocks bud cell growth at 1 ng/ml (Perantoni et al., 1991a; Perantoni et al., 1991b). Furthermore, when concentrations above 10 ng/ml were applied to MM explants, we also observed inhibition of morphogenesis. The basis for such a biphasic response is not understood, but mechanisms involving heterogeneous receptors with varying avidities for ligands (Armes and Smith, 1997) or the presence of non-signal-transducing accessory receptors (Massague, 1998) may provide possible explanations.

In the current studies, LIF and TGFβ2 expression was sustained or upregulated in LIF-and TGFβ2-treated MMs. These findings are consistent with published studies, which describe possible crossregulation. CNTF induces TGFβ3 expression in ciliary ganglionic neurons (Krieglstein et al., 1998); furthermore, TGFβ2 can stimulate its own expression and that of other TGFβ family members in certain cell lines (O'Reilly et al., 1992). Conversely, TGFβ1 induces expression

of LIF in cultured Schwann cells (Matsuoka et al., 1997) or lung fibroblasts (Elias et al., 1994). Thus, the activity of one cytokine/growth factor may be reinforced through induced expression of the complementary signaling ligand.

Determination of the initial source of TGFβ signaling in the metanephros is somewhat complicated by the apparent upregulation of various family members in responding MM at the time of induction. The ureteric bud is shown here to express and presumably secrete multiple TGFβs. The MM, when analyzed prior to bud penetration in 12 dpc MMs, does not express the gene for TGFβ2, but it is upregulated in 13-dpc MM, suggesting it is initially elaborated in a paracrine manner. Of course, expression alone does not assure biological activity since some of these factors are secreted as latent inactive proteins. In the case of TGFβ2, we found an active form in conditioned medium from ureteric bud cells. For TGFβ proteins expressed in MMs, secreted processing factors, perhaps originating in the ureteric bud, may be required to produce active and thus inductive TGFβ molecules in those tissues (Pedrozo et al., 1999). For other inductive factors, the existence of proteins that modulate biological activity is a significant issue. Cellular responses to LIF, for example, may be blocked by a naturally occurring soluble receptor (Tomida, 1997). Additionally, interactions of follistatin with activins neutralize activin-mediated responses. Induction might be significantly regulated through modulation of these suppressive co-factors.

Finally, among the most intriguing aspects of the current studies is the involvement and possible links among Stat, Smad and Tcf/Lef1 activation. LIF-mediated activation of Stat3 is rapid and transient, often dissipating within minutes of ligand-receptor binding, and yet capable of inducing branching morphogenesis many hours after exposure (Boccaccio et al., 1998). LIF also acts synergistically with Bmp2 to enhance cell differentiation and transcription from a Stat-bearing promoter (Nakashima et al., 1999). This cooperative signaling is believed to occur through formation of a p300-bridged complex of Stat3 and Smad1. As Smad2/3 also interacts with CBP/p300 (Feng et al., 1998), a similar complex involving Stat3 and Smad2/3 might enhance Stat activation. In the current study, relative levels of Stat3/DNA complex formation from explants with various treatments were comparably low at 24 hours; however, binding activity was elevated considerably at 72 hours with most treatments and especially with combinations of cytokines. As FGF2/TGFβ2 treatment was also effective in this regard, it may suggest that endogenous LIF or another gp130-binding ligand has been upregulated in these explants to activate Stat3. Thus, the current studies indicate that Stat activation is not necessarily transient and that crosstalk from other signaling pathways may be important in augmenting or sustaining Stat signaling.

Co-regulation has also been demonstrated between TGFβ and Wnt family members. In *Xenopus*, activin/Vg-1 cooperates with Xwnt8/β-catenin to enhance transcription from both Wnt- and activin-induced genes, and activity is dependent upon Smad2 signaling (Crease et al., 1998). More recently, Smad4 has been shown to interact directly with β-catenin and to complex with Lef1 to facilitate transcription from Wnt-induced genes (Nishita et al., 2000), although Smad2 involvement has not been clearly demonstrated. In our studies, LIF activated Tcf1/Lef1 binding, which was further increased with the cooperation of TGFβ2 and FGF2 in a manner consistent with the ability of the cytokines to accelerate tubulogenesis. Furthermore, the suggested natural presence of Smad4 in the Tcf complexes demonstrated here using preparations of explanted MM is consistent with the nature of complexes reported in HEK293 cells transfected with expression constructs for these transcription factors (Nishita et al., 2000).

While enhanced Tcf-DNA binding suggests a role for Wnt signaling, direct antagonism with an sFRP clearly demonstrates Wnt involvement in cytokine induction. Tubule formation was blocked in both LIF- and FGF2/TGFβ2-treated MM explants with sFRP1, and in fact LIF-treated explants showed poor survival with treatment. The exact mechanism for LIF-mediated Wnt signaling/Tcf activation is unclear. The human Tcf-1 promoter contains potential Stat-binding motifs (Roose et al., 1999), but their functionality is not known. Alternatively, LIF may induce Wnt signaling through its ability to upregulate *Wnt4* expression, which can be sufficient to induce epithelial conversion (Kispert et al., 1998). A third possibility is that p300 serves as a coactivator to assemble the various cofactors (Stats and/or Smads) in a transcriptional complex that binds Tcf. In this regard, it was recently demonstrated that p300 potentiates β-catenin-induced activation of a Wnt-dependent target by interacting directly with β-catenin and complexing with Tcf (Hecht et al., 2000).

In conclusion, the current studies implicate LIF and TGFβ2 as significant inductive factors for metanephric mesenchyme and provide evidence that each cytokine invokes Wnt signaling in stimulating morphogenesis of the mesenchyme. Cooperativity of soluble ligands is a common phenomenon in several cell systems. In combination with a TGFβ family member, CNTF (a gp130-binding ligand) or FGF2 significantly enhance neuronal survival (Krieglstein et al., 1998). Additionally, TGFβ family member activin A accelerates LIF-induced astroglial differentiation (Satoh et al., 2000). As our studies indicate, cytokine/growth factor cooperation in tubulogenesis correlates positively with activation of transcription factor binding from relevant signaling pathways. Such cooperativity may well prove to be the norm for inductive signaling in tissue differentiation, as it provides not only for a measured developmental output, but also a redundancy in signaling to ensure a successful morphogenetic outcome.

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