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# Analysis of growth factor and receptor mRNA levels during development of the rat seminal vesicle and prostate

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## SUMMARY

**Development of the mammalian male accessory sexual organs requires both androgens and mesenchymal/epithelial interactions. Paracrine acting factors whose expression is mesenchymal and androgen dependent have been proposed to regulate development of these organs, although the identity of these paracrine mediators is unknown. Keratinocyte growth factor (Kgf) has been shown to play an important role in the development of the mouse seminal vesicle and rat ventral prostate. Also, Kgf is expressed in mesenchymal cells and has been shown to be regulated by androgens in prostatic cells grown in vitro. Thus Kgf has been proposed as a mediator of androgen action. We have investigated the expression of Kgf mRNA during development of the rat seminal vesicle and prostate, both in vitro and in vivo. Additionally we have examined mRNAs for Kgf receptor (KgfR), transforming growth factor alpha (Tgfα), epidermal growth factor receptor (EgfR) and cytokeratin 19 (CK19). The levels of growth factor and receptor mRNAs fluctuated during androgen-regulated development; however, these changes reflected variations in the mesenchymal/epithelial ratio rather than regulation by testosterone. Expression of Kgf is mesenchymal, while KgfR is epithelial and Tgfα is predominantly epithelial. The changes in the levels of mRNAs for these factors correlated well with changes in the level of an epithelial marker, CK19, suggesting they were due to alterations in the relative abundance of tissue compartments in which they were expressed. Kgf has been shown to mimic androgen action in explant cultures of seminal vesicle and prostate. We demonstrate here that anti-androgens are able to block Kgf stimulated development, suggesting that Kgf and androgen receptor signalling pathways may interact. Taken together our data suggest that, in vivo, Kgf may interact with androgen receptor signalling but it is not a direct target of androgen action.**

Key words: keratinocyte growth factor, seminal vesicle, prostate, androgens, rat

## INTRODUCTION

Development of the male reproductive tract in rodents is directed by androgens and is dependent upon mesenchymal/epithelial interactions. Paracrine factors, which are produced by the mesenchyme and regulated by androgens, have been proposed to be crucial in controlling the organogenesis of the male reproductive tract. Androgens regulate gene expression via the androgen receptor (AR), a member of the nuclear receptor superfamily of transcription factors, which most likely control the synthesis of secreted or surface bound factors. To date, the majority of genes identified as being activated by androgens have been secretory proteins of the seminal fluid, while the identity of androgen-regulated paracrine factors has remained unknown.

Development of the coagulating gland (CG, also known as anterior prostate), seminal vesicle (SV) and ventral prostate (VP) is dependent upon androgens (Cunha et al., 1987). These, and other secondary male reproductive tissues, fail to develop when the function of the AR is inhibited by naturally occurring mutations (Wilson et al., 1983). The CG and VP arise from the urogenital sinus, while the SV is derived from the Wolffian duct (Cunha, 1986). During adult life these organs contribute proteins to the seminal fluid and without androgens the synthesis of these proteins is greatly reduced. Furthermore, following androgen deprivation the epithelial parenchyma of the prostate and seminal vesicle regress by apoptotic cell death (Rouleau et al., 1990), and the epithelial content is markedly reduced, changing the mesenchymal/epithelial ratio. Thus, androgens are required for development of male accessory sex organs and subsequently for maintaining tissue architecture and secretory function.

The paracrine nature of mesenchymal signals important in androgen induced development has been well established through analysis of tissue recombinants composed of wild-type and testicular feminization (Tfm) tissues that lack functional androgen receptors (Cunha and Chung, 1981). Such Tfm/wild-type experiments demonstrated that the presence of functional androgen receptors in the mesenchyme, but not epithelium, was important for androgen-regulated development. Further evidence suggesting the involvement of androgen dependent signals from the mesenchyme comes from studies on the distribution of the androgen receptor during the development of the male reproductive tract (Cooke et al., 1991; Hayward et al., 1996b; Shannon and Cunha, 1983; Takeda et al., 1985). Initially, the AR is detected in the mesenchyma of androgen

target organs during early development and morphogenesis. Thus it has been proposed that androgens regulate organogenesis via mesenchymal paracrine acting factors.

Several molecules have been suggested to be mediators of mesenchymal/epithelial interactions. In particular, Kgf (keratinocyte growth factor, also known as fibroblast growth factor 7, Fgf7) is an excellent candidate. Kgf is expressed broadly during organogenesis and is restricted to the mesenchymal cells of organs in which it is expressed (Finch et al., 1995; Mason et al., 1994). The receptor for Kgf (KgfR) is a splice variant of Fgf receptor 2 (FgfR2 type IIIb), which is expressed in epithelia. Thus the expression patterns of the ligand and receptor strongly suggest an important role in mediating paracrine interactions between mesenchyma and epithelia. Administration of Kgf to rodents results in proliferation and hyperplasia of several epithelia (Ulich et al., 1994a,b). Although Kgf has been shown to be mitogenic for several epithelia it is not mitogenic for mesenchyma since mesenchymal cells do not express the Kgf receptor.

Inhibition of Kgf action by gene knockout has yielded different results compared to expression of dominant negative receptors. Inactivation of Kgf by gene knockout results in mice which are phenotypically normal but have defects in hair follicle differentiation (Guo et al., 1996) although the status of the reproductive tract was not reported. Blocking Kgf action by using dominant negative forms of the KgfR has suggested important roles for Kgf in dermal wound healing and lung branching morphogenesis (Peters et al., 1994; Werner et al., 1993). Progesterone has been shown to regulate Kgf in primate endometrium suggesting that Kgf may be a paracrine mediator of progesterone action (Koji et al., 1994). Other studies have focused on the regulation of Kgf by glucocorticoids (Brauchle et al., 1995), interleukin1 (Chedid et al., 1994) and during wound healing (Werner et al., 1992).

Kgf has been demonstrated to be important in the development of the mouse SV (Alarid et al., 1994) and rat VP (Sugimura et al., 1996). Studies on the regulation of Kgf mRNA in rodent prostatic stroma grown in vitro suggested that testosterone might increase Kgf mRNA levels (Yan et al., 1992). Additionally, the promoter of the rat Kgf gene has been shown to be regulated by testosterone in transfection studies (Fasciana et al., 1996). Therefore, it has been suggested that Kgf might be an important androgen regulated mediator of development (Peehl and Rubin, 1995). However, as yet there have been no studies addressing androgen regulation of Kgf in vivo. Since Kgf has been proposed to be an important mediator of androgen action, we have examined the regulation of Kgf mRNA by androgens in vitro and in vivo.

Tgfα and Epidermal growth factor (Egf) have been detected in human and rodent prostate, and both are mitogenic for prostatic epithelial cells in vitro (McKeehan et al., 1984). The mitogenic signal of either Tgfα or Egf is mediated by the Egf receptor (EgfR) which has been reported to be down regulated by androgens in intact prostatic tissue (Traish and Wotiz, 1987). However, it was not determined if changes in EgfR in the whole prostate reflected variations in the ratio of epithelial to stromal cells expressing EgfR, or direct androgen regulation of EgfR levels. Overexpression of Tgfα in transgenic mice resulted in mammary carcinoma and hyperplasia of the CG (Sandgren et al., 1990). Thus Tgfα has been proposed to be a growth stimulator in the prostate. Inactivation of Tgfα by gene knock out does not appear to significantly alter the growth of the CG, but inactivation of the EgfR by gene knock out dramatically reduces prostatic growth (A. Donjacour, unpublished observations).

We have examined the levels of growth factor and growth factor receptor mRNAs to determine if they showed patterns of regulation suggestive of a role in the development of the prostate and seminal vesicle. Since development of these organs is dependent upon androgens and mesenchymal/epithelial interactions it is likely that molecules directing this process will be androgen-regulated and mesenchymally expressed, thus functioning as paracrine regulators. However, epithelially expressed molecules and autocrine regulators may also play important roles in androgen-regulated growth and development. We have analysed the mRNAs for Kgf, KgfR, Tgfα and EgfR during development of CG, SV and VP after development in an organ culture system in vitro, in primary cultures of mesenchyme, and during normal development in vivo. Also, we have measured changes in the epithelial content of these organs during development in vitro and in vivo. This was essential for interpretation of changes in transcript levels of genes that showed epithelial- or mesenchymal-specific expression patterns. Finally, we have examined the effect of an anti-androgen on Kgf-induced development in VP grown in vitro, to determine if the effects of Kgf on development were mediated via the androgen receptor. Interactions between growth factor signalling and the androgen receptor have recently been demonstrated by Culig et al. (1994). These authors were able to show that growth factors could regulate androgen responsive promoters in transfection studies. Androgen receptor was required for these interactions, and the effects of growth factors could be blocked by the addition of anti-androgens (Culig et al., 1995, 1994). An interpretation of these results was that growth factor signalling resulted in activation of the androgen receptor, perhaps by phosphorylation. Kgf was one of the growth factors which was able to regulate an androgen responsive reporter gene, although other factors such as insulin-like growth factor were shown to be a more effective stimulator of reporter activity.

## MATERIALS AND METHODS

### Cell and organ culture

Neonatal (0 days) CGs, SVs and VPs were microdissected from Fisher 344 (Simonsen Inc., Gilroy, California) rats and placed in organ culture as described previously (Sugimura et al., 1996). Organ rudiments were grown in vitro on Millipore CM filters floating on 50:50 v/v DMEM: Hams F12 medium supplemented with insulin (10 μg/ml) and transferrin (10 μg/ml). Cultures were treated with $1{\times}10^{-8}$ M testosterone or 50 ng/ml KGF and grown for 6 days prior to harvesting and preparation of RNA. CGs, SVs and VPs were dissected from 2-day, 20-day and adult Sprague Dawley rats and directly snap frozen on dry ice for subsequent total RNA preparation.

Urogenital mesenchyme was isolated from embryonic day 17/18 Sprague Dawley rats, the day of observation of the copulatory plug was taken as day zero. Urogenital sinuses were obtained by microdissection and treated with collagenase (187 U/ml) for 2 hours at 37 °C with continuous shaking. Cells were collected by centrifugation and washed twice with medium prior to plating on 10 cm tissue culture plates in 50:50 v/v DMEM: Hams F12 medium supplemented with 10% v/v fetal bovine serum, fungizone (2.5 μg/ml), penicillin (10 U/ml), and streptomycin (10 μg/ml). Typically, 10-15 sinuses or 15-20 CGs and SVs were plated on five 10 cm plates and were grown for

approximately 1 week, with or without testosterone, until about 80% confluent. Prior to harvesting, cells were washed three times with phosphate-buffered saline and then removed from the dish with a cell scraper into RNA stat 60 solution (Tel Test B, Freindswood, Texas).

### RNA preparation

Total RNA was prepared using the RNA Stat 60 reagent according the manufacturer's instructions (Tel Test B, Freindswood, Texas). Cells grown on plates were lysed directly into the reagent while organs were homogenised using a plastic pestle in a microcentrifuge tube followed by 3 passages through a 16 gauge and then a 25 gauge needle to ensure complete cell lysis.

### RNAse protection assay

5 or 10 μg of total RNA was used in all experiments. Total RNA was incubated with [$^{32}$P]UTP-labelled antisense riboprobes overnight at 45°C in hybridisation buffer (80% formamide, 0.4 M NaCl, 40 mM Pipes pH 6.6, 1 mM EDTA pH 8.0). After hybridisation, 350 μl of digestion buffer (0.6 M NaCl, 10 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 5 U RNAse A, 200 U RNAse T1, Ambion, Austin, Texas) was added and samples were incubated at 30-37°C for 45 minutes. After the addition of 30 μg Proteinase K and 20 μl 10% SDS the samples were further incubated for 15 minutes at 37°C. Samples were extracted with phenol/chloroform and then chloroform followed by ethanol precipitation. Samples were loaded onto a 6% acrylamide 8M urea sequencing gel after denaturation at 94°C for 4 minutes in 80% formamide gel loading buffer.

Antisense riboprobes were synthesized using T7 or T3 polymerases to transcribe fragments of cDNAs cloned into the pBluescript II vector (Stratgene, La Jolla, California). Probes were labeled with [$^{32}$P]UTP (Amersham, Arlington Heights, Illinois) and stored in hybridisation buffer prior to use. The sizes of expected protected fragments in the RNAse protection assay for the different growth factor and receptors are as follows, Kgf 361nt, KgfR 150 nt, Tgfα 385 nt, EgfR 266 nt, cytokeratin 19 350 nt, β-actin 126 nt and cyclophilin 103 nt. All sequences are from rat cDNA sequences.

To ensure probe excess an aliquot of the probes added to the samples was taken, diluted 1 in 200, and run in a neighboring lane. This band was always determined to be equal to or considerably stronger than the intensity of the most abundant protected fragment. Therefore probe excess was confirmed, and was at least 200-fold or greater. Century markers of 100 nt, 200 nt, 300 nt, 400 nt and 500 nt (Ambion, Austin, Texas) were also run to size protected fragments. To confirm probe sizes in all experiments a freshly diluted aliquot of probe was run to ensure that protected bands were significantly smaller than the full length (undigested) probe. Included in all experiments was an internal control probe which was either rat β-actin or rat cyclophilin (Ambion, Austin, Texas) to ensure equal amounts or RNA were used and to control for differences in gel loading.

To analyse amounts of protected bands, gels were directly exposed to a Phosphorimager screen which was quantitated using a Molecular Dynamics Phosphorimager and ImageQuant software (Molecular Dynamics, Sunnyvale, California). Values were calculated by subtracting a background figure (from the tRNA control), followed by division by the internal control value (actin or cyclophilin). These were used to calculate fold differences between samples shown in Tables 1 and 2.

The study is based on the averages of 30-50 organ rudiments per experiment, each experiment was performed between 2-5 times.

## RESULTS

To investigate regulation of growth factor and receptor mRNAs during prostatic development we analysed mRNAs for Kgf, KgfR, Tgfα and EgfR in organs from zero day old rats grown in vitro for 6 days. In the presence of testosterone CG, SV and VP will grow and branch in a similar pattern to that seen in vivo. When Kgf was added to the cultures in the absence of testosterone, SV and VP developed in a manner similar to that seen with testosterone but to a slightly lesser extent (Alarid et al., 1994; Sugimura et al., 1996). CG grew in the presence of both Kgf and testosterone, although the organs grown in the presence of Kgf showed considerably less branching morphology than those grown with testosterone (B. Foster, unpublished results). To enable sufficient quantities of RNA to be harvested, pools of 15-20 organs were grown under three different conditions; no additions, with testosterone ($1\times10^{-8}$ M) or with Kgf (50 ng/ml). After culture, organs were homogenised and total RNA prepared. To measure the levels of Kgf and KgfR mRNAs 10 μg of total RNA from CGs, SVs and VPs cultured under the different conditions (above) were analysed by RNAse protection assay. Antisense riboprobes to Kgf, KgfR and β-actin were added to each sample of total RNA, and the results of the RNAse protection assay are shown in Fig. 1.

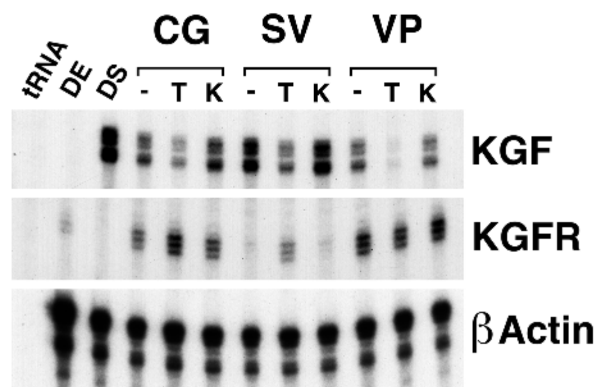


**Fig. 1.** Analysis of transcripts for Kgf and KgfR in organ cultures of neonatal rat CG, SV and VP. Zero-day old CG, SV and VP were isolated and grown in serum free medium for 6 days with no treatment (–), $1\times10^{-8}$ M testosterone (T) or 50 ng/ml Kgf (K). After culture, total RNA was prepared and 10 μg were used in an RNAse protection experiment with $^{32}$P-labelled antisense riboprobes for Kgf, KgfR and β-actin (as a control for RNA integrity and loading differences). After treatment with testosterone, a decrease in Kgf mRNA was observed in all three organs, relative to no treatment or treatment with Kgf. In contrast, KgfR mRNA showed an increase in CG and SV, but no change in VP. As controls for probe specificity RNA from Dunning epithelial cell line (DE) and Dunning stromal cells (DS) was used. Dunning epithelial cells express KgfR mRNA only (not Kgf mRNA) and Dunning stromal cells express only Kgf mRNA (not KgfR mRNA).

After culture with testosterone, Kgf mRNA levels decreased in all three organs grown in vitro (Fig. 1). In CG and SV Kgf transcripts decreased slightly, while in VP the decrease in Kgf mRNA was much larger (see later for quantitative data). Conversely, the KgfR mRNA showed a small increase in the CG and a large increase in the SV after culture with testosterone while no change in the VP was observed. Comparison of Kgf and KgfR mRNAs in untreated and Kgf treated organs indicated that growth in the presence of Kgf had no effect on the levels of these mRNAs. Analysis of mRNAs for Tgfα and EgfR in organs grown in vitro revealed a different pattern of regulation than those observed for Kgf and KgfR mRNAs (Fig. 2). There was an increase in Tgfα mRNA in the SV after

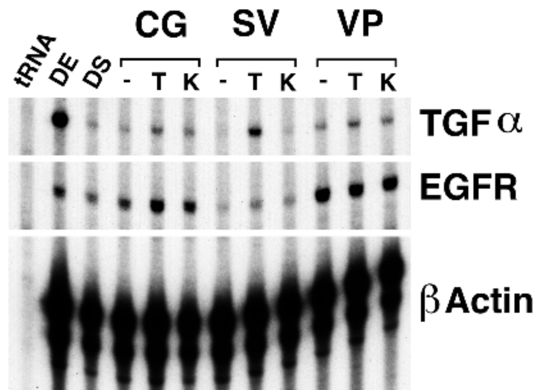


**Fig. 2.** Analysis of transcripts for Tgfα and EgfR in organ cultures of neonatal rat CG, SV and VP. Zero-day old CG, SV and VP were isolated and grown in serum free medium for 6 days with no treatment (–), $1\times10^{-8}$ M testosterone (T) or 50 ng/ml Kgf (K). After culture total RNA was prepared and 10 μg were used in an RNAse protection experiment with $^{32}$P-labelled antisense riboprobes for Tgfα, EgfR and β-actin. After treatment with testosterone there was an increase in Tgfα mRNA levels in the SV, and very little increase or no change in the CG and VP. EgfR mRNA levels were unchanged by treatment with testosterone. As controls, Dunning epithelium (DE) and stromal (DS) cell lines were included, which showed higher levels of Tgfα mRNA in the epithelial cell line compared to the stromal cell line, and similar levels of EgfR mRNA in both.

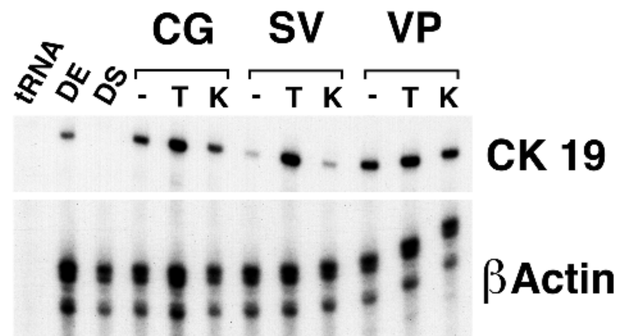


**Fig. 3.** Analysis of cytokeratin 19 mRNA levels in organ cultures of neonatal rat CG, SV and VP. Zero-day old CG, SV and VP were isolated and grown in serum free medium for 6 days with no treatment (–), $1\times10^{-8}$ M testosterone (T) or 50 ng/ml Kgf (K). After culture total RNA was prepared and 10 μg were used in an RNAse protection experiment with $^{32}$P-labelled antisense riboprobes for CK19 and β-actin. CK19 mRNA levels increase in all three organs after culture with testosterone, which most likely represents growth of the epithelium relative to the mesenchyme. Increases in CK19 mRNA levels in the CG and VP were small, however, a larger increase was observed in the SV. To control for probe specificity Dunning epithelium (DE) and stromal (DS) cell lines were used, CK19 mRNA is only expressed in DE and not in DS.

culture with testosterone, and little change in Tgfα mRNA expression in CG or VP after similar treatment (Fig. 2). No change in the expression of EgfR mRNA was observed in any of the organs under any of the culture conditions. Culture in the presence of Kgf had no effect on either Tgfα or EgfR mRNAs relative to control untreated organs.

Since expression of Kgf is mesenchymal and KgfR expression is epithelial it was important to determine if there were any changes in the relative amounts of these tissue components during development of these organs. We used an epithelial marker to measure epithelial content, from which the mesenchymal/epithelial ratio could be inferred. Cytokeratin 19 mRNA was chosen as a suitable marker of the prostatic epithelium as it is expressed throughout the epithelium, in both basal and luminal epithelial cell subtypes (Hayward et al., 1996a). It is expressed in the CG, SV and VP from the earliest appearance of the epithelia through neonatal, pubertal and adult periods. Analysis of CK19 mRNA levels in CG, SV and VP after culture in vitro showed different increases in this epithelial marker in the three organ types depending on the culture conditions (Fig. 3). After culture with testosterone, in the CG there was a 2-fold increase in CK 19 transcript levels while the SV showed a 6-fold increase and the VP a 1.5-fold increase. Surprisingly, after culture with Kgf there was no increase in the level of CK19 mRNA in any of the organs, despite the observed morphological increase in the epithelium.

To accurately measure changes in growth factor and receptor mRNAs we quantitated the RNAse protection experiments using a Phosphorimager and normalised mRNA expression to the β-actin controls. Changes in mRNA levels are shown in Table 1 and are reported as fold increase (or decrease) of testosterone-treated organs relative to untreated and are the average of three experiments. In the VP there was a 13.7-fold drop in the level of Kgf mRNA in the presence versus the absence of testosterone, while transcripts for KgfR, Tgfα, EgfR and CK19 remained unchanged in the presence or absence of testosterone. In the CG there were small changes in all mRNAs tested, while in the SV there were significant increases in mRNAs whose expression is completely or partially epithelially restricted. The mRNA levels of KgfR, Tgfα and CK19 all increased approximately 5-fold in the SV during development in the presence of testosterone. This suggested that a change in the mesenchymal/epithelial ratio was likely to account for the apparent increases in mRNA expression observed after testosterone treatment in the SV. Treatment of organs with Kgf resulted in similar patterns of mRNA expression to control untreated organs. This was quite suprising as all organs showed some epithelial development (especially SV and VP) in response to Kgf treatment, and thus increases in CK19 mRNA might have been expected.

To overcome the problem of changes in mesenchymal/epithelial ratio we analysed the effect of testosterone on growth

**Table 1. Average changes in mRNA levels after treatment with testosterone in CG, SV and VP organs grown in vitro**

| | CG | SV | VP |
|---|---|---|---|
| Kgf | −2.5 | −2.0 | −13.7 |
| KgfR | 1.8 | 6.5 | 1.3 |
| Tgfα | 1.5 | 4.3 | 1.2 |
| EgfR | 1.1 | 2.0 | 1.2 |
| CK19 | 1.8 | 4.6 | 1.3 |

Changes in mRNA levels are shown as fold increase (or decrease) of testosterone treated organs relative to untreated, and were determined by quantitation of RNAse protection experiments using a phoshoimager (molecular dynamics). Values assigned for a particular mRNA were normalized for differences in gel loading and amount of RNA by dividing the observed value by the β-actin value. All points represent the average of three experiments.

factor and receptor mRNAs in mesenchyme isolated and grown in primary cultures. CGs and SVs were microdissected from 2-day old rats and dispersed by digestion with collagenase. Cells were then washed, plated on plastic dishes and cultured for one week in the presence or absence of testosterone. Urogenital sinuses from embryonic day 17 animals were also microdissected and treated similarly. Analysis of total RNA from cultures of CG and SV mesenchyme (CGM/SVM) or urogenital sinus mesenchyme (UGM) with the CK 19 probe showed that almost all of the epithelial cells were lost during the culture period, perhaps due to the presence of serum in the medium (data not shown). As CK 19 mRNA was almost undetectable we inferred that the cultures were almost pure mesenchymal cells. The levels of Kgf mRNA remained unchanged in CGM/SVM and UGM after culture with testosterone. Similarly, the levels of Tgfα and EgfR mRNAs were unchanged in CGM/SVM or UGM after growth in the presence of testosterone (Fig. 4). To address the possibility that androgen treatment resulted in rapid or transient changes in Kgf mRNA levels we used primary cultures of UGM. Treatment with testosterone for periods between 2-24 hours did not result in any significant changes relative to untreated or anti-androgen treated controls (data not shown).

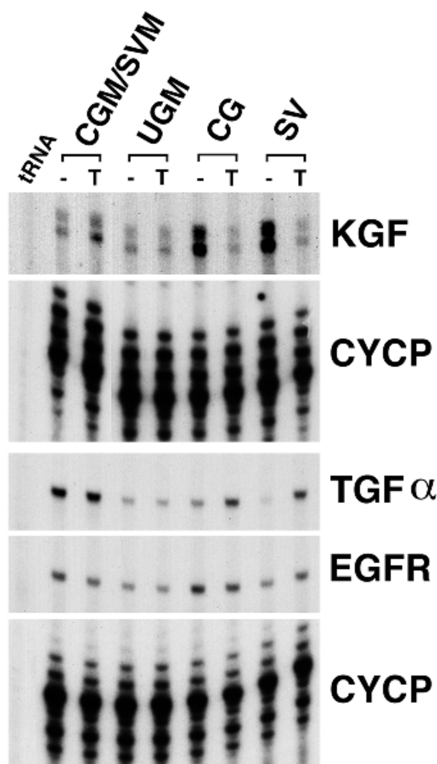


**Fig. 4.** Analysis of Kgf, Tgfα and EgfR mRNAs in primary cultures of mesenchyme from CG/SV and urogenital sinus. Mesenchymal cells from both CG and SV (CGM/SVM) and the urogenital sinus (UGM) were isolated and grown for 2 weeks in the presence of $10^{-8}$ M testosterone (T) or without treatment (–). After culture, total RNA was prepared and 10 μg were used in RNAse protection experiments with $^{32}$P-labelled antisense riboprobes for Kgf and cyclophilin (CYCP, a control for RNA integrity and loading differences) or with antisense riboprobes to Tgfα, EgfR and cyclophilin. No change in the levels of Kgf, Tgfα or EgfR mRNAs were observed after culture in the presence of testosterone in either CGM/SVM or UGM. As controls, RNA from CG and SV organs grown in vitro were included, the level of Kgf mRNA was decreased in both these controls after treatment with testosterone. In similar controls, Tgfα mRNA levels increased while EgfR mRNA levels were unchanged after treatment with testosterone.

Next, Kgf and KgfR mRNAs were quantitated during neonatal, pubertal and adult periods to determine if they showed any changes during these androgen regulated phases of prostatic and seminal vesicle growth in vivo. A mediator of androgen action might be expected to show some changes in level during these periods since they represent actively growing tissue in the neonatal and pubertal ages, while in the adult these tissues are androgen-dependent but growth quiescent. Testosterone levels in the neonate are low, rise at puberty and then remain high during adult life (Corpechot et al., 1981). After the onset of puberty the CG, SV and VP all become secretory and produce very high levels of seminal fluid proteins. The mRNAs for these seminal fluid proteins are so abundant that they appear to dominate the mRNA synthesis of these organs, and thus levels of control mRNAs (such as cyclophilin) appeared to decrease between neonatal and adult periods. Fig. 5 shows the levels of Kgf and KgfR mRNAs in 2-day, 12-day, 20-day and adult tissues. Interestingly the levels of both Kgf and KgfR mRNAs appeared to decrease in all organs as a function of age and rising androgen levels. The decrease in Kgf and KgfR mRNAs was more pronounced in the VP than the CG or SV. Analysis of Tgfα and EgfR mRNAs during in vivo development of CG, SV and VP is shown in Fig. 6. Levels of Tgfα mRNA did not change significantly in any of the organs at any of the different developmental time points. In contrast, EgfR mRNA levels appeared to decrease with age in all three organs.

The epithelial content of CG, SV and VP, during development in vivo, was measured by quantitating CK19 mRNA levels (Fig. 7). The epithelial content (and by inference the

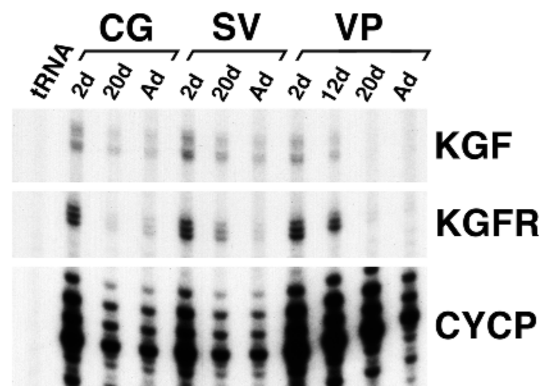


**Fig. 5.** Analysis of transcripts for Kgf and KgfR during development of the CG, SV and VP. Neonatal (2-day old; 2d), pubertal (20-day old; 20d) and adult (Ad) CG, SV and VP were isolated and total RNA was prepared. 10 μg of RNA were used in an RNAse protection experiment with $^{32}$P-labelled antisense riboprobes for Kgf, KgfR and cyclophilin. Kgf mRNA levels showed a small decrease during the development of the CG and SV, and a large decrease during the development of the VP. KgfR mRNA levels showed a decrease in all three organs during development from neonate to adult. The decreases in the cyclophilin control RNA are most likely due to the onset of secretory protein synthesis, which is initiated at puberty.

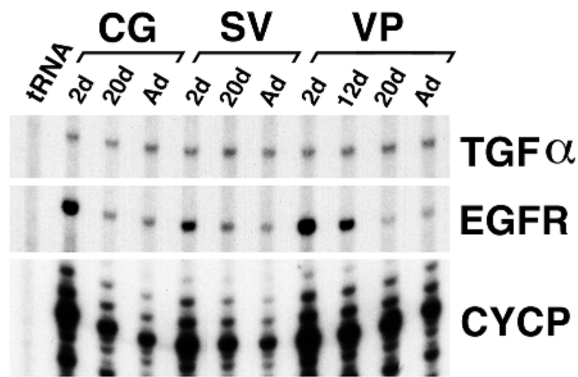


**Fig. 6.** Analysis of Tgfα mRNA and EgfR mRNA levels during development of the CG, SV and VP. Neonatal (2-day old; 2d), pubertal (20-day old; 20d) and adult (Ad) CG, SV and VP were isolated and total RNA was prepared. 10 µg of RNA were used in an RNAse protection experiment with $^{32}$P-labelled antisense riboprobes for Tgfα, EgfR and cyclophilin. The levels of Tgfα mRNA did not change during the development of the CG, SV and VP, however the levels of EgfR mRNA decreased in all three organs during development from neonate to adult.

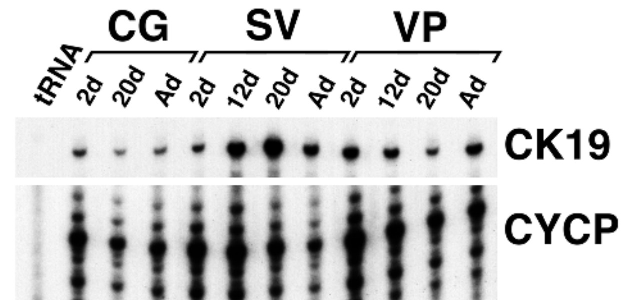


**Fig. 7.** Analysis of cytokeratin 19 mRNA levels during development of the CG, SV and VP. Neonatal (2-day old; 2d), pubertal (20-day old; 20d) and adult (Ad) CG, SV and VP were isolated and total RNA was prepared. 10 µg of RNA were used in an RNAse protection experiment with $^{32}$P-labelled antisense riboprobes for CK19 and cyclophilin (CYCP, a control for RNA integrity and loading differences). The levels of CK19 mRNA increased during development of the SV, but did not change in the CG or VP during development from neonate to adult.

mesenchymal/epithelial ratio) in the CG and VP remains similar between the neonate and adult, while the SV shows an increase of the epithelium. The mesenchymal/epithelial ratio in the SV appears to vary during the neonatal, pubertal and adult stages of development. There was a small increase in the epithelium between the neonatal and adult periods, but a greater increase between the neonatal and pubertal periods. It was interesting to note that the changes in the mesenchymal/epithelial ratio during development in vivo were similar to those observed in the neonatal organs grown in vitro.

Table 2 shows the average changes in mRNAs for Kgf, KgfR, Tgfα, EgfR and CK19 during development between neonatal and adult stages. Kgf and KgfR decreased in all organs during development, the largest decreases were observed in the VP. Tgfα mRNA levels remained relatively constant during development while EgfR mRNA levels decreased in all organs. CK19 mRNA levels in CG and VP were unchanged during development, while SV showed some developmental variation. In the SV, CK19 mRNA levels increased 4.6-fold between neonatal and pubertal periods, but then declined to only a 2.1-fold difference between neonatal and adult periods.

Kgf has been shown to mimic the effects of testosterone on the development of SV and VP grown in explant cultures. To further investigate the role of Kgf in the development of the VP we examined the effects of an anti-androgen on Kgf mediated growth. In the absence of testosterone, or in the presence of an anti-androgen (cyproterone acetate, CA), there was no development of the VP in explant culture (Fig. 8). The addition of testosterone or Kgf to the VP resulted in significant development. In the presence of both Kgf and CA development was inhibited, indicating that this anti-androgen could block Kgf mediated growth (Fig. 8).

**Table 2. Average changes in mRNA levels during development of CG, SV and VP organs in vivo**

| | CG | SV | VP |
|---|---|---|---|
| Kgf | −2.5 | −2.8 | −5.0 |
| KgfR | −4.4 | −4.6 | −13.3 |
| Tgfα | 2.2 | −1.4 | 1.1 |
| EgfR | −6.1 | −8.1 | −15.7 |
| CK19 | 1.3 | 2.1 (4.6) | −1.4 |

Changes in mRNA levels are shown as fold increase (or decrease) of 2-day old organs relative to adult, and were determined by quantitation of RNAse protection experiments using a phoshoimager (molecular dynamics). All points represent the average of two experiments.

## DISCUSSION

Development of CG, SV and VP is controlled by androgens, which most likely regulate organogenesis via paracrine factors produced in the mesenchyma of these organs. Androgen receptors in the mesenchyme are likely to regulate the expression of genes for factors which are secreted and act upon the epithelium. The identity of these paracrine acting factors is currently unknown, although Kgf has been suggested to possess many of the properties of such a paracrine regulator (Peehl and Rubin, 1995).

To determine whether Kgf or Tgfα might play important regulatory roles in development of the prostate and seminal vesicle, we examined the mRNAs for these molecules (and their receptors) to determine if they might exhibit some of the characteristics suggestive of an androgen-regulated paracrine factor. We observed changes in mRNA levels for Kgf, KgfR, Tgfα, EgfR and CK19 in the prostate and seminal vesicle, during development in vitro or in vivo. Both in vitro and in vivo, changes in levels of growth factor and receptor mRNAs correlated well with changes in the epithelial marker (CK19) levels suggesting that the observed variations were due to changes in the mesenchymal/epithelial ratio. Kgf mRNA levels decreased in all organs after development in the presence of testosterone, and increases in Tgfα and KgfR mRNAs observed in SV correlated with increases in the epithelial content. In vivo, levels of growth factor and receptor mRNAs were either unchanged or decreased during development from neonatal to adult periods. Androgen levels rise at puberty, and

development of the reproductive tract is completed (Corpechot et al., 1981). In the adult, androgens stimulate the production of seminal fluid proteins and maintain the tissue architecture of reproductive tract organs. Therefore it is unlikely that genes for Kgf, Tgfα or their respective receptors are directly regulated by androgens since their expression showed either no change or decreased as androgen levels increased.

Kgf has been shown to be expressed throughout development in almost all organs of the body (Finch et al., 1995; Mason et al., 1994). A mediator of androgen action might be expected to show some restriction to androgen target tissues as well as a localisation to the mesenchyme. While Kgf expression is mesenchymal it does not appear to show any androgen regulation in our studies. Furthermore, inactivation of Kgf by gene knock-out has revealed that Kgf plays an important role in hair follicle development, although the effect of inactivation of Kgf on the male reproductive tract was not reported (Guo et al., 1996). No problems of male fertility were noted suggesting that the secondary sex organs were likely to be functional.

Kgf has been proposed to be regulated by androgens in studies of cells grown in vitro (Yan et al., 1992). Furthermore the promoter of the Kgf gene has been shown to be regulated by androgens using transient transfection studies (Fasciana et al., 1996). In our studies we have not observed any increases of Kgf mRNA levels by androgens, either in organs grown in vitro or in vivo. Immunohistochemical studies of Kgf protein levels have not shown any changes in response to androgen withdrawal (J. Nemeth and C. Lee, personal communication). The large decrease in Kgf mRNA levels observed in the VP, both in vitro and in vivo, might suggest that androgens negatively regulate Kgf mRNA in this organ. However it is possible that the decrease is due to a loss of mesenchyme, although we did not observe a corresponding increase in the epithelial content of the organ. The decrease in Kgf may result from differentiation of the mesenchyme into fibroblasts and smooth muscle. This process takes place in vivo during the neonatal period and may occur during the course of the explant organ cultures. Fibroblasts are known to express Kgf but whether smooth muscle produces Kgf is unknown, thus the decrease in Kgf transcripts observed in the VP may be due to differentiation of the mesenchyme into one population of cells which express Kgf (fibroblasts) and one population which does not (smooth muscle). Decreases in Kgf mRNA in the CG and SV following culture with testosterone in vitro might be explained by the increase in the epithelial content of these organs, or by the differentiation of the mesenchyme although smooth muscle development in the VP is earlier than the CG or SV. The CG shows a 2-fold increase in epithelial content and a 2-fold reduction in the level of Kgf suggesting that the reduction in Kgf is a result of increased epithelial content of the organs. The SV shows a 2-fold reduction in Kgf mRNA but a 6-fold increase in the level of epithelium, which might suggest androgen regulation of Kgf mRNA, however, without a suitable mesenchymal marker with which to correlate this change in Kgf transcripts, the data must be interpreted with caution.

While Kgf has been shown to be regulated by progesterone (Koji et al., 1994) and glucocorticoids (Brauchle et al., 1995), in vivo, we have not observed regulation of Kgf mRNA by testosterone in vivo. It has been shown that the promoter of the Kgf gene can be regulated by androgens (Fasciana et al., 1996), this might be explained by the fact that receptors for androgen, progesterone and glucocorticoids all bind to a similar DNA sequence, known as a steroid response element. Thus it is possible that the putative steroid response element(s) present in the Kgf promoter might mediate responses to all three steroids under transient transfection conditions, although other tissue-specific promoter elements may control the regulation of the Kgf gene in vivo.

Development of organs in vitro in the presence of Kgf gave suprising results. While Kgf treatment resulted in development of the SV, VP and to a lesser extent the CG it had no effect upon any of the mRNAs tested, relative to untreated controls. This finding suggests that although Kgf is able to mimic growth induced by testosterone it does so without some of the associated changes in gene expression, and in particular changes in CK19 mRNA. As Kgf treatment stimulates epithelial development in SV and VP, it was suprising not to see an increase in the level of the epithelial marker, CK19. There are two possible interpretations of this result. It is possible that the Kgf treatment failed in these experiments; however, morphological inspection of the organs prior to RNA preparation indicated that significant development had been induced by KGF. A more

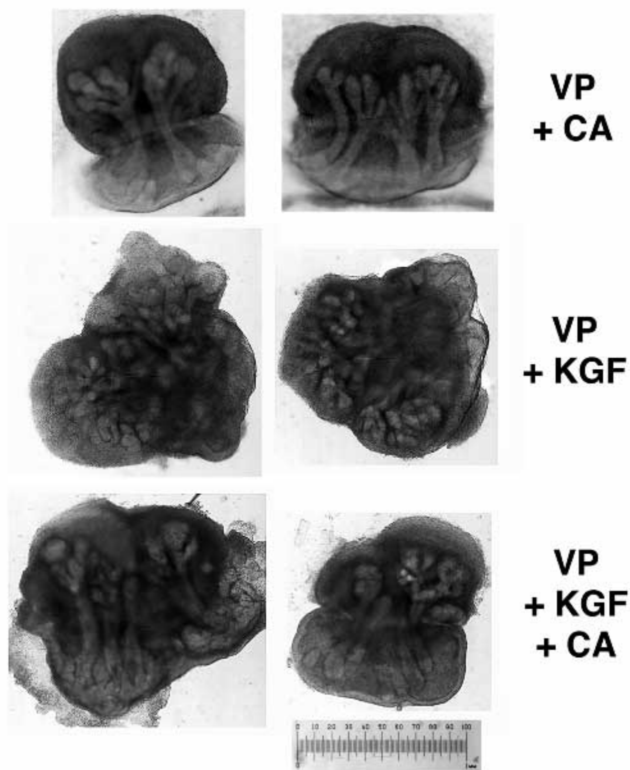


**Fig. 8.** Explant cultures of neonatal rat ventral prostates. VPs from zero-day old rats were microdissected and placed in serum-free organ culture for 6 days. Organs were treated with cyproterone acetate (CA), Kgf (K), or cyproterone acetate and Kgf. Organs treated with CA (top panels) show very similar development to those grown in the absence of testosterone (not shown). Addition of Kgf resulted in significant development (middle panels) while organs cultured in the presence of Kgf and CA showed very little development (bottom panels) and were very similar to organs grown in the presence of only CA. The scale bar shown corresponds to 1 mm.

likely interpretation might be that the epithelium induced by Kgf treatment was not differentiated in the same way as that induced by testosterone treatment. Indeed, if Kgf were stimulating the epithelial androgen receptor (see below) this might result in a different pattern of growth compared to that induced by mesenchymal androgen receptors.

Changes observed in CK19 mRNA levels in vitro correlated well with changes observed in vivo, suggesting that the in vitro model accurately reflects in vivo development with regard to androgen induced development. Furthermore, it was interesting to note that the SV showed differences in mesenchymal/ epithelial ratio during development (both in vitro and in vivo) compared to the CG and VP. The SV is derived from the Wolffian duct while the CG and VP originate from the urogenital sinus, which could account for the difference in the changes in mesenchymal/epithelial ratio observed between these organs.

The observation that Kgf mediated growth could be blocked with an anti-androgen was particularly important for the interpretation of results suggesting that Kgf might be a mediator of androgen-regulated growth. Treatment of neonatal VP and SV with Kgf has been shown to result in growth and development similar to that induced by testosterone (Alarid et al., 1994; Sugimura et al., 1996). This supported the hypothesis that Kgf might be a mediator of androgen action. However, since we have shown that treatment with an anti-androgen is able to block Kgf-induced development, an alternative interpretation is possible. Kgf and other growth factors have been demonstrated to increase expression of androgen regulated reporter gene constructs (Culig et al., 1995, 1994). This effect can be blocked by anti-androgens and is thought to result from growth factor induced phosphorylation of the androgen receptor. Therefore, it is possible that the growth and development induced by Kgf, in organs grown in vitro, arises from Kgf stimulating genes normally regulated by androgens. Indeed, we have recently shown that Kgf is able to affect the expression of androgen regulated secretory proteins in the prostate, indicating that Kgf is able to regulate endogenous androgen target genes (E. Lopes, personal communication). Thus it seems likely that although Kgf is not a direct mediator of androgen action, it is able to mimic androgen action perhaps by activation of the androgen receptor. In our experiments we propose that development induced by testosterone or Kgf may be arising through different mechanisms. While testosterone stimulates development via mesenchymal androgen receptors and presumably via paracrine acting factors, development elicited by Kgf treatment may result from KgfR activation of the androgen receptor in the epithelial compartment. It is tempting to speculate that in vivo the androgen receptor and growth factor pathways (perhaps including Kgf) may interact. Neonatal development of the male sex accessory organs occurs during periods of low circulating testosterone levels, and thus interaction between growth factor and testosterone signalling might provide a mechanism to maintain androgen regulated development during periods of low androgen concentration.

Anti-Kgf antibodies have been shown to block testosterone induced growth and branching morphogenesis in the SV and VP. This observation has been interpreted as suggesting that Kgf is therefore a mediator of androgen action, however an alternative interpretation is that by blocking Kgf action mesenchymal/epithelial interactions are sufficiently impaired to inhibit androgen-induced development. Indeed, Kgf may be required for mesenchymal/epithelial interactions in many organs of the body and thus inhibition of Kgf action could disrupt organogenesis in a non-specific manner. In support of this, inhibition of Kgf and KgfR has been shown to disrupt lung branching morphogenesis (Post et al., 1996).

In conclusion, Kgf has been implicated as a mediator of mesenchymal/epithelial interaction, and has been proposed to act as an androgen regulated mediator of development in the male reproductive tract. Our results presented here show no evidence for regulation of Kgf transcripts by testosterone in vivo or in organs grown in explant culture, and that an anti-androgen can inhibit Kgf-induced development suggesting that the androgen receptor may act as an effector of Kgf under our experimental conditions. Future studies to determine if Kgf and androgen receptor signalling interact in vivo will be required.

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