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Effects of Various Androgens and Growth Factors
on Prostatic Development In Vitro

by

Barbara Ann Foster

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Anatomy

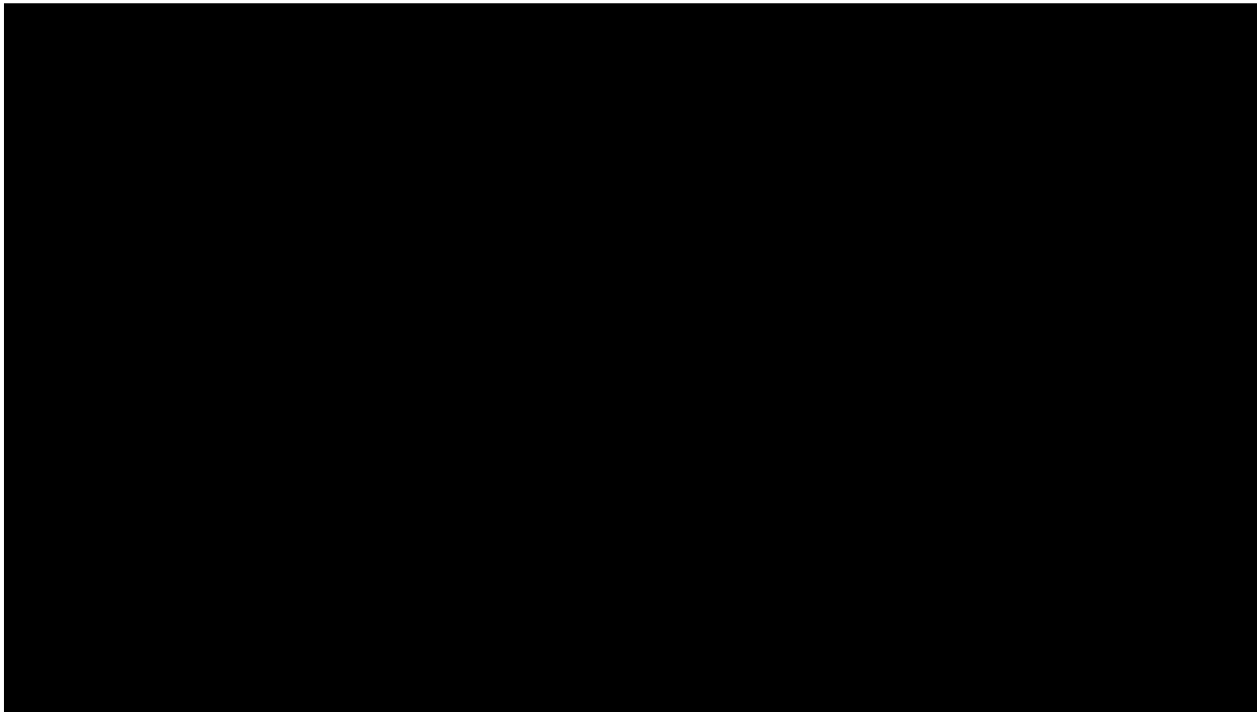
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Barbara Ann Foster

Forward

A river may be compared to life; always moving, ever changing. The rapids represent the obstacles and hardships we all encounter from time to time. The challenges to be faced squarely and taken with enthusiasm. The slow peaceful stretches are like the quiet uneventful days we never see enough of.

Often the river breaks up into several channels and one must make a decision as to which course is best. In life, as on the river, we can't be sure what lies ahead around the bend. But we can look forward to the unknown as a challenge and an opportunity rather than as a possible disaster.

Watch and study the river. It has much to teach about life.

James M. Quinn

Handbook to the Rogue River Canyon

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I wish to express my appreciation to the Outdoor Unlimited boating community and all the rivers I have run. White water rafting got me through some "interesting times".

Abstract

**Effects of Various Androgens and Growth Factors on Prostatic
Development *in vitro***

The effect of androgens and growth factors on ductal branching morphogenesis of neonatal anterior and ventral prostate (AP and VP) were assayed *in vitro*. Four parameters were measured: epithelial area, epithelial perimeter, node number, and form factor. At all concentrations testosterone (T), dihydrotestosterone (DHT) and 5 α -androstane-3 α ,17 β -diol (3 α -diol) were equally effective at inducing ductal branching morphogenesis. Metabolism studies indicated that while there was conversion of T to DHT during the culture period, this alone was insufficient to account for the activity of T. However, the conversion of 3 α -diol to DHT was sufficient to account for the activity of 3 α -diol. Metribolone and 7 α -methyl-19-nortestosterone, synthetic androgens which cannot be 5 α -reduced to DHT, were also potent inducers of ductal branching morphogenesis. Androstanedione, androsterone, 5 α -androstane-3 β ,17 β -diol, and 5 β -androstane-3 α ,17 β -diol were relatively ineffective in inducing ductal branching morphogenesis. Exogenous transforming growth factor-beta inhibited T-induced ductal branching morphogenesis and reduced epithelial area. Neutralizing antisera to transforming growth factor-alpha (TGF- α) completely inhibited T-induced ductal branching morphogenesis. However, neither exogenous epidermal growth factor nor TGF- α alone could induce ductal branching, and both factors inhibited T-induced ductal branching morphogenesis. Exogenous hepatocyte growth factor, FGF-1/aFGF (fibroblast growth factor-1/acidic FGF) and FGF-2/bFGF (basic FGF) had no effect on ductal branching morphogenesis in the presence or absence of T. In the VP, FGF-7/KGF (keratinocyte growth factor) induced ductal branching morphogenesis in the absence of T, and neutralizing anti-KGF inhibited T-induced ductal branching morphogenesis. In contrast, in the AP, FGF-7 at high doses elicited increased epithelial area but did not induce ductal branching morphogenesis in the

absence of T, and anti-KGF did not affect T-induced development of the AP. Neutralizing antisera to TGF- α blocked the effects of FGF-7/KGF in the ventral prostate. Reverse transcriptase-polymerase chain reaction and RNase protection assay indicate that TGF- α , FGF-7/KGF and their receptors are expressed in AP and VP in the presence and absence of T.

Gerald R. Cunha

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INTRODUCTION

Significance

Proliferative diseases of the prostate, benign prostatic hyperplasia (BPH) and prostate cancer are prevalent among elderly men. Prostatic adenocarcinoma is the most common cancer in men with 120,000 new cases and an estimated 35,000 deaths per year in the United States (Boring et al., 1992; Chiarodo, 1991). The incidence of prostatic cancer is expected to continue to increase yearly, having already surpassed lung cancer as the most commonly diagnosed form of cancer in men in the United States. The incident of BPH increases from 23% in men at 40 years of age to 88% by the ninth decade (Berry et al., 1984). BPH afflicts 75% of men over the age of 50 years, and approximately 30% of men over 60 years will require surgical therapy for BPH (Coffey and Pienta, 1987; McNeal, 1983). The prevalence of BPH and prostatic cancer is on the rise due to increased longevity. Unfortunately, treatment of these diseases, especially of prostate cancer, has not significantly altered the morbidity or mortality of patients with these diseases.

J. E. McNeal postulated that the etiology of BPH is due to re-expression of embryonic inductive capability of the prostatic stroma. BPH develops initially as stromal nodules, which are subsequently invaded by proliferating glandular epithelium (McNeal, 1984). Experimental support for this hypothesis comes from the ability of adult prostatic epithelium to respond to embryonic prostatic mesenchyme of the urogenital sinus (Chung et al., 1984; Cunha et al., 1987; Cunha et al., 1980b; Norman et al., 1986). It has been postulated that in malignancy, cells inappropriately express growth and differentiation properties characteristic of some earlier point in development. This has lead to the idea that return of a malignant cell to its embryonic environment can influence its growth and differentiative properties (Pierce, 1974; Pierce and Cox, 1978; Pierce et al., 1978). This concept is the basis for differentiation therapy, whereby inducers of differentiation might be able to cause proliferating tumor cells to differentiate, and thus, reduce their growth rate

and metastatic potential. Studies suggest that malignant cells can participate in normal development and that tissue environment can influence the behavior of tumor cells (Cooper and Pinkus, 1977; De Cosse et al., 1973; De Cosse et al., 1975; Fujii et al., 1982; Fukamachi et al., 1986; Fukamachi et al., 1987; Mackenzie and Hill, 1984; Mackenzie et al., 1979; Mintz, 1978; Pierce and Cox, 1978). For example, in the prostate, embryonic mesenchyma, which are able to induce prostatic epithelial development, are also able to regulate growth and differentiation of the Dunning prostatic adenocarcinoma (Hayashi and Cunha, 1991; Hayashi et al., 1990; Wong et al., 1992). The isolation and characterization of factors that influence and regulate prostatic growth and differentiation could lead to a better understanding of the etiology of BPH and could be instrumental in developing better and more effective means of treatment or prevention of BPH and prostatic cancer.

Normal Development

During normal development, the reproductive tract goes through an ambisexual stage in which the gonad is undifferentiated and the internal genitalia are composed of the mesonephric tubules, urogenital sinus (UGS), and Wolffian and Müllerian ducts (Cunha, 1986; Cunha, 1994). The timing of the ambisexual stage differs for the various anlagen, such that the various structures of the so-called ambisexual stage form and undergo sex differentiation at different times. The gonad is the first structure to undergo sex differentiation. In males the gonad differentiates into the testes which produces two hormones critical for normal male development: testosterone and Müllerian inhibiting substance (MIS) (Jost, 1970; Jost, 1972). Under the influence of these two testicular hormones the male genital tract develops from two embryonic anlagen: the Wolffian ducts and UGS. MIS produced by Sertoli cells induces regression of the Müllerian ducts in males (Donahoe et al., 1987). The first effect of testosterone (or its metabolites) is the prevention of programmed cell death in the Wolffian duct. Androgens subsequently induce the mesodermally derived Wolffian ducts to give rise to the epididymis, ductus deferens,

ampullary gland, seminal vesicle and ejaculatory duct (Wilson et al., 1981b). The mesonephric tubules associated with the Wolffian ducts develop into the efferent ducts. Under the influence of testicular androgens the UGS, whose epithelium is endodermally derived, gives rise to the prostate, bulbourethral gland, urethra, periurethral glands and contributes to the bladder. The differentiation of the reproductive tract into the female phenotype is not dependent on hormonal stimulation during fetal periods (Jost, 1953). In the female the Wolffian ducts regress without the stabilizing effects of androgens, and the Müllerian duct survives because MIS is not produced. In females the Müllerian duct develops into the oviduct, uterus, cervix, and upper vagina, while the UGS gives rise to the lower vagina, urethra, and contributes to the bladder. Recently an estrogen receptor knockout (ERKO) transgenic mouse was described. These ERKO mice are not able to respond to estrogens, and yet sex differentiation in female mice is normal (Korach, 1994). Thus, estrogens are not required for sex differentiation in females, although postnatal growth of the female genital tract is dependent upon estrogens.

Development and growth of the male accessory sexual glands are androgen-dependent. Production of androgens by the fetal testes begins early during the ambisexual stage of sex differentiation and remains high until birth, after which androgen levels fall and remain low until puberty (Corpechot et al., 1981; Pointis et al., 1980; Winter et al., 1981). Fetal castration during the ambisexual stage of development blocks male sex differentiation (Jost, 1953). Likewise, chemical castration during sex differentiation by treatment of pregnant females with anti-androgens suppresses masculine development (Elger et al., 1974; Neumann and Elger, 1984; Neumann et al., 1970; Neumann et al., 1974). Development of the prostate, seminal vesicle and bulbourethral gland continues postnatally and is dependent on androgens during this stage even though androgen levels are low (Cooke et al., 1987; Corpechot et al., 1981; Donjacour and Cunha, 1988a; Shima et al., 1990).

The prostate gland is dependent on androgens for development, differentiation and function (Cunha et al., 1987). The prostate develops from the urogenital sinus as solid cords of epithelium that bud into the surrounding mesenchyme just below the bladder. Prostatic buds appear in the rat at 19 days and in mouse at 17.5 days of gestation. At this stage, androgen receptors (AR) are barely detectable in the urogenital sinus epithelium (UGE), while urogenital sinus mesenchyme (UGM) is intensely positive for AR (Cooke et al., 1991; Hayward et al., 1995; Prins and Birch, 1993; Shannon and Cunha, 1983; Takeda and Chang, 1991; Takeda et al., 1985). AR are undetectable by immunocytochemistry in prostatic buds emerging from the UGS (Hayward et al., 1995; Takeda et al., 1985), and are not detectable in prostatic epithelial cells until shortly after birth (Cooke et al., 1991; Hayward et al., 1995; Prins and Birch, 1995; Shannon and Cunha, 1983; Takeda and Chang, 1991). Throughout prostatic development the mesenchyme remains intensely AR positive. During early post-natal development, rat and mouse prostatic epithelium becomes intensely AR-positive as the prostatic buds undergo extensive ductal branching morphogenesis, a process involving epithelial proliferation, extracellular matrix remodeling, lumen formation, epithelial cytodifferentiation and ultimately the expression of lobe-specific patterns of branching morphogenesis (Aumuller et al., 1994; Hayashi et al., 1991; Lee et al., 1990).

The rat prostate is composed of 5 lobes: dorsal, lateral type 1 and lateral type 2 (These three lobes are often collectively referred to as the dorsolateral prostate), ventral prostate and the anterior prostate (also called the coagulating gland). Each lobe of the rat prostate has a specific pattern of secretory proteins and a characteristic pattern of epithelial ductal branching (Aumuller et al., 1994; Hayashi et al., 1991; Lee et al., 1990). The main secretory products of the dorsal, lateral type I and anterior prostate are DP-1 and DP-2 (Hayashi et al., 1991; Kinbara and Cunha, 1994; Wilson et al., 1981a). Probasin (M-40) is the main secretory product of the lateral type 2 prostate (Hayashi et al., 1991; Kinbara and Cunha, 1994). The predominant secretory product of the ventral prostate is prostatein,

which is a tetrameric protein known by many different names: estramustine binding protein, prostatic steroid binding protein, or α -protein. Prostatein is composed of three subunits (C1, C2, C3) (Heyns and DeMoor, 1977; Lea et al., 1979). When androgen levels rise at puberty prostatic epithelium undergoes functional differentiation and expresses secretory products. Prostatic secretions are rich in fructose, zinc ions and proteins which contribute to the semen. In rodents, these secretions are responsible for the formation of the copulatory plug which is thought to prevent the female from being fertilized by subsequent partners (Aumuller et al., 1994). However, the prostate is not required for male fertility, since removal of the anterior prostate from guinea pigs or ventral prostate from rats does not prevent insemination and subsequent fertilization (Engle, 1926; Gunn and Gould, 1958).

Each lobe of the rat prostate also has a characteristic pattern of epithelial ductal branching (Aumuller et al., 1994; Hayashi et al., 1991; Kinbara and Cunha, 1994; Lee et al., 1990). Dorsal prostate has a simple elm tree pattern of branching with 5-6 main ducts and moderate epithelial infolding (Hayashi et al., 1991; Kinbara and Cunha, 1994). Lateral type I has a palm tree pattern of branching with 5-7 main ducts per gland and few epithelial infoldings (Hayashi et al., 1991; Kinbara and Cunha, 1994). Lateral type II has a bushy pattern of branching with 5-6 main ducts per gland and many epithelial infoldings (Hayashi et al., 1991; Kinbara and Cunha, 1994). Ventral prostate has a complex elm tree pattern of branching with 2-3 main ducts per gland and few epithelial infoldings (Hayashi et al., 1991). Anterior prostate has a coral pattern of branching with 1 main duct and extensive epithelial infoldings (Hayashi et al., 1991).

Androgen Action

Androgens exert their biological activity by acting through the androgen receptor (AR). The AR is a hormone-activated transcriptional regulatory protein that mediates androgen-regulated gene expression. Hormone binding is required for transcriptional

activation and regulation of targeted genes (Liao et al., 1985). Upon ligand binding, the AR undergoes a conformational change in the nucleus which allows it recognize and bind to cis-acting enhancer sequences, called androgen response elements (ARE), associated with the flanking regions of androgen regulated genes. In the nucleus the AR forms a transcriptional complex with other proteins and regulates gene transcription (Zhou et al., 1994).

The AR is a member of the large family of DNA-binding zinc-finger proteins which have a common structural arrangement of their major functional domains (O'Malley, 1990; Zhou et al., 1994). The AR consists of three structural domains: 1.) a variable N-terminal domain, 2.) a highly conserved DNA-binding domain, and 3.) a moderately conserved C-terminal ligand binding domain. The DNA-binding domain contains nine highly conserved cysteine residues common to the family of steroid receptors which are involved in the zinc finger tertiary structure of the receptor (Freedman and Luisi, 1993). The N-terminal domain is important in transcriptional activation of the activated receptor; while the carboxy-terminal region is responsible for high affinity androgen binding and inhibits transcriptional activity of the receptor in the absence of ligand (Zhou et al., 1994). While a great deal is known about the mechanism of action of other steroid receptors as transcriptional factors, the AR is a recently cloned member of the steroid receptor family and considerable work is still needed to determine the mechanism of action of AR. Comparison with the other members of the steroid receptor family may give insight into how the AR may work, but these mechanisms of action in many cases still need to be proven for the AR.

Transcriptional factors in general and steroid receptors in particular require dimerization in order to become activated (Jones, 1990). Dimerization is required for transcriptional activity for most of the steroid receptors which form homodimers, even though this has not yet been confirmed for the AR (Zhou et al., 1994). Data suggests that the AR can heterodimerize with an androgen receptor binding protein (ARBP) (Zhou et al.,

1994). Such protein-protein interactions involving steroid receptors are thought to be important for specific DNA binding and transcriptional complex activation. There is a potentially large number of nuclear proteins that could interact with AR. These factors or co-regulators could be cell-type specific and may be important in cell-specific gene activation accounting in part for the cell specific effects that androgens have on their target cells (Glass et al., 1990; Zhang et al., 1992).

The NH₂-terminal domain is the most variable domain of the steroid receptor. This domain is required for transcriptional activation (Simental et al., 1992). The suggested mechanism of action of transcriptional activation is that the NH₂-terminal domain interacts with nuclear factors that are involved in transcriptional initiation. It is believed that the steroid-binding domain acts to repress activation of the NH₂-terminal domain in the absence of hormone by inhibiting its binding to other nuclear regulatory proteins required for activation (Zhou et al., 1994). Further work is needed to identify the transcriptional regulatory proteins with which the AR interacts during androgen-induced gene transcription. It is possible that some of these proteins will vary depending on the cell type and state of differentiation of the cell.

Phosphorylation is a key regulatory mechanism by which cell function is controlled. Presently steroid receptor phosphorylation is under intense investigation (Ignar-Trowbridge et al., 1993). Progesterone receptor (PR) and glucocorticoid receptor (GR) are phosphorylated primarily on serine and threonine residues, while estrogen receptor (ER) is phosphorylated on tyrosine residues. Phosphorylation of PR enhances hormone binding and is linked to transcriptional activation. While, seven phosphorylation sites have been identified on GR, phosphorylation does not appear to be necessary for GR function. Recent studies indicate that the AR is also phosphorylated during nuclear processing, and it has been suggested that phosphorylation is required for hormone binding to the AR (van Laar et al., 1991). Further studies are needed to determine the role of phosphorylation in steroid receptor function.

It has recently been proposed that the steroid response pathway may be coupled with other cellular signal transduction mechanisms (such as growth factors) to confer an additional layer of control on hormone-mediated transcriptional responses (Ignar-Trowbridge et al., 1993; Nordeen et al., 1994). Specifically in the mouse uterus, epidermal growth factor (EGF) and transforming growth factor- α (TGF- α) elicit estrogen receptor (ER)-dependent transcription of a reporter gene containing an estrogen response element (ERE) (Ignar-Trowbridge et al., 1993). A recent report by Culig et al (1994) suggests that a similar convergence of signaling pathways may exist between the AR and growth factors (Culig et al., 1994). This is an intriguing idea that warrants further investigation.

Androgen Metabolism

Testosterone (T) is the principle circulating androgen produced by the testes and is readily taken up by the prostate. T is metabolized in accessory sex tissues (Isaacs and Coffey, 1981) including the prostate where it is converted to several androgenic metabolites (Baulieu et al., 1968; Krieg et al., 1975; Krieg and Voigt, 1976; Orlowski and Clark, 1985a; Orlowski and Clark, 1985b; Robel et al., 1971; Tenniswood et al., 1982; Warren and Ahmad, 1978). (See Figure 1) The liver is a major site of androgen metabolism and is responsible for clearance of androgens from the body (Isaacs and Coffey, 1981).

There are several key aspects of testosterone metabolism in the prostate. Testosterone is irreversibly metabolized to dihydrotestosterone (DHT) by the enzyme 5 α -reductase (Isaacs and Coffey, 1981). There are two isoforms of the 5 α -reductase enzymes, type I and type II. 5 α -reductase type II is the active form found in the prostate (Thigpen et al., 1992a; Thigpen et al., 1992b; Thigpen et al., 1993) and has an optimal pH of about 5.5. Finasteride is a 4-azasteroid which specifically inhibits the activity of 5 α -reductase type II, but not type I (Andersson et al., 1991; Andersson and Russell, 1990; Jenkins et al., 1992; Thigpen et al., 1992b). DHT is thought to be more potent than T, having about a 10-fold

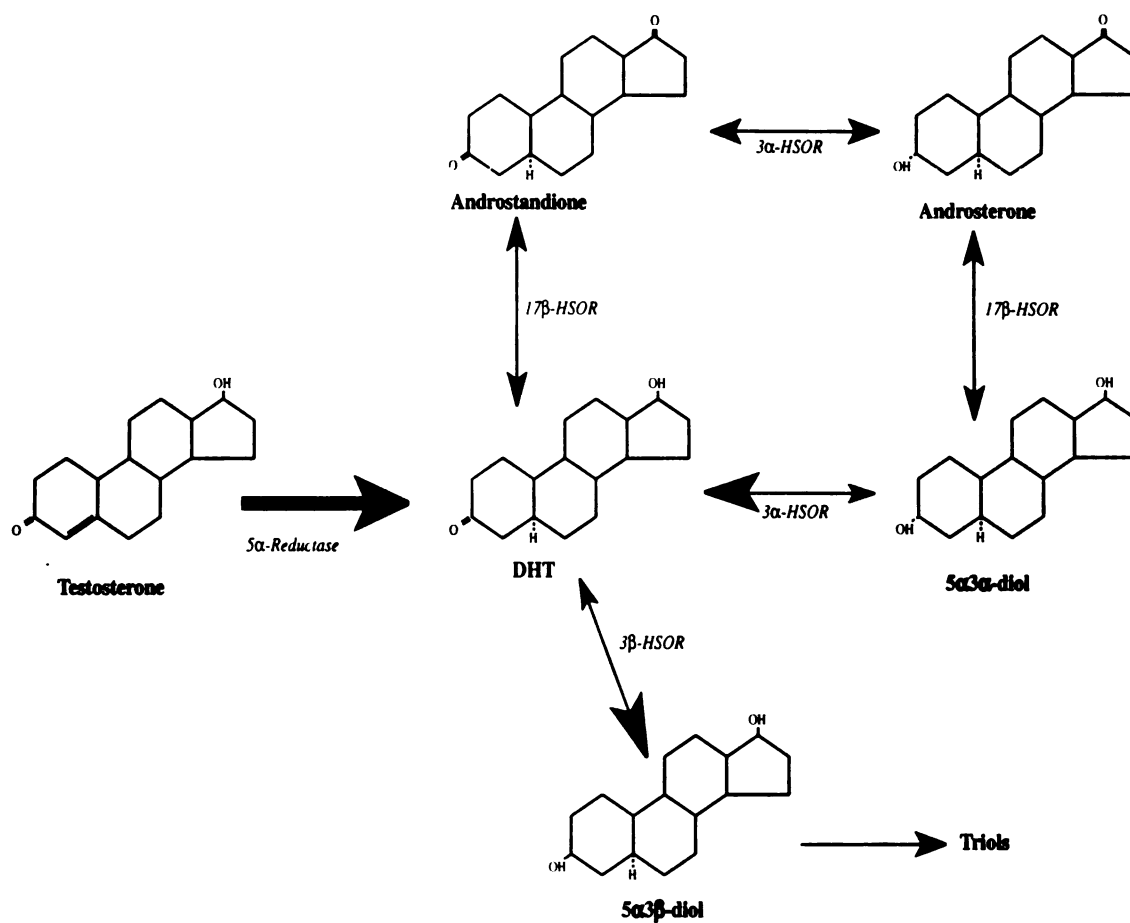
higher affinity for the AR than T (Baulieu, 1970; Carlson and Katzenellenbogen, 1990; Grino et al., 1990b; Liao et al., 1973; Maes et al., 1979; Mainwaring, 1969; Wilbert et al., 1983; Wilson and French, 1976; Wilson and Gloyna, 1970). DHT is also more potent than T in restoring prostatic weight following castration than T in restoring prostatic weight following castration (Morali et al., 1994; Walvoord et al., 1976). DHT can be metabolized by three enzymes into two pathways. One leads to a circular pathway in which the products can be interconverted to reform DHT (Fig. 1). All of these reactions are reversible and all intermediates of this pathway have been reported to have androgenic properties. The interconversion of DHT to 5 α -androstane-3 α ,17 β -diol (3 α -diol) is favored in the direction of DHT (Krieg et al., 1975). It is believed that the metabolites of this pathway exert their androgenic effect by conversion to DHT (Bruchovsky and Wilson, 1968; Tenniswood et al., 1982). This pathway is thought to be involved in the tight local regulation of the availability of DHT in the prostate (Anderson and Liao, 1968; Bruchovsky and Wilson, 1968).

The other pathway of DHT metabolism results in non-androgenic products (triols) that cannot be back converted to DHT. DHT is reduced to form 5 α -androstane-3 β ,17 β -diol (3 β -diol), which is rapidly and irreversibly hydroxylated at either the 6 or 7 position to form 6 α - or 7 α -triol (Hammond, 1978; Isaacs et al., 1979a; Isaacs et al., 1979b). The triol metabolites do not have androgen activity when tested by classical bioassays in rats (Isaacs et al., 1979a). It is believed that this pathway is responsible for clearing androgenic metabolites from the prostate.

It has been hypothesized that the effects of testosterone are not due to the activity of testosterone alone but to the summation of effects of all of its metabolites. The action of testosterone is elicited through the local formation of androgenic metabolites in target cells (Baulieu, 1970; Baulieu, 1973). T is metabolized to several compounds in the prostate, and thus, T acts as a "prohormone" in this tissue (Baulieu, 1970; Walsh et al., 1974;

Figure 1

Metabolism of Testosterone by Adult Rat Prostate



HSOR = hydroxysteroid oxidoreductase

Wilson and Gloyna, 1970). There has been extensive research into the androgenic potency of testosterone metabolites in the prostate with an emphasis on DHT, 3 α -diol and 3 β -diol.

In addition to testosterone and DHT, both of which bind with a high affinity to the AR, 3 α -diol is an important androgen even though it has low affinity for the androgen receptor (Baulieu et al., 1968; Walsh et al., 1974; Wilson and Gloyna, 1970). Administration of 3 α -diol to castrated male rats increases the weight of the ventral prostate and seminal vesicle. Thus, 3 α -diol mimics the effects of T and DHT. Unexpectedly, 3 α -diol is more potent than either T or DHT in restoring ventral prostate and seminal vesicle weight following castration (Isaacs and Coffey, 1981; Morali et al., 1994). In rats 3 α -diol is also able to restore male sexual behavior with better copulatory performance than either T or DHT. In rat ventral prostate organ cultures, the epithelium proliferates in response to 3 α -diol, but has a less differentiated phenotype (Robel et al., 1971). In dogs, chronic administration of 3 α -diol or DHT can induce benign prostatic hyperplasia with 3 α -diol being slightly more effective than DHT. Both DHT and 3 α -diol are even more effective in inducing BPH when given in combination with estradiol. Indeed, the highest incidence of BPH is achieved when 3 α -diol is administered with estradiol (DeKlerk et al., 1979; Gloyna et al., 1970; Isaacs and Coffey, 1981; Walsh and Wilson, 1976). The interpretation of these studies is that 3 α -diol is back-converted to DHT, which binds to the AR to elicit the androgenic response (Bruchovsky and Wilson, 1968). This interpretation is based on the finding that following 3 α -diol treatment, DHT is the androgen found in the nucleus (Anderson and Liao, 1968; Bruchovsky and Wilson, 1968; Gloyna and Wilson, 1969; Mainwaring, 1969; Robel et al., 1971; Wilson and Gloyna, 1970). Steroid metabolism studies using radiolabeled 3 α -diol show that the majority of 3 α -diol is back converted to DHT (Morfin et al., 1977; Robel et al., 1971). However, why 3 α -diol is more potent than DHT itself has not been adequately explained.

Unlike 3 α -diol, 3 β -diol is not a potent androgen. 3 β -diol does not effect the weight of ventral prostate (VP) or seminal vesicle when injected into castrated male rats and

is unable to restore male sexual behavior (Morali et al., 1994). In VP organ cultures 3β -diol does not induce the epithelium to proliferate, but 3β -diol does maintain the differentiated phenotype and secretory activity of the epithelium. In this system little DHT or 3α -diol is produced when 3β -diol is added to the culture medium (Robel et al., 1971). 3β -diol binds with low affinity to AR and is not able to compete with DHT for the androgen receptor (Baulieu and Jung, 1970). 3β -diol is not converted back to DHT because it is rapidly metabolized to the triols. This rapid metabolism is thought to account for the weak androgenic properties of 3β -diol (Hammond, 1978; Isaacs et al., 1979a; Isaacs et al., 1979b) compared to the potent androgenic effects of 3α -diol which can be converted back to DHT.

It is believed that 5α -reductase type II activity and the formation of DHT are essential for the development of the prostate and that DHT is the active androgen in the developing prostate and external genitalia (Cantu et al., 1980; Fisher et al., 1978; George and Peterson, 1988; Imperato-McGinley, 1984; Imperato-McGinley et al., 1985; Imperato-McGinley et al., 1992a; Imperato-McGinley et al., 1974; Imperato-McGinley et al., 1991; Imperato-McGinley et al., 1984; Imperato-McGinley et al., 1980; Imperato-McGinley et al., 1992b; Kuttann et al., 1979; Perez-Izquierdo et al., 1990; Peterson et al., 1977; Price et al., 1984; Raber and D'Ambrosio, 1986; Saenger et al., 1978; Siiteri and Wilson, 1974; Walsh et al., 1974; Wilson and Lasnitzki, 1971). Prostatic development is partially or completely inhibited when T is unable to be metabolized to DHT such as when the 5α -reductase enzyme is defective or when an inhibitor of 5α -reductase is administered (Cantu et al., 1980; Fisher et al., 1978; George and Peterson, 1988; Imperato-McGinley et al., 1985; Imperato-McGinley et al., 1974; Imperato-McGinley et al., 1991; Imperato-McGinley et al., 1980; Imperato-McGinley et al., 1992b; Kuttann et al., 1979; Peterson et al., 1977; Price et al., 1984; Saenger et al., 1978; Walsh et al., 1974). Human male patients with 5α -reductase deficiency have a defect in the type II enzyme, inefficiently convert T to DHT, and have feminized external genitalia. Prostates are small or absent,

even though patients with 5 α -reductase deficiency have normal Wolffian derivatives (epididymis, vas deferens, seminal vesicle). Such findings have led to the conclusions that: 1.) Masculinization of the external genitalia and development of the prostate are dependent on DHT, and 2.) masculine development of Wolffian-derived structures is dependent on T (Imperato-McGinley, 1984; Imperato-McGinley et al., 1974; Imperato-McGinley et al., 1984; Peterson et al., 1977; Wilson et al., 1993; Wilson, 1992). Supporting these conclusions are the findings that treatment of pregnant rats with 5 α -reductase inhibitors elicits feminization of the external genitalia and inhibits prostatic development while development of the seminal vesicle is not affected (George and Peterson, 1988; Imperato-McGinley et al., 1985). However, such prenatally treated male rats in adulthood have normal reproductive capacity and normal reproductive tract morphology (Iguchi et al., 1991), suggesting that acute inhibition of prostatic development by 5 α -reductase inhibitors early in development (prenatally) can be overcome by adulthood. Additionally, DHT restores prostatic development when given to pregnant rats in combination with a 5 α -reductase inhibitor (George and Peterson, 1988).

Supporting evidence that DHT is the active androgen in the prostate comes from the ontogenic pattern of 5 α -reductase activity. 5 α -reductase activity and DHT formation in the UGS are detectable both before and during masculinization of the external genitalia and UGS (prostatic development) (Siiteri and Wilson, 1974; Wilson and Lasnitzki, 1971). In contrast, 5 α -reductase activity is undetectable during initial sex differentiation of the Wolffian duct, that is, during the period when androgen prevents programmed cell death in the Wolffian duct. However, 5 α -reductase is detectable during actual organogenesis of the Wolffian duct into seminal vesicles, vas deferens and epididymis (Siiteri and Wilson, 1974). Recent studies by Tsuji and collaborators (1994) demonstrate a cranial-caudal gradient of 5 α -reductase in the Wolffian duct. Production of DHT by 5 α -reductase is lowest in the cranial region of the Wolffian duct that develops into the epididymis and highest in the caudal region of the Wolffian duct that develops into the seminal vesicle

(Tsuji et al., 1994). Thus, the concept, that T is the hormone for the Wolffian duct and DHT is the hormone for the prostate and external genitalia, is currently being re-examined.

In adulthood, DHT is the major hormone identified in prostatic nuclei, which supports the idea that DHT is the active androgen in the adult prostate (Anderson and Liao, 1968; Bruchovsky and Wilson, 1968; Gloyna and Wilson, 1969; Mainwaring, 1969; Robel et al., 1971; Wilson and Gloyna, 1970). In VP organ cultures DHT is found to be more active than T in maintaining epithelial differentiation and secretory activity. DHT in this system also induces epithelial hyperplasia (Robel et al., 1971). While both T and DHT restore prostatic function and size in castrated rats, DHT is more potent than T at restoring prostatic weight (Morali et al., 1994; Walvoord et al., 1976). Clearly, DHT production plays a critical role in androgen action.

Synthetic analogs of T that cannot be 5α -reduced and converted to DHT have been developed, e.g., 17β -hydroxy- 17α -methyl-estra-4,9,11-trien-3-one (R1881 or methyltrienolone) and 7α -methyl-19-nortestosterone (MENT) (Bonne and Raynaud, 1975; Kumar et al., 1992). Both bind to the AR with high affinity and elicit androgenic responses in target organs (Agarwal and Monder, 1988; Bonne and Raynaud, 1975; Bonne and Raynaud, 1976; Bonne and Raynaud, 1977; Carlson and Katzenellenbogen, 1990; Kumar et al., 1992; Pousette et al., 1979). MENT increases the weight of VP and seminal vesicle in castrated rats. The activity of MENT is not blocked by 5α -reductase inhibitors but is inhibited by anti-androgens that block binding to the AR (Agarwal and Monder, 1988; Kumar et al., 1992). The androgenicity of non- 5α -reducible T analogs is thought to be due to their binding to the AR. Androgenicity of synthetic androgens in the prostate correlates with their binding affinity for the AR, indicating that androgens do not necessarily have to be 5α -reduced to be active (Liao et al., 1973). The affect of these synthetic androgens on the developing prostate is unknown.

Ductal Branching Morphogenesis

Development of the prostate is androgen-dependent. It is the epithelium which exhibits the most dramatic morphogenetic changes during perinatal and postnatal development. However, little is known about the mechanism of ductal branching morphogenesis of the developing prostate beyond its dependence on androgens. The mechanism of ductal branching morphogenesis has been studied much more extensively in the developing salivary gland and to a lesser extent the developing lung and mammary gland (Hardman and Spooner, 1992).

Ductal branching morphogenesis is a complex process involving many molecular events including: epithelial-mesenchymal interactions, cell proliferation, changes in cell shape mediated by intracellular microfilaments, cell-extracellular matrix (ECM) interactions, as well as, ECM deposition, degradation and remodeling (Hardman and Spooner, 1992). Experiments have demonstrated that the mesenchyme directs the pattern of ductal branching morphogenesis (Kratochwil, 1969; Lawson, 1974; Nogawa and Mizuno, 1981). Furthermore, the epithelium is dependent on both the ECM and other factors produced by the mesenchyme for ductal branching morphogenesis. Changes in cell shape have been suggested to be involved in cleft formation and are known to effect cell behavior and gene expression. Contraction of microfilaments located in bands at the epithelial cell basal and apical surfaces has been proposed as a mechanism of initiation of cleft formation (Spooner and Wessells, 1972).

An intact basal lamina is essential for maintenance of epithelial morphology and ductal branching morphogenesis in developing salivary gland. In the absence of an intact basal lamina microfilaments disorganize, suggesting that the basal lamina acts to stabilize microfilaments within the cell (Bernfield and Wessells, 1970). Proteoglycans are a major component of the basal lamina. Proteoglycans are composed of a protein core with glycosaminoglycans (GAG) attached and are thought to function as a coupling molecule between ECM components and cytoskeletal components within the cell. Proteoglycans

are produced by the epithelium, but the mesenchyme mediates GAG turnover via a neutral hyaluronidase (Bernfield et al., 1984). During ductal branching morphogenesis of the developing salivary gland, GAG turnover is greater on the lobules than in the clefts. This is thought to be due to an increase degradation of GAGs by the mesenchyme surrounding the lobules (Bernfield et al., 1972; Bernfield et al., 1984; Bernfield et al., 1973). Thus, remodeling of the basal lamina by the mesenchyme has been proposed as a key feature in the control of ductal branching morphogenesis.

Interstitial collagen (collagen type I and III) have been implicated in the stabilization of cleft formation during development of the salivary gland. Collagen has been proposed to function in cleft formation by stabilizing basal lamina components. Inhibition of collagen synthesis inhibits new cleft formation, but does not effect established clefts (Spooner and Faubion, 1980). During ductal branching morphogenesis of the developing salivary gland the epithelial surface continuously forms and reabsorbs small "practice" clefts, only a few of which are stabilized. Treatment of developing salivary glands with collagenase inhibitors results in enhanced ductal branching of the glands, presumably because branch points are non-selectively stabilized (Nakanishi et al., 1986). In contrast, treatment with collagenase reduces ductal branching in both the salivary gland and the lung (Nakanishi et al., 1986; Nogawa and Mizuno, 1981). This data suggests that collagenase is important in the regulation of cleft formation. Thus, during ductal branching morphogenesis regulation of both collagen deposition and degradation is important in cleft formation.

The mechanism coordinating the complex events which result in ductal branching morphogenesis is unknown, but growth factors are likely to be involved (Hardman and Spooner, 1992). EGF, TGF- α , FGF-1/aFGF (acidic FGF), FGF-2/bFGF (basic FGF) and TGF- β have been implicated in ductal branching morphogenesis. EGF and TGF- α has been implicated in epithelial proliferation and ductal outgrowth (Goldin and Opperman, 1980; Seth et al., 1993; Snedeker et al., 1991; Warburton et al., 1992). Recently EGF and TGF- α have also been implicated in the regulation of matrix-degrading metalloproteinases

(Ganser et al., 1991; Matrisian et al., 1992). FGF-1/aFGF can elicit ductal branching in lung epithelium grown in Matrigel in the absence of mesenchyme (Nogawa and Takaaki, 1995), suggesting that FGF-1/aFGF is important in ductal branching in the developing lung. FGF-2/bFGF interacts with the ECM via interaction with heparin and heparin sulfate proteoglycans. It has been suggested that FGF-2/bFGF is stored in the ECM and is released by hydrolysis during tissue remodeling (Gospodarowicz et al., 1986). Thus, FGF-2/bFGF could act as a local growth regulator during ductal branching morphogenesis.

TGF- β has been strongly implicated in the process of ductal branching morphogenesis, partly because of its ability to regulate ECM formation. TGF- β modulates cell-ECM interactions by increasing synthesis of major components of the ECM, controlling proteolytic degradation of ECM and regulating the expression of cell adhesion receptors (integrins) (Matrisian et al., 1992; Sporn et al., 1986). In the developing lung TGF- β colocalizes with interstitial collagen (collagen type I and III), fibronectin and GAGs in the mesenchyme during formation and stabilization of clefts (Heine et al., 1990). Moreover, exogenous TGF- β inhibits ductal branching morphogenesis in the developing mammary gland and lung, presumably through regulation of collagen and ECM formation, and regulation of ECM degrading enzymes (Matrisian et al., 1992; Silberstein, 1990; Silberstein and Daniel, 1987; Silberstein et al., 1992). Thus, ductal branching morphogenesis is a complex highly orchestrated series of interactions of which the exact mechanism are unknown. Cell shape changes involving the cytoskeleton, cell-ECM interactions, collagen and ECM deposition and degradation, proteoglycan turnover and growth factors have all been implicated as important factors in this complex event. More research is needed to understand how these events are controlled and coordinated.

Epithelial-Mesenchymal Interactions

It is well documented that prostatic development is androgen-dependent. The effects of androgens are most dramatically seen in the development of prostatic epithelium

and are mediated by the mesenchyme. Under androgenic influence the UGS epithelium (UGE) proliferates and forms prostatic buds that elongate and undergo ductal branching morphogenesis. During prostatic ductal morphogenesis the epithelium expresses AR, undergoes columnar cytodifferentiation and ultimately expresses prostatic specific secretory proteins. While fetal UGE is weakly AR-positive, AR in prostatic buds emerging from the UGS are undetectable until birth or shortly thereafter (Cooke et al., 1991; Prins and Birch, 1993; Shannon and Cunha, 1983; Takeda and Chang, 1991; Takeda et al., 1985). There has been considerable debate on the exact timing of AR expression in developing prostatic epithelium. Differences in sensitivity of detection methods or antibodies probably account for these discrepancies. It is believed that androgens act on the epithelium via the underlying AR-positive mesenchyme. The most compelling evidence of the role of mesenchyme as the mediator of androgens on epithelial development comes from tissue recombination experiments using tissues from the testicular feminized (Tfm) mice that lack functional AR due to a mutation in the gene encoding the AR (Cunha et al., 1981; Cunha and Lung, 1978; Donjacour and Cunha, 1993; Lasnitzki and Mizuno, 1980).

Tissue recombinant studies show that the mesenchymal component of the developing prostate mediates the effects of androgens on the epithelium. Homotypic recombinants of wild-type (wt) urogenital sinus mesenchyme (UGM) and wt epithelium develop into prostatic tissue (Cunha and Lung, 1978; Lasnitzki and Mizuno, 1980), that is, the epithelium undergoes branching morphogenesis, proliferates, expresses AR and differentiates into tall columnar secretory epithelium which expresses prostate specific secretory proteins (Donjacour and Cunha, 1993). Tissue recombinants of Tfm UGM + Tfm epithelium or Tfm UGM + wt epithelium do not develop into prostate (Cunha et al., 1980a). These recombinants are small and have a stratified vaginal epithelial phenotype. In heterotypic recombinants of wt UGM + Tfm epithelium, prostatic tissue develops, even though the Tfm epithelium is AR-negative. The Tfm epithelium undergoes extensive ductal branching morphogenesis, proliferates, and differentiates into ducts lined with columnar

epithelium (Cunha and Chung, 1981; Cunha and Lung, 1978; Lasnitzki and Mizuno, 1980). While these wt UGM + Tfm epithelium recombinants formed prostatic tissue as judged morphologically, they never express prostatic secretory proteins (Cunha and Chung, 1981; Cunha and Lung, 1978; Donjacour and Cunha, 1993; Lasnitzki and Mizuno, 1980). These tissue recombinant studies demonstrate that prostatic development (i.e. ductal morphogenesis, epithelial proliferation, and change in cytodifferentiation) requires functional AR in the mesenchyme but not in the epithelium. However, functional epithelial AR is required for expression of prostatic secretory proteins.

While mesenchyme induces prostatic epithelial development, these interactions are truly reciprocal as the epithelium in turn induces mesenchymal differentiation. Indeed, differentiation of prostatic mesenchyme is dependent on both androgens and the epithelium. In the rat, fetal UGS mesenchyme is initially a loose embryonic connective tissue which later differentiates into thin smooth muscle sheaths surrounding the epithelial ducts. Between individual prostatic ducts the stroma is a loose connective tissue (Flickinger, 1972; Hayward et al., 1995; Ichihara et al., 1978). For internal organs the differentiation of smooth muscle in the mesenchyme is dependent on the epithelium (Cunha et al., 1992b; Cunha et al., 1989). Only small amounts of smooth muscle form (mostly associated with the vasculature) when UGM is grafted alone and grown in intact male hosts. However, if UGM is grafted with either bladder epithelium or UGE into intact male hosts, prostatic ducts form and the mesenchyme differentiates into sheaths of smooth muscle surrounding the epithelial ducts (Cunha et al., 1992b). The differentiation of smooth muscle in the prostate is also androgen-dependent. If human fetal prostates are grafted into castrated hosts, prostatic buds present at the time of grafting are maintained but do not grow and only small amounts of smooth muscle are detected (Hayward et al., 1995). Thus, androgen-dependent development of the prostate occurs via reciprocal interactions between the epithelium and the mesenchyme. The paracrine signaling molecules that mediate such interactions are at present unknown, but growth factors are likely candidates.

Growth Factors

Although it has been known for many years that development of the prostate is mediated by mesenchymal-epithelial interactions, the molecular mechanisms are uncharacterized by which androgens influence the mesenchyme to induce and direct epithelial ductal morphogenesis, epithelial cytodifferentiation and to elicit differentiation of mature prostatic glandular acini. There is strong evidence for the existence of factor(s) (andromedins) that are produced by the mesenchyme in response to androgens which act in a paracrine manner on the epithelium to mediate androgen action. Growth factors have been postulated to act as mediators of stromal-epithelial interactions in the adult prostate.

Growth factors are generally made and act locally. Growth factors are known to control not only cell proliferation, but other events such as differentiation, chemotaxis, and matrix deposition and remodeling. Growth factors exert their biological affects on cells via specific receptors. This can allow growth factors to mediate cell-cell interactions in a paracrine manner as well as via autocrine interactions. Growth factors act by binding to their receptor at the cell surface. Growth factor receptors are transmembrane glycoproteins and are characterized by an extracellular ligand binding domain; a hydrophobic transmembrane domain; and a carboxy terminal intracellular catalytic domain. This intracellular domain can have ATP-binding domains, phosphorylation sites, and tyrosine kinase domains (McKeehan, 1991). The pattern of expression of growth factors and their receptors by either the mesenchyme or the epithelium suggests a possible role of growth factors as mediators of mesenchymal-epithelial interactions in many tissues including the prostate. Overexpression or knock out of growth factor genes or their receptors have been shown to perturb development in some cases.

The extensive literature on growth factors in the prostate is mostly based upon analysis of growth-quiescent adult rodent prostate or human BPH (Cunha et al., 1994; Davies and Eaton, 1991; McKeehan, 1991; Story, 1991). Many of these studies used adult

rat prostatic tissue. For studies on human BPH the growth status of the specimen is unknown but must be presumed to be growth quiescent. BPH tissue has been shown to go through periods of proliferation and growth quiescence (Coffey et al., 1987), and studies using BPH tissue have never addressed whether the tissue utilized was actually in a period of growth. Growth factors have been studied in the adult prostate by examining the expression of growth factors and by assaying the response of adult prostatic cells in culture to exogenous growth factors. The relevance of such studies to prostatic growth *in vivo* is unclear, since high rates of proliferation achieved *in vitro* are in stark contrast to growth quiescence of the prostate *in vivo*. In the adult prostate growth factors may play a role in tissue homeostasis and not growth. Growth factors have also been implicated in benign and malignant growth of the prostate. Again the relevance of these studies to growth in normal developing prostate is unclear. This review will focus on three families of growth factors to critically assess their role in prostatic growth: epidermal growth factor (EGF)/transforming growth factor-alpha (TGF- α), transforming growth factor-beta (TGF- β), and fibroblast growth factor (FGF).

EGF/TGF- α Family

Epidermal growth factor (EGF) was discovered by Stanley Cohen in 1962 by its ability to accelerate tooth eruption and eyelid opening in newborn mice (Cohen, 1962). EGF is synthesized as a precursor and acts by binding to its receptor at the cell surface. EGF *in vitro* can influence a wide variety of mesenchymal and epithelial cells. When given *in vivo* in pharmacological levels many effects are seen: thickening of the skin, retardation of hair growth, stimulation of gastrointestinal tract function (Goodlad et al., 1987), modulation of thyroid hormone secretion (Ahren, 1987), stimulation of craniofacial morphogenesis, premature eye lid opening (Cohen, 1962; Hoath and Pickens, 1987; Imada et al., 1987), enhancement of the rate of wound healing (Schultz et al., 1987), acceleration

of vaginal opening and early estrus (Imada et al., 1987) (Reviews: (Burgess, 1989; Davies and Eaton, 1991; McKeehan, 1991; Story, 1991).

Transforming growth factor-alpha (TGF- α) is a member of the EGF family. It has structural homology with EGF (Derynck, 1986), but limited sequence homology (Marquardt et al., 1984). TGF- α mimics EGF and acts through the EGF receptor (EGFR). TGF- α is expressed by many tissues during development and was originally thought to be the embryonic ligand for EGFR. It is now known that TGF- α is also expressed in adult tissues and a variety of malignant cells (Hsuan et al., 1989; Snedeker et al., 1991; Yasui et al., 1992).

The EGFR family consists of three gene products: EGFR (also called c-erb-B and HER-1), *neu* (also called erbB-2, HER-2, and NGL), and erbB-3 (Carpenter and Cohen, 1990; McKeehan, 1991; Schlessinger, 1986; Ullrich and Schlessinger, 1990). Several ligands have been identified for this family of receptors: EGF, TGF- α , heparin-binding EGF, amphiregulin, cripto, and heregulin (Prigent and Lemoine, 1992). The EGFR binds EGF and TGF- α with equal affinity. The *neu* gene product does not bind EGF or TGF- α , however, several potential ligands for *neu* have recently been identified (Prigent and Lemoine, 1992). Some information is available on the expression of *neu* in the prostate. By immunocytochemistry McCann and collaborators were unable to detect *neu* in 23 prostatic cancer and 10 BPH specimens (McCann et al., 1989). In contrast, other groups have reported moderate to strong immunostaining of prostatic cancers by anti-*neu* (Ware et al., 1989) and diffuse staining of BPH tissue (Giri et al., 1993). Possible explanation for the contradictory results may be due to differences in the antibodies used and the variability found in both BPH tissue and prostatic cancers. However, in the developing rat prostate *neu* staining is not observed at 8 to 42 days of age (Taylor and Ramsdell, 1993).

EGFR is a plasma membrane glycoprotein containing four distinct regions: an extracellular portion containing the ligand binding domain; a transmembrane domain which spans the plasma membrane; a cytoplasmic domain containing protein-tyrosine kinase

activity; and a C-terminal regulatory segment. Binding of ligand to the EGFR activates the receptor's protein-tyrosine kinase activity, which elicits autophosphorylation of the receptor and phosphorylation of a variety of cellular proteins. EGFR is subject to complex regulatory controls, including ligand activation, downregulation by internalization, autophosphorylation, autoregulation, and transmodulation involving phosphorylation by kinase C (Martin, 1986).

EGF or TGF- α has been implicated in ductal branching morphogenesis of the developing mammary gland, lung and salivary gland. When pellets containing either EGF or TGF- α are implanted into regressed mammary glands of ovariectomized mice, end buds reappear and ductal branching occurs. TGF- α is localized to the epithelial cap-cell layer of mammary end buds and to the stroma surrounding the end bud (Snedeker et al., 1991). EGF absorbed microbeads are able to induce supernumerary budding and stimulate local DNA synthesis in chick trachea in culture (Goldin and Opperman, 1980).

Bronchial branching morphogenesis of embryonic lungs is stimulated by EGF *in vitro*. When EGF was added to the culture media, Warburton et al (1992) reported an increase in branching morphogenesis of the lung along with an increase in DNA, RNA, and protein content (Warburton et al., 1992). However, since the number of branch points was not actually counted, it is difficult to determine if branching morphogenesis increased in these experiments. In contrast, Ganser et al (1991) reported that organ cultures of embryonic lungs treated with exogenous EGF or TGF- α increased in size, but the number of ductal branch points decreased. Thus, exogenous EGF and TGF- α inhibited normal lung branching (Ganser et al., 1991). In these experiments EGF and TGF- α stimulated activity of metalloproteinases which are involved in degradation of the extracellular matrix. Branching morphogenesis of embryonic lung in organ culture was also inhibited when synthesis of EGF was blocked by an antisense oligodeoxynucleotide directed against the EGF precursor mRNA (Seth et al., 1993).

During development of the salivary gland the epithelium undergoes mesenchymal-dependent ductal branching morphogenesis. Branching morphogenesis occurs when salivary gland epithelium is cultured in Matrigel transfilter from its mesenchyme (Takahashi and Nogawa, 1991). Surprisingly, a single factor, either EGF or TGF- α , is able to substitute for the mesenchyme in promoting typical epithelial branching morphogenesis (Nogawa and Takahashi, 1991). Thus, findings from the mammary gland, lung and salivary gland suggest that EGF and/or TGF- α may be involved in branching morphogenesis.

Epidermal growth factor (EGF) is a mitogen for prostatic epithelial cells in culture (McKeehan et al., 1984; Peehl and Stamey, 1986). EGF has been detected in rat and human prostatic fluid (Elson et al., 1984; Gregory et al., 1986; Hirata et al., 1987; Jacobs and Story, 1988; Kishi et al., 1988; Pesonen et al., 1987), prostatic extracts (Elson et al., 1984; Hiramatsu et al., 1988; Jacobs and Story, 1988; Rubin and Bradshaw, 1984; Shaikh et al., 1990) and conditioned media from a human prostatic cell line, DU-145 (Connolly and Rose, 1989). EGF receptor (EGFR) has been detected in normal rat prostatic epithelium, human BPH epithelium, and prostatic adenocarcinoma cells (Davies and Eaton, 1989; Eaton et al., 1988; Fiorelli et al., 1989; Hofer et al., 1991; Lubrano et al., 1989; Maddy et al., 1987; St Arnaud et al., 1988; Traish and Wotiz, 1987).

In the rodent, prostatic EGF and EGFR levels have been reported to be androgen-dependent. Castration causes a marked reduction in EGF (Hiramatsu et al., 1988) and a two to six fold increase in EGF binding. EGF binding decreases to precastration levels following androgen replacement in castrated rats (St Arnaud et al., 1988; Traish and Wotiz, 1987). However, since these EGF binding studies used whole prostatic tissue homogenates, the interpretation of the results is unclear. The overall change in the amount of EGFR in whole prostatic tissue could be due to a change in the amount of EGFR per cell or due to a change in the ratio of EGFR expressing cells in the total prostatic tissue. In this regard, the ratio of epithelial to stromal cells in adult prostate is known to change

dramatically following castration (DeKlerk and Lombard, 1986). In homogenous cultures of an androgen-responsive prostatic epithelial cancer cell line, LNCAP, androgens have clearly been shown to increase EGFR levels (Mulder et al., 1989; Schuurmans et al., 1988a; Schuurmans et al., 1988b; Sehgal et al., 1994). Adult prostatic tissue *in vivo* can respond to EGF stimulation. When EGF is mixed with Matrigel and orthotopically injected into the ventral prostate of intact adult rats, EGF stimulates growth of the prostate *in vivo* as measured by wet weight, DNA content, protein content, and incorporation of BrdU into prostatic epithelium. EGF's stimulatory effect was inhibited by neutralizing antibodies to EGF. Histological examination did not reveal any gross changes in the ratio of stroma to epithelium. Surprisingly, the same dose of EGF that was stimulatory in intact rats had no effect in castrated rats (Marengo and Chung, 1994).

TGF- α is expressed by several human prostatic carcinoma cell lines (Connolly and Rose, 1990; Kim et al., 1991; Wilding et al., 1989a), normal rat prostate, and human BPH tissue (Ching et al., 1993; Harper et al., 1993; Myers et al., 1993; Taylor and Ramsdell, 1993; Wu et al., 1993). In explants of human BPH, TGF- α is mitogenic for the epithelium and has little effect on stromal cells (Hofer et al., 1991; Sherwood et al., 1992). TGF- α and EGFR are detectable in the ventral and lateral prostate of rats from 28 days into adulthood. TGF- α immunoactivity is restricted to the epithelium of the prostate and is not found in the stroma. TGF- α is also not detectable in the dorsal lobe at any age nor in ventral and lateral lobes of rat prostates at 10 or 21 days of age. The antibody used in these studies recognizes the mature form of TGF- α and has no cross reactivity with EGF (Taylor and Ramsdell, 1993). Sandgren describes a line of transgenic mice that overexpress TGF- α . These mice develop mammary carcinoma and also have hyperplastic and dysplastic anterior prostates (coagulating gland) (Sandgren et al., 1990). However, disruption of the TGF- α gene does not effect prostatic growth (Donjacour, unpublished).

TGF- β Family

Transforming growth factor-beta (TGF- β) was discovered along with TGF- α because these two molecules induce morphologic transformation of fibroblastic cells in monolayer culture and stimulate colony formation in soft agar (Barnard et al., 1990; Goustin et al., 1986; Todaro et al., 1980). TGF- β and TGF- α are structurally and biologically distinct molecules. TGF- β is primarily an inhibitor of cell growth. TGF- β family members have been divided into two groups. In mammals there are three closely related members, TGF- β 1, TGF- β 2, and TGF- β 3 (Derynck et al., 1985; Derynck et al., 1988; Madisen et al., 1988; Miller et al., 1989). Other more distantly related members of the TGF- β family include Müllerian inhibiting substance, inhibins, activins, bone morphogenetic proteins, and products of the decapentaplegic gene complex in *Drosophila* (Roberts and Sporn, 1988; Roberts and Sporn, 1990; Sporn and Roberts, 1988b). These molecules share a 25-40% sequence homology with TGF- β and have 7 of the 9 conserved cysteine residues. This discussion will focus on the three mammalian TGF- β s and their receptors.

TGF- β has been implicated in a wide variety of processes such as wound healing and tissue repair, bone formation and remodeling, embryogenesis, branching morphogenesis, and cell differentiation (Barnard et al., 1990; Roberts and Sporn, 1990). TGF- β is synthesized by a wide variety of tissues and cultured cell lines. TGF- β is synthesized as a latent precursor molecule which is further processed to yield the smaller active form (B 5 (Derynck et al., 1985). TGF- β in its latent form does not bind to the receptor (Barnard et al., 1990). The mechanism of TGF- β activation *in vivo* is unclear. Several chemical treatments activate latent TGF- β . These include acidification below pH 4, alkalization above pH 9, treatment with chaotropic agents such as sodium dodecyl sulfate and urea (Pircher et al., 1986) and proteinase treatment (Lyons et al., 1988). Proteolysis of latent TGF- β is the likely mechanism of activation *in vivo* (Barnard et al., 1990).

TGF- β receptors are ubiquitously expressed by almost all normal and neoplastic cells (Frolik et al., 1984; Tucker et al., 1984a). There are 3 classes of receptors: type I (TGF- β R-I), type II (TGF- β R-II) and type III (TGF- β R-III or betaglycan) (Barnard et al., 1990; Chieftetz et al., 1987). TGF- β R-I and TGF- β R-II are both transmembrane serine/threonine kinases. TGF- β binds directly to TGF- β R-II which is a constitutively active kinase. This complex is recognized by TGF- β R-I which is then recruited into the complex and becomes phosphorylated by receptor II. Phosphorylation allows receptor I to propagate the signal to down stream substrates (Lopez-Casillas et al., 1994; Massague et al., 1992; Ventura et al., 1994; Wieser et al., 1993). TGF- β R-III is a cell surface heparin/chondroitin sulfate proteoglycan that binds TGF- β via the receptor's NH₂-terminal extracellular domain and can also interact with the extracellular matrix (Lopez-Casillas et al., 1994). TGF- β R-III presents TGF- β to the type II receptor resulting in increased binding of TGF- β to the signaling receptor, enhanced cell responsiveness to TGF- β and elimination of marked biological differences between the TGF- β isoforms by overcoming differences in isoform binding affinity for TGF- β R-II. The extracellular domain of TGF- β R-III can be shed by cells (Lopez-Casillas et al., 1994; Lopez-Casillas et al., 1993). The soluble form of the type III receptor can still bind TGF- β but cannot present TGF- β to the type-II receptor. In fact, recombinant soluble type III receptor acts as a potent inhibitor of TGF- β binding to membrane receptors and blocks TGF- β action. Thus, TGF- β R-III acts as a regulator of TGF- β access to the signaling receptors (TGF- β R-I and TGF- β R-II) (Lopez-Casillas et al., 1994).

The biologic effects of TGF- β are diverse. Depending on the cell type, TGF- β can inhibit or stimulate cell proliferation, stimulate extracellular matrix (ECM) formation, and promote or inhibit cell migration (Barnard et al., 1990; Roberts and Sporn, 1990; Sporn and Roberts, 1988a). Differences in the effect of TGF- β 1, TGF- β 2 and TGF- β 3 on proliferation of cells in culture appear to be quantitative and not qualitative (Graycar et al., 1989). TGF- β inhibits proliferation of epithelial cells, while promoting proliferation of

some mesenchymal cells (fibroblasts and osteoblasts) (Barnard et al., 1990; Shipley et al., 1985; Tucker et al., 1984b). TGF- β is considered to be an "indirect" mitogen for fibroblasts (Leof et al., 1986). After TGF- β treatment there is a 12 hour lag time before proliferation is induced. Platelet-derived growth factor-like molecules have been implicated as potential mediators of TGF- β 1-induced proliferation of fibroblasts (Leof et al., 1986; Soma and Grotendorst, 1989). TGF- β promotes ECM formation not only by stimulating ECM protein synthesis, but by also inhibiting proteinase synthesis, stimulating synthesis of proteinase inhibitors, and increasing synthesis of cell surface membrane proteins that bind ECM proteins (Barnard et al., 1990).

Cell differentiation is usually correlated with decrease or inhibition of cell proliferation. The effect of TGF- β on adipogenesis and myogenesis, as well as, the response of epithelial cell types to TGF- β demonstrates the dichotomy between the differentiating effects and the proliferative effects of TGF- β . TGF- β inhibits differentiation of murine 3T3 preadipocytes (Ignotz and Massague, 1985) and suppresses myogenic differentiation by blocking formation of myotubes in myoblast cultures (Massague et al., 1986; Olson et al., 1986) without inhibiting proliferation of these cell types. Conversely, TGF- β induces terminal differentiation of intestinal crypt epithelium (Kurokawa et al., 1987) and stimulates squamous differentiation of bronchial epithelial cells (Jetten et al., 1986; Masui et al., 1986) while inhibiting proliferation of both of these cell types (Chakrabarty et al., 1989; Kimchi et al., 1988; Rizzino, 1988) (Reviews: (Barnard et al., 1990; Pelton and Moses, 1990). Thus, the differentiating and proliferative effects of TGF- β appear to be mutually exclusive.

Localization of transcripts for TGF- β 1 (Lehnert and Akhurst, 1988), TGF- β 2 (Pelton et al., 1989) and TGF- β 3 (Pelton et al., 1990a; Pelton et al., 1990b) by in situ hybridization suggests that TGF- β is important during embryogenesis. TGF- β protein or TGF- β transcripts have been detected during embryogenesis in a variety of tissues including hematopoietic cells, liver, megakaryocytes, osteoclasts, intestine, lung, kidney,

bone, cartilage, tendon, tracheal submucosa, blood vessels, skin, placenta, heart, perichondrium, palate, and central nervous system (Heine et al., 1987; Pelton et al., 1990a; Pelton et al., 1990b; Pelton et al., 1989; Pelton et al., 1991). The timing and pattern of expression for the different isoforms of TGF- β are highly variable during embryogenesis. These studies suggest that TGF- β plays an important role in the development of a diverse number of tissues (Barnard et al., 1990; Lehnert and Akhurst, 1988).

TGF- β inhibits epithelial branching morphogenesis in the developing mammary gland and lung *in vivo*. Slow release pellets containing TGF- β 1 in the developing mammary gland inhibit ductal growth. This inhibitory effect of TGF- β 1 is epithelium-specific. TGF- β 1 inhibits DNA synthesis of rapidly proliferating mammary end bud epithelium, but has no effect on DNA synthesis of the stroma surrounding the end buds (Daniel et al., 1989; Silberstein and Daniel, 1987). TGF- β 2 and TGF- β 3 likewise inhibit ductal branching morphogenesis in the developing mammary gland (Robinson et al., 1991). TGF- β 1 immunostaining is faint on end buds which are actively proliferating and intense in non-growing proximal ducts and growth-quiescent ductal tips (Silberstein et al., 1992). TGF- β 1 inhibits branching morphogenesis of embryonic lungs in culture. This inhibition is dose dependent and reversible after removal of TGF- β 1 from the culture medium (Serra et al., 1994). TGF- β is expressed during lung development and immunostaining colocalizes TGF- β 1 with matrix proteins during cleft formation and tissue stabilization (Heine et al., 1990). These studies suggest TGF- β is involved in epithelial branching morphogenesis.

TGF- β has been detected in both normal and neoplastic prostatic tissue (Kyprianou and Isaacs, 1988; Kyprianou and Isaacs, 1989b; Kyprianou et al., 1991; Pai et al., 1991; Shain et al., 1990). TGF- β induces cell death (apoptosis) in ventral prostatic glandular cells in culture, as well as *in vivo* when administered directly into the rat ventral prostate. TGF- β has no affect when administered to the regressed prostate (Martikainen et al., 1990). TGF- β activity has been isolated from immature rat ventral prostates in culture, and

this activity can be abolished by anti-TGF- β antibodies (Atfi et al., 1994). Expression of all three isoforms of TGF- β (β 1, β 2, β 3) have been detected in the embryonic anlagen of the rat prostate (UGS) by Northern analysis. Expression is located primarily in the mesenchyme. In contrast, TGF- β 2 and TGF- β 3 expression is barely detectable in the adult prostate while TGF- β 1 expression remains elevated (Timme et al., 1994).

TGF- β receptor levels increase after castration in the prostate and return to precastration levels with androgen replacement (Kyprianou and Isaacs, 1988). This correlates with an increase in TGF- β 1 expression. TGF- β 1 transcript levels increase 10 to 40-fold after castration in the rat prostate, and androgen replacement restores expression to precastration levels (Kyprianou and Isaacs, 1989b). In regressing prostates TGF- β levels increase, suggesting that androgens down regulate TGF- β (Kyprianou et al., 1991). In the human prostatic tumor line PC-82, TGF- β is also down-regulated in response to androgens. Normal and malignant prostatic tissue have TGF- β receptors and generally respond to exogenous TGF- β with decreased proliferation (Kyprianou and Isaacs, 1988; McKeehan and Adams, 1988; Shain et al., 1990; Wilding et al., 1989b). Unexpectedly, expression of TGF- β has been associated with transition from benign to malignant phenotype in prostatic tumors *in vivo*. Immunostaining for TGF- β 1 is greater in prostatic cancer than BPH tissue (Thompson et al., 1993), and TGF- β 1 transcript levels are elevated in rat prostatic cancer relative to normal rat prostate (Steiner et al., 1994), suggesting that in malignancy, TGF- β is no longer growth inhibitory for prostatic epithelial cells.

FGF Family

A hallmark of the fibroblast growth factor (FGF) family is their binding to heparin, which merits the name heparin binding growth factors (HBGF). Nine FGF family members encoded by nine different genes have been identified (Benharroch and Birnbaum, 1990; Mason, 1994). These FGFs elicit their effects following binding to different receptors which are encoded by four alternatively spliced genes (Givol and Yayon, 1992;

Johnson and Williams, 1993; Mason, 1994). FGFs evoke a variety of responses in cells including cell proliferation, changes in cell motility and differentiation. FGFs are able to affect the function of a remarkable number of cell types including mesenchymal, epithelial, endocrine, and neural crest derivatives. FGFs have been implicated in normal physiological functions *in vivo* such as: embryonic and fetal development, vascularization, tissue interactions, spatial patterning, and wound healing (Mason, 1994). The cell types that FGFs can affect are as varied as the responses they can evoke.

FGF-1 (acidic FGF) and FGF-2 (basic FGF) were the first members of the FGF family to be identified and cloned (Jaye et al., 1986; Kurokawa et al., 1987). FGF-1 and FGF-2 share 55% amino acid sequence homology (Esch et al., 1985) and lack a signal sequence. They are widely distributed throughout the body. FGF-1 or FGF-2 has been identified in almost every tissue that has been reported to contain "FGF-like" activity. Because of the ubiquitous nature of FGF-1 and FGF-2, many researchers studying "growth promoting activity" in a variety of tissues have found the activity to be due to either FGF-1 or FGF-2 (Story, 1991).

In situ hybridization studies indicate that FGFs are widely expressed during development. FGFs have been implicated in ventral mesoderm induction in *Xenopus* (Slack, 1994). FGF-3 (also known as int-2) and FGF-4 are involved in spatial patterning during development. FGF-3 and FGF-8 transcripts exhibit a dynamic and transient expression pattern in the developing central nervous system (Crossley and Martin, 1995; Lumsden, 1990; Mason, 1994). In the developing limb bud FGF-4 is synthesized in the apical ridge and can substitute for the apical ridge (Niswander et al., 1993; Vogel and Tickle, 1993). FGF-5 knockout transgenic mice have sustained hair growth. In these mice the basal region of the hair follicle fails to degenerate (Herbert et al., 1994). FGF-7, also known as KGF, has been implicated as an andromedin and as a paracrine mediator of mesenchymal-epithelial interactions (Aaronson et al., 1991; Yan et al., 1992). FGF-7 has been detected in the developing mammary gland, salivary gland, and lung during epithelial

development at a time when these glands are undergoing branching morphogenesis (Mason, 1994) (Finch et al, in press).

Although nine FGF genes have been identified, a variety of different isoforms can be generated (Mason, 1994). FGF-2 (Borja et al., 1993) and FGF-8 (Tanaka et al., 1992) can be alternatively spliced to give different isoforms. FGF-2 and FGF-3 can use alternative initiation codons for translation resulting in different gene products (Acland et al., 1990; Powell and Klagsbrun, 1991). Further diversity can be obtained with post translational modifications such as glycosylation and proteolytic cleavage (Mason, 1994).

FGF-1, FGF-2 and FGF-9 lack a classical leader sequence for secretion (Burgess and Maciag, 1989) and, thus, it is unclear how they are released into the extracellular space and exert their biological activity. It was originally thought that FGF-1 and FGF-2 were only released from dead cells. However, there is growing evidence that FGFs are released by cells via a novel secretory mechanism (Mignatti et al., 1992). Following transfection into COS cells, FGF-9 is constitutively secreted even though it lacks a signal sequence (Miyamoto et al., 1993). In contrast, FGF-3, which has a signal sequence, is released very slowly by COS cells (Kiefer et al., 1993; Mason, 1994), suggesting that FGF-9 and FGF-3 are secreted from COS cells via different mechanisms.

FGFs transduce their signal to the cytoplasm through transmembrane tyrosine kinase receptors (FGFR) (Givol and Yayon, 1992; Johnson and Williams, 1993; Mason, 1994; McKeehan, 1991). FGFRs can be activated by dimerization which results in autophosphorylation and recruitment of intracellular signaling proteins (Mason, 1994). Four FGFR genes have been cloned: FGFR-1, also called *Flg* (*Fms like gene*) (Dionne et al., 1990; Hou et al., 1991); FGFR-2, also called *Bek* (*bacterially expressed kinase*) (Dionne et al., 1990); FGFR-3, also called *cek-2* (*chicken embryo kinase*) (McKeehan, 1991; Pasquale, 1990) and FGFR-4 (Stark et al., 1991). FGFRs contain an intracellular tyrosine kinase domain, a transmembrane domain and an extracellular ligand binding domain. The extracellular domain contains three immunoglobulin like loops (I, II, III).

These loops have been implicated in specificity of ligand binding. Modifications, such as phosphorylation, in other domains of the FGFR can also alter its binding affinity (McKeehan, 1991).

Alternative splicing of the FGFR genes can yield a diverse population of receptors. FGFRs can be alternatively spliced in the extracellular domain resulting in changes in ligand affinity and specificity. FGF receptors can also be spliced in the intercellular domain resulting in truncation of the receptor, or possibly changing the internal signaling pathways with which the receptor can interact. This activity can result in a given ligand having different biological activities depending on the intracellular domain of the receptor with which the ligand is bound. It has been suggested that differences in the COOH-terminal domain of the receptor can determine which internal signaling pathway is activated (Wang et al., 1994). Additionally, the signaling pathway available for activation in the cell may in part depends on the differentiated state of the cell. Truncated secreted forms of the FGFR have been identified (Johnson et al., 1991). These forms lack the transmembrane domain and intercellular domain. This form of the receptor may act as a dominant negative receptor by binding ligand and, thus, competing with active integral membrane receptors. Alternatively, this truncated form of the receptor may act as an extracellular reservoir for FGFs (Johnson et al., 1991). Expression of truncated receptors can have dominant negative effects. Peters and collaborators expressed a membrane-bound truncated form of FGFR-2 (FGFR-2IIIb, also known as the KGFR) using the surfactant promoter. Tissue specific expression of this truncated form of the FGFR-2/KGFR lacking the intracellular domain inhibited branching morphogenesis of the lung buds (Peters et al., 1994). Thus, a dominant negative FGFR/KGFR completely blocked epithelial branching morphogenesis and differentiation.

It has become apparent that alternative splicing of the third Ig-like loop of the extracellular domain is involved in both ligand binding and specificity of FGFR-1 (Johnson et al., 1990; Johnson et al., 1991), FGFR-2 (Champion-Arnaud et al., 1991; Johnson et

al., 1991; Miki et al., 1992; Yayon et al., 1992) and FGFR-3 (Chellaiah et al., 1994). The differential use of exons encoding the C-terminal half of the third Ig-like loop results in two isoforms of the FGFR-2 (Champion-Arnaud et al., 1991; Johnson et al., 1991) displaying markedly different affinities for FGF-2/bFGF and FGF-7/KGF (Miki et al., 1992; Yayon et al., 1992). If exon IIIb is used, the resulting FGFR-2IIIb has a high affinity for FGF-1/aFGF and FGF-7/KGF, but does not bind FGF-2/bFGF. However, if exon IIIc is used, the FGFR-2IIIc binds FGF-1/aFGF and FGF-2/bFGF, but not FGF-7/KGF. Exon IIIa for the FGFR-1 lacks a transmembrane domain, and this form is secreted (Johnson et al., 1991). Although a secreted form of the other FGFRs has not yet been identified, such forms are thought to exist. Orr-Urtreger and collaborators examined the developmental expression of the FGFR-2(IIIb) (KGFR) and FGFR-2(IIIc) (bFGFR) forms by *in situ* hybridization (Orr-Urtreger et al., 1993). They found that the two forms differ in localization during both early development and later during organogenesis. During this later period FGFR-2IIIb (KGFR) was localized to the surface ectoderm and epithelium of the internal organs, whereas the FGFR-2IIIc (bFGFR) form was localized to the mesenchyme. This pattern of expression is in agreement with the results of others (Miki et al., 1992; Rubin et al., 1989) (Finch et al, in press).

Several members of the FGF family have been implicated in studies on the prostate. Both FGF-1/aFGF and FGF-2/bFGF have been implicated in prostatic growth (Cunha et al., 1994; Gelmann, 1991; Mansson et al., 1989; McKeehan, 1991; McKeehan et al., 1991; Story, 1991). Jacobs and collaborators (1979) have shown that extracts from normal, BPH and differentiated prostatic tissue are mitogenic for cultured osteoblasts and fibroblasts. The purified growth factor was identified as FGF-2/bFGF (Jacobs et al., 1979). FGF-2/bFGF has been detected in prostatic tissue by radiolabeled binding assays, immunoreactivity, amino terminal sequence analysis, and message levels (Katz et al., 1989; Mansson et al., 1989; Mori et al., 1990; Mydlo et al., 1988a; Story, 1991; Story et al., 1987a; Story et al., 1987b). Story reports that BPH has 2-3 times more FGF-2/bFGF per

mass of tissue than normal prostatic tissue (Story, 1991), and transcript levels for FGF-2/bFGF are increased in BPH (Mori et al., 1990; Story, 1991). In castrated rats, androgens elevate FGF-2/bFGF transcript levels, but the increase in FGF-2 transcripts occurs after the peak of DNA synthesis (Katz et al., 1989). Stromal cells are the main source of FGF-2/bFGF in the prostate, and it is the stromal cells that proliferate in response to FGF-2/bFGF (Story, 1991; Story et al., 1989). Cultured epithelial cells have also been reported to synthesize FGF-2/bFGF, but at very low levels (Story, 1991). Mydlo and collaborators were only able to detect FGF-2/bFGF and not FGF-1/aFGF in BPH tissue and prostatic carcinoma by Western and Northern blot analysis (Mydlo et al., 1988a; Mydlo et al., 1988b). Yan and collaborators report that accompanying stromal independence and progression to malignancy, prostatic epithelial cells switched from exclusive expression of FGFR-2IIIb (KGFR), which binds FGF-7/KGF and FGF-1/aFGF, to exclusive expression of FGFR-2IIIc, which binds FGF-1/aFGF and FGF-2/bFGF. Concomitantly, the epithelium also expressed the FGFR-1 gene, which is normally expressed only in the stromal cells, as well as, FGF-2/bFGF, FGF-3 (int-2) and FGF-5 genes. With these molecular changes the epithelial cells progress to malignancy (Yan et al., 1993a).

In contrast to the abundant literature on the presence of FGF-2/bFGF in BPH and prostatic carcinoma, scarce information is available on FGF-2/bFGF in normal or developing prostate. Yan and collaborators report that FGF-2/bFGF is expressed in the adult prostate (Yan et al., 1993a). Furthermore, FGF-2/bFGF stimulates prostatic growth *in vivo* when administered orthotopically into the ventral prostate of an adult rat. Wet weight, DNA content and protein content increase. However, the epithelium to stroma ratio did not change. This effect of FGF-2/bFGF could be blocked with neutralizing antibodies. FGF-2 administration has no effect in castrated rats (Marengo and Chung, 1994). In the developing prostate FGF-2/bFGF does not seem to be important (Cunha et al., 1992a). Neutralizing antibodies to FGF-2/bFGF did not block development of the

prostate (Alarid et al., 1991) even though this antibody has been shown to inhibit development of the epididymis (Alarid et al., 1991) and growth and differentiation of rat embryos (Liu and Nicoll, 1988). FGF-1/aFGF has been implicated in the late pubertal developing prostate. Mansson showed that FGF-1/aFGF transcripts were detectable in the epithelium of 6-8 week old rats, declined at 14 weeks, and was undetectable by 35 weeks (Mansson et al., 1989).

Transgenic mice overexpressing FGF-3 (int-2) using the MMTV promoter have been reported to develop hyperplastic mammary glands and prostates (Muller et al., 1990; Tutrone et al., 1993). It was suggested that these mice could be used as an animal model to study BPH. However, careful dissection and histological examination of the "enlarged prostates" of these mice reveals that the hyperplastic tissue is not prostate. Instead, these mice have hyperplastic ampullary glands (Donjacour and Cunha, unpublished). The ampullary gland is derived from the caudal region of the ductus deferens and is a derivative of the Wolffian duct. The ampullary gland is generally a discrete gland in rodents that encircles the ductus deferens, but in some inbred strains of mice these glands are vestigial. Ampullary glands are absent from many mammalian orders (Price and Williams-Ashman, 1961). While the hyperplastic ampullary glands of Int-2 mice are clear examples of glandular hyperplasia in the male urogenital tract, the significance (if any) of this model to BPH is questionable.

FGF-7/KGF has some intriguing qualities that make it a likely candidate to be a mediator of androgen-dependent epithelial-mesenchymal interactions in the developing prostate. The pattern of expression of both the ligand and its receptor make it ideally suited to be involved in mesenchymal-epithelial interactions (Mason et al., 1994; Rubin et al., 1992; Yan et al., 1992). Transcripts for FGF-7/KGF are localized to the mesenchyme of developing glands while the receptor FGFR2-IIIb (KGFR) is found exclusively in the epithelium (Mason et al., 1994) (Finch et al, in press). FGF-7/KGF has been implicated in the development of several tissues whose development is known to depend on

mesenchymal-epithelial interactions. FGF-7/KGF has been implicated in the proliferation and differentiation of keratinocytes (Marchese et al., 1990; Pierce et al., 1994), type II pneumocytes of the lung (Ulich et al., 1994), and mucin producing cell lineages of the gut (Housley et al., 1994). FGF-7/KGF can also induce hyperplasia in the developing mammary gland (Ulich et al., 1994).

FGF-7/KGF has been suggested to act as an andromedin in adult rat prostate (Yan et al., 1992). Yan and collaborators examined the androgenic response of adult prostatic epithelial and mesenchymal cells in culture. Neither of these cell types respond to androgens when cultured alone. However, in co-cultures of prostatic epithelium and stroma separated by a permeable membrane, the epithelium proliferates in response to androgens, while the stroma does not. Northern analysis and nuclease protection analysis of the mRNA reveals that FGF-7/KGF is made by prostatic stromal cells and that androgens stimulated expression of FGF-7/KGF transcripts by 4-7 fold in this system. FGF-7/KGF added to isolated cultures of prostatic epithelium or stroma stimulates epithelial growth but has no effect on the stromal cells. Thus, FGF-7/KGF is synthesized by adult prostatic stromal cells *in vitro* in response to androgens and signals to the epithelium via FGFR-2IIIb (KGFR), which is expressed exclusively in the epithelium.

FGF-7/KGF has also been implicated in the androgen-dependent development of the neonatal mouse seminal vesicle (SV) (Alarid et al., 1994). Development of the SV is very similar to the development of the prostate. Indeed, SV mesenchyme is able to induce prostatic development in urogenital sinus epithelium and bladder epithelium in tissue recombination studies (Donjacour and Cunha, 1988b; Donjacour and Cunha, 1994). In organ cultures of neonatal SV, T induces epithelial growth. A neutralizing antibody to FGF-7/KGF inhibits androgen-induced epithelial ductal branching morphogenesis and reduces DNA content when compared to controls. Addition of exogenous FGF-7/KGF to cultures of neonatal SVs induces approximately 58% of the growth elicited by T. Expression of FGF-7/KGF is detectable in neonatal and cultured SVs by RT-PCR and

Northern analysis (Alarid et al., 1994). Thus FGF-7/KGF has been implicated in the androgen-dependent development of the SV.

HGF

Hepatocyte growth factor (HGF), also known as scatter factor, has been implicated as a mediator of epithelial-mesenchymal interactions in the liver and kidney (Cantley et al., 1994; Johnson et al., 1993; Montesano et al., 1991a; Montesano et al., 1991b; Santos et al., 1994; Santos and Nigam, 1993). HGF is produced by stromal cells (Rubin et al., 1991). The receptor for HGF, identified as the c-met proto-oncogene (Bottaro et al., 1991; Naldini et al., 1991), is expressed primarily in epithelial cells (Gherardi et al., 1993; Prat et al., 1991). Madrin-Darby canine kidney (MDCK) cells grown in collagen gels form branching tubules in the presence of fibroblasts or fibroblast conditioned media (Montesano et al., 1991b). HGF was identified as the fibroblast-derived morphogen which mediates MDCK epithelial tubulogenesis (Montesano et al., 1991a). The prostate is immunoreactive for HGF (Wolf et al., 1991). Primary fibroblast cultures from prostate produce HGF (Seslar et al., 1993). However, further research is needed to determine if HGF is involved in the development of the prostate.

Conclusions

At present it is known that prostatic development is androgen dependent. However, it is unclear as to which androgen is the active agent in prostatic development. T, DHT, 3 α -diol and 3 β -diol have all been implicated in prostatic development, growth and differentiation. A serum-free organ culture system was devised to test the potency and efficacy of T and its metabolites to induce ductal branching morphogenesis and growth of the developing prostate.

There is ample evidence that during prostatic development the mesenchyme mediates androgen induced-differentiation of the epithelium. Yet, the mediators of such

mesenchymal-epithelial interactions in the prostate are presently unknown. Growth factors are likely to be involved in such interactions. The families of growth factors discussed above (EGF, TGF- β and FGF) have been implicated in development, branching morphogenesis, and differentiation in other systems, as well as, in epithelial-mesenchymal interactions. Adult prostatic tissues can respond to EGFs, TGF- β s and FGFs, and either the ligand or receptor is present in prostatic epithelium or mesenchyme (stroma). Our serum-free organ culture system is ideal to test the effects of various androgens, growth factors or neutralizing agents to growth factors on ductal branching morphogenesis in the developing prostate. By this method some of the growth factors involved in androgen-induced prostatic ductal branching morphogenesis have been elucidated.

MATERIALS and METHODS

Androgens

The following androgens were used in this study:

4-Androstene-17 β -ol-3-one (Testosterone)	Sigma (Culture grade)
5 α -androstane-17 β -ol-3-one (Dihydrotestosterone)	Steraloids
5 α -androstane-3 α , 17 β -diol (3 α -diol)	Steraloids
5 α -androstane-3 β , 17 β -diol (3 β -diol)	Sigma
5 β -androstane-3 α , 17 β -diol (5 β 3 α -diol)	Steraloids
5 α -androstane-3, 17-dione (Androstanedione)	Steraloids
5 α -androstane-3 α -ol-17-one (Androsterone)	Steraloids
Metribolone (R1881, Methyltrienolone)	Roussel UCLAF
7 α -methyl-19-nortestosterone (MENT)	The Population Council

Growth Factors

The following growth factors were used int this study:

Keratinocyte growth factor (recombinant human)	gift from Jeff Rubin (NIH)
Hepatocyte growth factor	gift from Jeff Rubin (NIH)
Epidermal growth factor (recombinant human)	Collaborative Research
Transforming growth factor alpha	Upstate Biotechnology, Inc.
Transforming growth factor beta one	gift from Dr. M Sporn
Transforming growth factor beta two	gift from Dr. M Sporn
Acidic fibroblast growth factor (FGF-1, recombinant human)	Upstate Biotechnology
Basic fibroblast growth factor (FGF-2, recombinant human)	Upstate Biotechnology

Anti-growth Factors

The following anti-growth factors were used in this study:

Anti-transforming growth factor alpha I	Biotope
Anti-transforming growth factor alpha II	Dr. M. Skinner
Anti-transforming growth factor beta	gift from Dr. Harold Moses
Anti-keratinocyte growth factor IG4	gift from Jeff Rubin (NIH)
Anti-keratinocyte growth factor peptide 412	gift from Jeff Rubin (NIH)
Control mouse IgG	Sigma
Control goat IgG	Biotope

Media

DME H-16 50%/F-12 50% mix with NEAA	UCSF Cell Culture
with 1.401 g/L Glucose, 0.11 g/L NaPyruvate, 0.365 g/L L-Glutamine, Linoleic Acid, 1.2 g/L NaHCO ₃	
Gentamycin 50µg/ml	UCSF Cell Culture
Insulin 10µg/ml	UCSF Cell Culture
Holo-transferrin (human)	Sigma
Millicell-CM 0.4 µm Culture Plate Insert	Millipore
4-well tissue culture plate, flat bottom	Nunc

Animals

Fisher 344 male rats less than 12 hours old	Simonsen Laboratories
Sprague-Dawley male rats 1-2 days old	

Organ Culture

Male Fisher 344 rat pups less than twelve hours old were used for organ culture experiments. The rats were sacrificed by decapitation. The reproductive tracts with

attached bladder were removed. Further dissection of the tract was done in a watchmaker's depression slide using number 5 biological forceps and a 27 1/2 gage needle as a scalpel using a dissection microscope. All dissections were done in DME H-16 50%/F-12 (1:1) medium.

To isolate the rat VP, the connective tissue and ganglionic tissue surrounding the prostatic complex was removed using forceps. The ventral prostate was removed from the urethra by a cut just above the VP and below the bladder. The dorsal urethral tissue was removed by two cuts lateral to the VP. The VP was then cut into its two lobes through the fascial plane separating them.

To isolate the AP, the SV, DLP, AP complex was cut into right and left halves through the midline. The vas deferens and DLP were removed and the AP was dissected from the SV.

Care was taken during the dissection to not crush the VP, SV, or AP, so that when the glands were placed in culture they were intact and undamaged. The AP cultures used for morphometric analysis were unbranched at the start of culture. Cultures for RNA analysis used 1 day old Sprague-Dawley rats, whose prostates were somewhat more developed than that of 0 day Fisher 344 rats used for organ culture.

Glands were cultured on floating Millicell-CM filters in 1 ml of culture media in four well culture dishes. Glands were transferred to floating filters using forceps in a drop of media. The drop of media was approximately 4 times larger than the gland. Under these conditions the glands grew two-dimensionally rather than three-dimensionally.

Androgens were dissolved in 100% ethanol and 10^{-4} M stocks were stored at -20° . Stocks were diluted in media to the working concentration. Ethanol concentrations were never greater than 1% in the culture media. Ethanol at 1% was tested and did not have an effect on development of the prostate in culture.

The cultures were grown at 37° C in an atmosphere of 5% CO₂ and 95% air. The media was changed every 2-3 days.

Image Analysis

Image analysis was performed on the glands using the procedures of Alarid et al. (Alarid et al., 1994). Whole-mount images of the glands were captured and digitized using a Dage-MT1 CCD-72 TV camera interfaced with a Macintosh Quadra 800 computer and processed with PRISM VIEW software (Dapple, Sunnyvale, CA). PRISM VIEW software calculated epithelial area and perimeter from computer-generated binary images of individual glands. Morphological complexity of epithelial shape was assessed using the node number parameter and the form factor parameter of PRISM VIEW. Node number is a topological measure whose value is proportional to the complexity of ductal branching. Node number is indicative of the number of branch points. The node number was determined from a computer-generated skeletonized epithelial outline and is defined as the number of internal connections between three or more links in the skeleton. Form factor is an indication of the complexity of the shape. A circle has a form factor of one. As shape becomes more complex its form factor decreases. The form factor is calculated based on the ratio of area to perimeter. (Form factor = $4\pi \text{ area/perimeter}^2$.) Inverse of the form factor was calculated so that as morphological complexity of ductal branching increased so did the values for 1/form factor.

Statistical Analysis

Statistical analysis on the dose response experiments as performed using ANOVA with differences determined using Fisher's protected least significance differences (LSD) at a 95% confidence interval using Abacus Concepts, Statview 4.02 software. Outliers, which were greater than two standard deviations from the mean, were removed from the data set. Statistical analysis of growth factor and anti-growth factor experiments used the two tailed Dunnett post-hoc procedure at a 95% confidence interval to compare treatment groups to positive and a negative controls using Abacus Concepts, SuperANOVA 1.11. The controls used in the Dunnett procedure were glands cultured with T 10^{-8} M (positive control) or in

the absence of T (negative control) which had been dissected and placed into culture at the same time as the treatment glands. Each experiment contained at least three glands per treatment and all experiments were performed at least three times. Unequal sample size among the different treatment groups was due to technical difficulties that occurred during the six days of culture. This study was based upon the analysis of 2072 APs and 442 VPs.

Reverse Transcriptase-Polymerase Chain Reaction

Tissues were dissected from the animal or harvested from culture, placed in 100 µl of guanidine extraction buffer (4 M guanidine thiocyanate, 0.5 M sodium acetate, 0.005 M EDTA pH 7.5, 1% sarcosyl, 1 M β-mercaptoethanol) and vortexed. RNA was purified by pelleting 100 µl of the sample through 100 µl of a CsCl cushion (5.7 M CsCl, 0.1 M EDTA, 0.01 M Tris-Cl pH 7.5) in a Beckman TL-100 centrifuge at 90,000 rpm at 22 °C for 70 minutes. The RNA pellet was resuspended in 40 µl of guanidine extraction buffer, and precipitated with 100 % ethanol. The purified RNA was dissolved in 50 to 100 µl DEPC-treated distilled water and stored at -70 °C prior to use.

For first strand synthesis 49 µl of the purified RNA solution was heated to 85 °C for 5 minutes, cooled on ice, and then added to 16 µl of 5x reverse transcriptase (RT) buffer (250 mM Tris pH 8.3, 30 mM MgCl₂, 200 mM KCl), 8 µl of a 10 mM mix of the four deoxynucleotide triphosphates (dNTPs, Pharmacia), 4 µl of random hexamer nucleotide primers (2 µg/µl stock, Pharmacia) and 1 µl of reverse transcriptase (NBL). Following a 90 minute incubation at 42 °C the nucleic acids were ethanol precipitated, washed 2 times with 70 % ethanol, and dissolved in 300 to 600 µl of DEPC-treated distilled water.

PCR was performed according to Rappolee et al with slight modifications (Rappolee et al., 1989). The reaction was run in tubes containing 10 µl of 10x PCR buffer (500 mM KCl, 100 mM TrisCl pH 8.3, 15 mM MgCl₂), 16 µl of dNTPs (1.25 mM each), 0.5 µl of Amplitaq (2.5 units, Perkin-Elmer) and 53.5 µl of the product from the RT

reaction. For all reactions a Ericomp thermocycler was used programmed as follows : 94 °C for 1 minute; 60 °C for 2 minutes; 72 °C for 3 minutes for 45 cycles.

Utilizing sequence published earlier, the following primers were used to detect rat KGF mRNA : 5'-AGAATTCCAAGTCCACAGTCATGATTTC and 5'-CAATCTAGATTACACAGATAGGAGGAGGC (Yan et al., 1991). This gave an amplified fragment of 423 base pairs. Oligonucleotide primers for rat KGFR were as follows: 5'-CACTCGGGGATAAATAGCTCCAAT and 5'-TATCCTCACCAGCGGGGTGTTGGA which gave an amplified fragment of 407 base pairs (Miki et al., 1991). Primers for GAP were 5'-TGATGACATCAAGAAGGTGGTGAAG and 5'-TCCTTGGAGGCCATGTGGGCCAT (ARCARI ET AL., 1984; ERCOLANI ET AL., 1988) which gave an amplified fragment of 244 base pairs. Oligonucleotide primers for TGF- α were as follows: 5'-TGCCCAGATTCCCACACTCAG and 5'-GAGGGCGCTGGGCTTCTC which gave an amplified fragment of 370 base pairs (Blasband et al., 1990). Products of PCR reaction were run on 2 % Seachem ME agarose gels (FMC) in 0.5 M TBE buffer containing ethidium bromide. Tissue specificity was investigated for all PCR assays.

RNase Protection Assay

RNase protection assays were performed by Dr. Axel Thomson as previously published (Sambrook et al., 1989). Briefly, antisense single stranded RNA probes for TGF- α (R. Coffey), EGFR (M. Skinner), KGF (W. McKeehan), KGFR (P. Finch), actin (Ambion Inc.) and cytokeratin 19 (A. Quaroni) were synthesized by T3 or T7 RNA polymerase. The sequences for the RNase probes RNA was isolated by RNA Stat 60 according to the methods of the manufacturer (Teltest B1, Houston, TX) from APs, SVs and VPs cultured six days under the various treatment conditions. RNA was incubated with the radiolabeled probe for 5 minutes at 95° then 12-16 hours at 45° to allow the probe to anneal to the mRNA. Single stranded RNA was digested with RNase A and RNase T1 which

specifically digests single stranded RNA. The reaction was stopped and the RNA/probe complex was phenol:chloroform extracted and recovered by ethanol precipitation. The RNA/probe complex was heated to 95° for 5 minutes and electrophoresed through a 6% polyacrylamide/ 7M urea gel with appropriate size standards (Sambrook et al., 1989). Gels were dried and autoradiographed.

Metabolism Studies

The metabolism studies were performed according to the methods of Siiteri and Wilson (Siiteri and Wilson, 1974). Briefly, glands were cultured 24 or 48 hours with [³H]-T, [³H]-DHT or [³H]-3 α -diol. Steroids were extracted from the culture media with 1 ml of 9:1 ether:ethylacetate, followed by 1 min. of vortexing and 10 minutes of centrifugation at 1500 rpm. The supernate containing the steroids was evaporated using a stream of nitrogen gas. The steroids were then brought up in 100 μ l chloroform and used for thin layer chromatography. A carrier mixture of reference steroids was added to each sample, such that 5 μ g of each carrier was loaded per sample. Next the sample was spotted on silica gel plastic plates and developed in a double ascent system of 98:2 chloroform:methanol at 4 C followed by 13:1 benzene:ethyl acetate at room temperature. The plates were air dried, sprayed with 25:1:1 glacial acetic acid:sulfuric acid:anisaldehyde mixture and heated to 80-90 C for 2-5 minutes. The steroids were marked, cut from the plate, extracted with 100% ethanol and radioactivity was counted using a scintillation counter. Percentage of conversion was based on total radioactivity added to the cultures. Control cultures lacking glands were included to determine breakdown of androgens in the absence of tissue.

RESULTS

General Observations: culture versus *in vivo*

The anterior prostates (AP) of newborn rats were cultured for six days, and various androgens were assayed for their ability to induce ductal branching morphogenesis. Nine androgens were tested: T, DHT, 3α -diol, R1881, MENT, androsterone, androstanedione, 3α -diol and $5\beta 3\alpha$ -diol. Four epithelial parameters were measured as an indication of prostatic ductal branching morphogenesis: epithelial area, epithelial perimeter, node number and form factor. Node number as assayed by Prism View software is a measure of the number of ductal branch points. Form factor is a numerical indication of the complexity of shape based on the area and perimeter of an object. A perfect circle, having the simplest shape, has a form factor of one. The form factor decreases as a shape becomes more complex. For this reason, shape complexity is reported as the inverse of form factor.

At birth (0 day) the rat AP is composed of a single duct without branch points surrounded by mesenchyme (Fig. 2A). Newborn rat APs had 0.186 ± 0.394 nodes/gland and an inverse form factor of 2.551 ± 1.289 . In contrast, APs from 6 day old rats had a highly branched ductal tree composed of a primary duct with secondary and tertiary branch points. The gland was elongated and secondary branching of the main duct emerged predominantly from one side resulting in asymmetrical branching of the gland (Fig. 2B). APs from 6 day old rats had 19.059 ± 4.205 nodes/gland and an inverse form factor of 29.683 ± 6.775 . Thus, between birth and 6 days postnatal, the AP undergoes striking morphogenetic changes *in situ*.

Androgen-dependent development of the AP in culture mimicked the development of the gland *in vivo* (Fig. 2). At the start of culture the AP had a single unbranched epithelial duct surrounded by mesenchyme and developed a highly branched epithelial ductal tree after 6 days of culture with androgens. As expected, epithelial branching morphogenesis was androgen-dependent *in vitro*. APs cultured for 6 days without

androgens survived, but the epithelium remained as a single unbranched duct (Fig. 2C). The pattern of epithelial branching induced by androgens in culture was different from that *in vivo* both qualitatively and quantitatively. AP cultured for 6 days with androgen had fewer nodes/explant, a reduction in the spacing between branch points along the main duct and thicker epithelial ducts in comparison to APs from 6 day old rats. The physical constraints acting on the developing AP in the organ culture system are different from those acting *in vivo*. *In vivo*, the AP is closely associated with the seminal vesicle, which is growing and elongating during this time of development. In organ culture the gland is isolated from the surrounding tissue and becomes much more rounded rather than elongated. Additionally, *in vivo* the gland is growing and branching in a 3-dimensional manner, while in organ culture the surface tension of the media, the physical limitation of tissue thickness, and access to nutrients in the culture system predisposes the gland to grow and branch in a much more 2-dimensional manner (Fig. 2). AP consistently had more ductal branches after 6 days of development *in vivo* than explants cultured for 6 days with any of the androgens tested (Fig. 2). This is likely due to a slight retardation of development while the glands recover from the micro dissection and manipulations involved in the culture procedure. It should be noted that the epithelium grows into and fills the mesenchymal compartment during the 6 days of growth either *in vivo* or *in vitro* with androgen. T was a potent inducer of epithelial branching morphogenesis in culture (Fig. 2D). However, the developmental response elicited by a maximal dose of T (10^{-7} M) was considerably less than that observed *in vivo* in comparably aged specimens (Fig. 2B and 2D). In response to 6 days of culture with T (10^{-7} M) the nodes/explant (Fig. 3A), inverse form factor (Fig. 3B) and epithelial perimeter (Fig. 3C) were 43%, 50.7% and 66%, respectively, of that observed in APs of 6 day old rats. However, the epithelial area of APs cultured with T (10^{-7} M) for 6 days was comparable (89.7%) to APs of 6 day old rats (Fig. 3D). Despite these differences in development of the AP *in vivo* versus *in vitro*, dramatic morphogenetic changes can be elicited by androgens in newborn rat APs during a

6 day culture period. Moreover, the developmental process observed *in vitro* is qualitatively comparable to that *in vivo*.

APs Cultured in the Absence of Androgens

APs cultured for six days in the absence of androgens survived but did not undergo epithelial ductal branching morphogenesis (Fig. 2C). Those glands that were completely unbranched at the start of culture remained unbranched after 6 days of culture in the absence of androgen (Fig. 2A and 2C). However, glands which were beginning to branch at the start of culture (1-2 branches) continued to branch for the first 24 hours of culture, even when cultured without androgen. This is likely due to "playing out" of the androgenic stimulus the gland received *in vivo*. The more branching present at the start of culture, the more ductal branching achieved in glands cultured without androgens. Thus, the androgenic response of the gland is not terminated immediately upon removal of androgens. The response was analogous to a ball rolling down a hill. The more branching at the start of culture the higher the hill and the farther the ball rolls, and, thus, the more branching observed at the end of culture without androgen. For this reason, only unbranched APs were used for all the experiments reported here. Nodes/explant, inverse form factor and epithelial perimeter were not different in glands grown in the absence of T from 0 day glands (Fig. 2A, 3B, and 3C). However, there was a 78.6% increase in the epithelial area during 6 days of culture in the absence of androgens compared to glands from newborn rats ($p < .0001$) (Fig. 3D). This increase in epithelial area was rather modest as epithelial area increased 454% during a comparable 6 days of development *in vivo*. Thus, after 6 days of culture in the absence of androgen APs grew slightly indicating that the explants were healthy, but the overall growth in culture in the absence of androgen was limited compared to APs at 6 days of development *in vivo*.

APs Cultured with Testosterone

APs cultured 6 days with testosterone (T) underwent extensive ductal branching morphogenesis (Fig. 2D). Based upon the analysis of 118 APs in 8 replicate experiments, an increase in ductal branching, relative to glands grown in the absence of androgen, was detectable as an increase in both node number and inverse form factor. APs cultured for 6 days with T concentrations as low as 10^{-10} M had 3.6 ± 2.914 nodes/explant and an inverse form factor of 7.705 ± 5.096 . Maximal response to T was observed at 10^{-9} M with 8.100 ± 3.985 nodes/explant and an inverse form factor of 12.758 ± 6.076 (Fig. 4). Thus, T was a potent inducer of prostatic development.

APs Cultured with DHT

DHT induced extensive epithelial ductal branching morphogenesis (Fig. 2E) similar in extent to that induced by T (Fig. 4). Based upon the analysis of 264 APs in 17 replicate experiments, branching was initially detectable as an increase in both node number and inverse form factor at DHT concentrations as low as 10^{-10} M with 3.543 ± 2.307 nodes/explant and an inverse form factor of 7.146 ± 3.295 . Maximal response was achieved with DHT at 10^{-8} M with 7.567 ± 2.515 nodes/explant and an inverse form factor of 12.527 ± 4.853 (Fig. 4). The degree of epithelial branching morphogenesis induced by T and DHT were comparable for nodes/explant, epithelial area and epithelial perimeter (Fig. 4). Surprisingly, the inverse form factor for DHT at 10^{-8} M was less than that of T at the same concentration ($p < .02$). Thus, T at 10^{-8} M induced greater complexity of the epithelial shape as measured by the inverse of form factor and a higher degree of ductal branching morphogenesis than DHT. While DHT is a potent androgen, the effect of T was equal or greater than that of DHT at all concentrations.

APs Cultured with 3 α -diol

3 α -diol induced extensive ductal branching morphogenesis (Fig. 2F) similar in extent to that induced by DHT and T (Fig. 4). Based upon the analysis of 346 APs in 21 replicate experiments, branching was initially detectable by both node number and inverse form factor at concentrations as low as 10^{-11} M ($p=.0058$ and $p=.0009$, respectively) with 1.469 ± 1.609 nodes/explant and an inverse form factor of 4.852 ± 2.871 . Maximal branching morphogenesis was induced at 10^{-7} M with 8.593 ± 3.434 nodes/explant and an inverse form factor of 13.006 ± 4.939 (Fig. 4).

The degree of ductal branching morphogenesis induced by T and 3 α -diol were comparable for node number, epithelial area and epithelial perimeter (Fig. 4). However, nodes/explant was slightly decreased with 3 α -diol at 10^{-8} M compared to T at the same concentration ($p<.009$) (Fig. 4A). Similarly, in cultures treated with 3 α -diol (10^{-8} M) inverse form factor ($p<.002$) (Fig. 4B) and epithelial perimeter ($p<.009$) (Fig. 4C) were consistently less than that observed in explants treated with T (10^{-8} M). Additionally, the inverse form factor of explants cultured with 3 α -diol 10^{-9} M was slightly decreased in comparison to explants cultured with T at the same concentration ($p<.023$) (Fig. 4B). Thus, T at 10^{-8} M and 10^{-9} M was more effective than 3 α -diol at the same concentrations and induced a greater complexity of the epithelial shape as measured by inverse form factor indicative of increased ductal branching morphogenesis. However, the amount of epithelial area induced by 3 α -diol was comparable to that induced by T (Fig. 4D). While 3 α -diol is a potent androgen, at higher concentrations 3 α -diol does not elicits the same degree of prostatic development as that elicited by T.

APs Cultured with Synthetic Androgens

R1881 and MENT are both synthetic androgens capable of binding to the androgen receptor, but cannot be 5 α -reduced (Bonne and Raynaud, 1975; Kumar et al., 1992). Both R1881 and MENT induced extensive ductal branching morphogenesis (Fig. 2G and

2H). Ductal branching was initially detectable by both node number and inverse form factor using R1881 at concentrations as low as 10^{-10} M with 5.000 ± 2.082 nodes/explants and an inverse form factor of 8.139 ± 1.155 (Fig. 5A and 5B). The maximal response to R1881 was observed at 10^{-8} M with 6.286 ± 0.488 nodes/explants and an inverse form factor of 10.922 ± 4.356 (Fig. 5A and 5B).

Ductal branching was initially detectable as an increase in both node number and inverse form factor using MENT at concentrations as low as 10^{-10} M with 5.000 ± 2.082 nodes/explants and an inverse form factor of 8.139 ± 1.155 (Fig. 5A and 5B). Maximal ductal branching was induced by MENT at concentrations of 10^{-9} M with 6.143 ± 1.069 nodes/explant and an inverse form factor of 10.409 ± 1.577 (Fig. 5A and 5B). Thus, the biological response, as measured by branching morphogenesis, elicited by either of these synthetic androgens, was comparable to that elicited by T (Fig. 5).

APs Cultured with Androsterone, Androstenedione, 3β -diol, $5\beta 3\alpha$ -diol

Androsterone, androstenedione, 3β -diol and $5\beta 3\alpha$ -diol were relatively ineffective in inducing ductal branching morphogenesis compared to T (Fig. 2I, 2J, 2K, 2L). At optimal doses (10^{-7} M) the maximal nodes/explant induced by these androgens were between 35.9-70.8% of the nodes/explant induced by T. With androsterone at concentrations as low as 10^{-9} M ductal branching was initially detectable with 2.857 ± 1.215 nodes/explant and an inverse form factor of 5.980 ± 1.368 (Fig. 6A and 6B). Maximal ductal branching was induced by androsterone at 10^{-7} M concentration with 6.250 ± 1.258 nodes/explant and an inverse form factor of 10.081 ± 2.754 (Fig. 6A and 6B). Androsterone at 10^{-7} M induced 78.1 % of the nodes/explant induced by T at the same concentration. Thus, at all concentrations androsterone was less effective than T.

Androstenedione induced detectable ductal branching at concentrations as low as 10^{-8} M with 4.300 ± 2.584 nodes/explant and an inverse form factor of 6.381 ± 2.353 (Fig. 6A and 6B). Maximal response of androstenedione was achieved at 10^{-8} M.

Androstenedione at 10^{-7} M induced only 55.5% of the nodes/explant induced by T at the same concentration.

3β -diol induced detectable ductal branching at concentrations as low as 10^{-9} M with 2.100 ± 2.025 nodes/explant and an inverse form factor of 5.206 ± 3.013 (Fig. 6A and 6B). Maximal ductal branching was induced by 3β -diol at 10^{-8} M with 3.889 ± 1.691 nodes/explant and an inverse form factor of 8.015 ± 3.182 (Fig. 6A and 6B). 3β -diol at 10^{-7} M induced only 48.6% of the nodes/explant induced by T at the same concentration.

$5\beta 3\alpha$ -diol was the least effective androgen tested (Fig. 2L). Ductal branching was initially detectable using $5\beta 3\alpha$ -diol at a concentration of 10^{-7} M with 2.875 ± 1.808 nodes/explant and an inverse form factor of 6.208 ± 1.655 (Fig. 6A and 6B). $5\beta 3\alpha$ -diol at 10^{-7} M was also the concentration at which the maximal response was induced. $5\beta 3\alpha$ -diol at 10^{-7} M induced only 35.9% of the nodes/explant induced by T at the same concentration. Thus, at all the concentrations tested androstenedione, androsterone, 3β -diol and $5\beta 3\alpha$ -diol were less effective than T at inducing ductal branching morphogenesis.

Metabolism of T, DHT, and 3α -diol in Culture

Metabolism studies were performed on cultured neonatal rat APs to determine the amount of conversion during culture of T, DHT, and 3α -diol to other metabolites. Following culture of APs with ^3H -T at 10^{-8} M for 48 hours, 34.9% of T remained unmetabolized, while 52.6% was metabolized to DHT, 5.7% was metabolized to androstenedione and 3.5% was metabolized to 3α -diol (See Fig. 7). Thus, T was extensively metabolized to DHT by the developing rat AP during culture. After 24 hours of culture with ^3H -DHT (10^{-7} M, 10^{-9} M, 10^{-11} M) 82.5%-89.0% of the DHT remained unmetabolized, while 6.7%-11.7% was metabolized to 3α -diol, 1.7%-2.3% was metabolized to 3β -diol, 1.0%-2.4% was metabolized to androstenedione and 1.2%-1.8% was metabolized to androsterone (See Fig. 8). Thus, DHT was only slightly metabolized to other androgens by the developing rat AP during culture. After 24 hours of culture with

^3H - 3α -diol (10^{-7} M, 10^{-9} M, 10^{-10} M) 63.4%-70.8% remained unmetabolized, while 18.9%-25.1% was metabolized to DHT, 4.1%-10.5% was metabolized to 3β -diol, 1.2%-1.7% was metabolized to androsterone, and 0.2%-1.5% was metabolized to androstanedione (Fig. 9). Thus, only about 20% of 3α -diol is metabolized back to DHT in the developing rat AP during culture. This backconversion of 3α -diol to DHT is such that the androgenic activity of 3α -diol can not be ruled out as being due to the activity of DHT.

Summary

The endpoint of the assay (ductal branching morphogenesis) is complex and has associated with it some degree of variation. However, these studies have utilized measurements of four epithelial parameters to quantitate the amount of ductal branching morphogenesis in the developing rat prostate during 6 days of culture. T, DHT and 3α -diol are potent androgens and able to induce ductal branching morphogenesis. However, while the potency of T, DHT and 3α -diol are comparable, surprising the efficacy of T is equal to or greater than either DHT or 3α -diol at all concentrations. The studies on the metabolism of T indicate that T is efficiently converted to DHT, while 3α -diol is a less abundant metabolite of T. The studies on 3α -diol metabolism do not rule out the possibility that the androgenic activity induced by 3α -diol is due to backconversion to DHT. The synthetic androgens R1881 and MENT are also potent inducers of ductal branching morphogenesis, suggesting that 5α -reduction is not required for potent androgen action in the developing prostate. However, T also has a greater efficacy than either of the synthetic androgens. Androsterone, androstanedione, 3β -diol and 3β - 3α -diol are less potent androgens at inducing prostatic ductal branching morphogenesis. At high concentrations all four of these androgens induce ductal branching but the efficacy of T is greater than all four of these androgens.

Figure 2

Rat APs at birth (A), at 6 days neonatal (B), or after 6 days of culture (C-L).

A.) 0 day AP at the start of culture. Epithelial branching morphogenesis has not begun.

B.) 6 day neonatal AP. Extensive epithelial branching morphogenesis has occurred. Note that ducts emerge preferentially from one side of the main duct.

C.) 0 day AP cultured 6 days in the absence of androgen. Epithelial branching morphogenesis has not occurred in the absence of androgens.

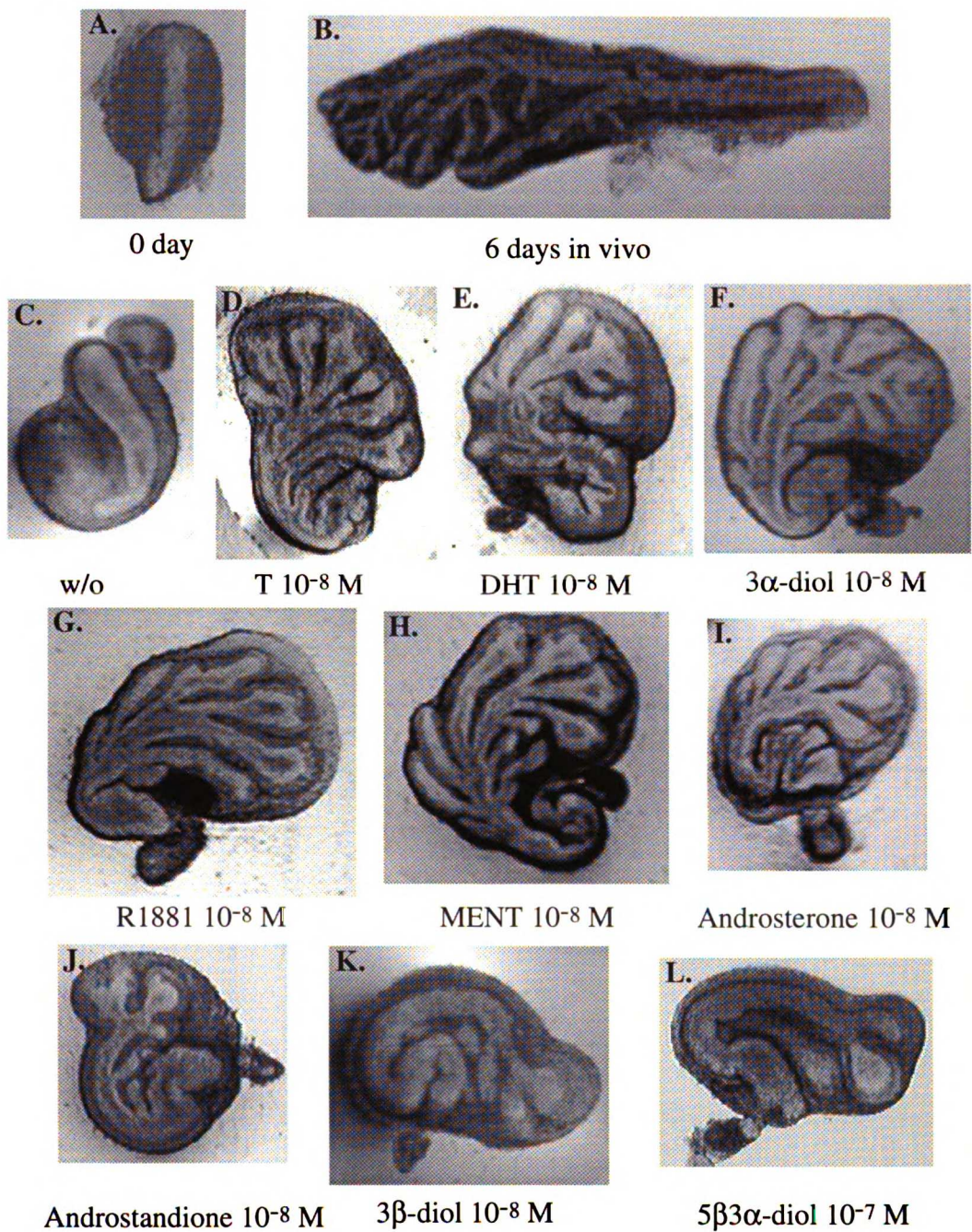
0 day APs cultured 6 days with T 10^{-8} M (D), DHT 10^{-8} M (E) or 3α -diol 10^{-8} M (F). All three androgens induced extensive epithelial branching morphogenesis.

0 day APs cultured 6 days with R1881 10^{-8} M (G) or MENT 10^{-8} M (H). Both of these synthetic androgens induced extensive epithelial branching morphogenesis comparable to T. Both androgens have a high affinity for the androgen receptor, but are not metabolized.

0 day APs cultured 6 days with Androsterone 10^{-8} M (I), Androstanedione 10^{-8} M (J), 3β -diol 10^{-8} M (K) or $5\beta3\alpha$ -diol 10^{-7} M (L).

All of these later androgens induced branching, but were less effective than T. Compare I-L with D.

Figure 2



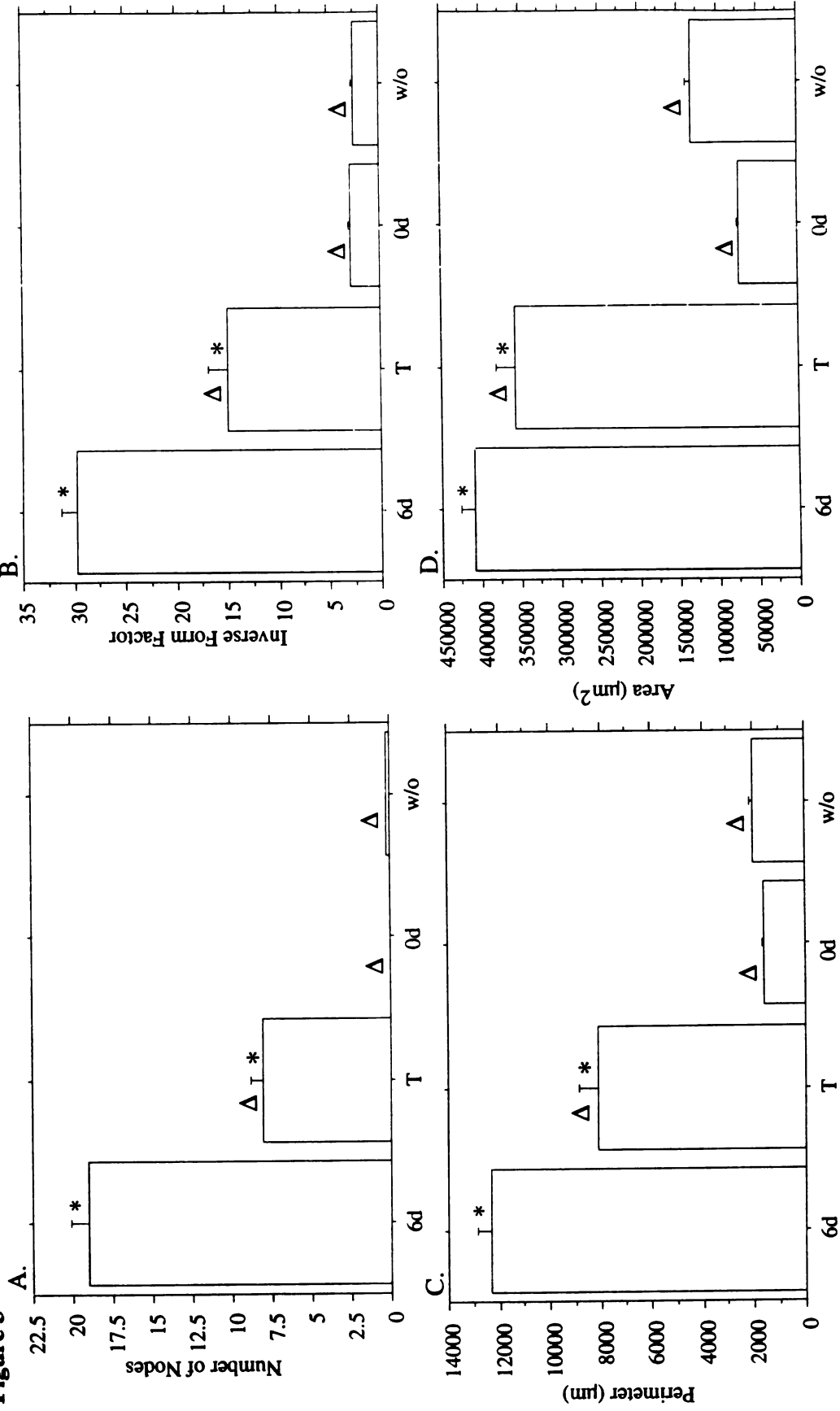
Representative images of rat APs after 6 days of culture with different androgen treatments.

Figure 3

Comparison of 0 day (0d) and 6 day (6d) neonatal rat APs with newborn (0 day) APs cultured for 6 days in the absence of T (w/o) or with T 10^{-7} M (T). Four parameters of epithelial morphogenesis were assessed: A. Number of nodes, B. Inverse form factor, C. Epithelial perimeter, D. Epithelial area. * Indicates significantly different from 0 day and Δ indicates significantly different from 6 days in vivo ($p < .01$).

Treatment	Count
6 day	n=17
T 10^{-7} M	n=10
0 day	n=19
w/o	n=43

Figure 3



* Different from 0 day ($p < .05$).
Δ Different from 6 days in vivo ($p < .05$).

Figure 4

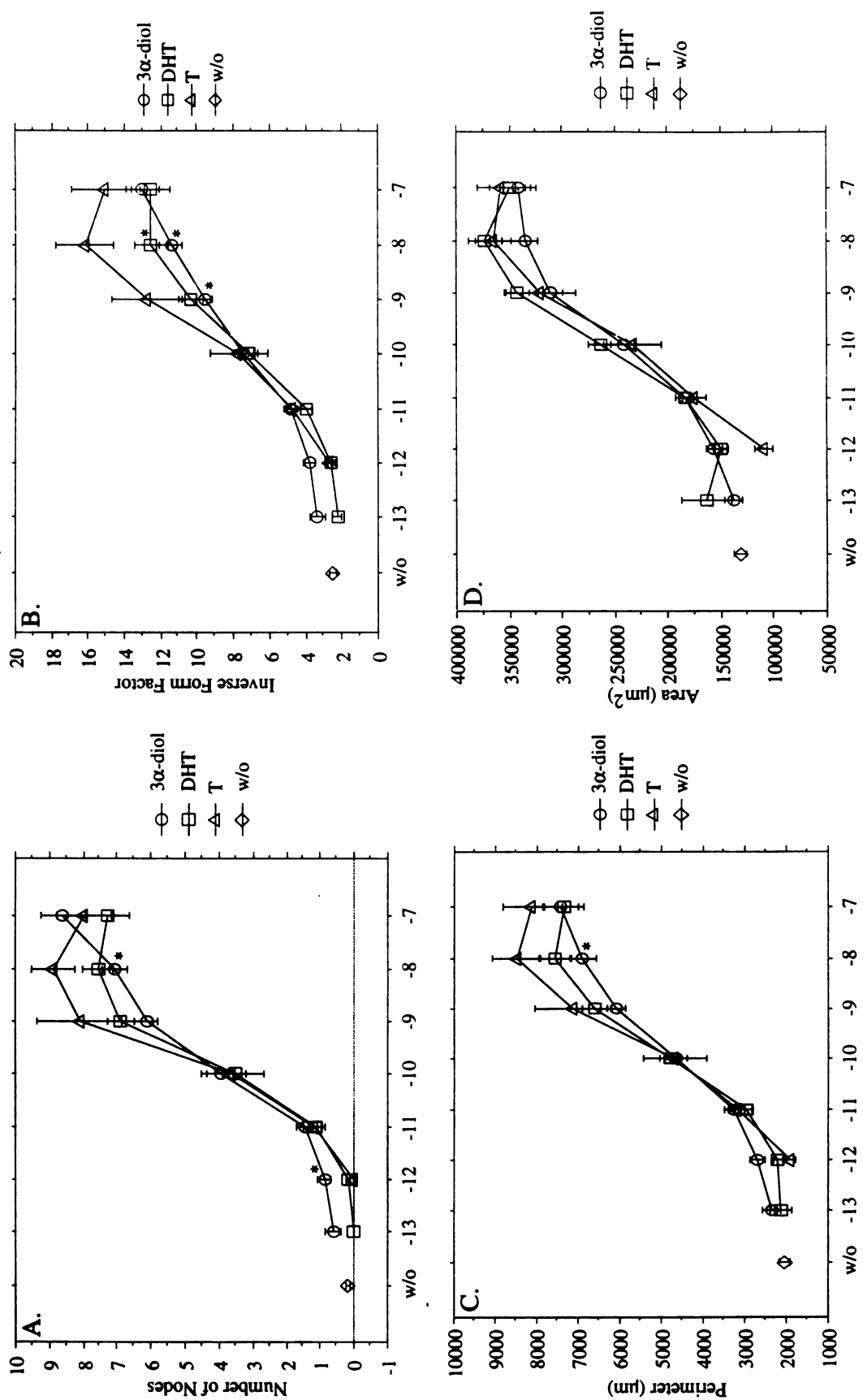
Dose response for APs cultured for 6 days with T, DHT, 3 α -diol and in the absence of androgen (w/o). Four parameters of epithelial morphogenesis were assessed: A. Number of nodes, B. Inverse form factor, C. Epithelial perimeter, D. Epithelial area. * Indicates significantly different from T at the same dose ($p<.05$).

Count of glands per data point on graph

w/o n=43	10 ⁻¹³ M	10 ⁻¹² M	10 ⁻¹¹ M	10 ⁻¹⁰ M	10 ⁻⁹ M	10 ⁻⁸ M	10 ⁻⁷ M
T	ND	n=6	n=11	n=10	n=10	n=25	n=10
DHT	n=6	n=39	n=40	n=46	n=39	n=30	n=22
3 α -diol	n=26	n=44	n=49	n=65	n=41	n=36	n=27

ND=Not done

Figure 4



* Different from T at the same concentration

Figure 5

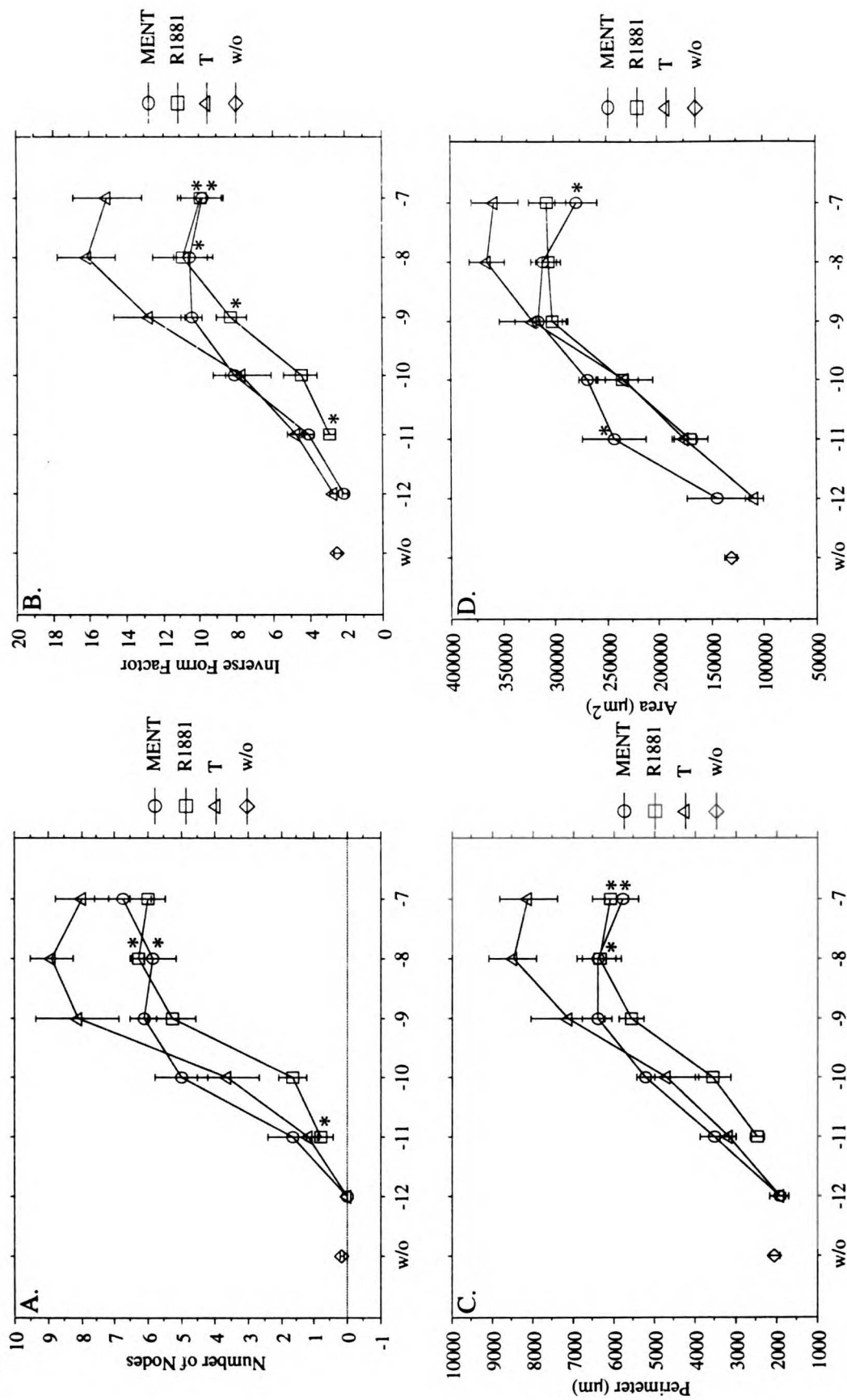
Dose response for APs cultured for 6 days with T, R1881, MENT and in the absence of androgen (w/o). Four parameters of epithelial morphogenesis were assessed: A. Number of nodes, B. Inverse form factor, C. Epithelial perimeter, D. Epithelial area. * Indicates significantly different from T at the same dose ($p<.05$).

Count of glands per data point on graph

w/o n=43	10 ⁻¹² M	10 ⁻¹¹ M	10 ⁻¹⁰ M	10 ⁻⁹ M	10 ⁻⁸ M	10 ⁻⁷ M
T	n=6	n=11	n=10	n=10	n=25	n=10
MENT	n=3	n=6	n=7	n=7	n=8	n=8
R1881	ND	n=6	n=6	n=7	n=7	n=8

ND=Not done

Figure 5



* Different from T at the same concentration

Figure 6

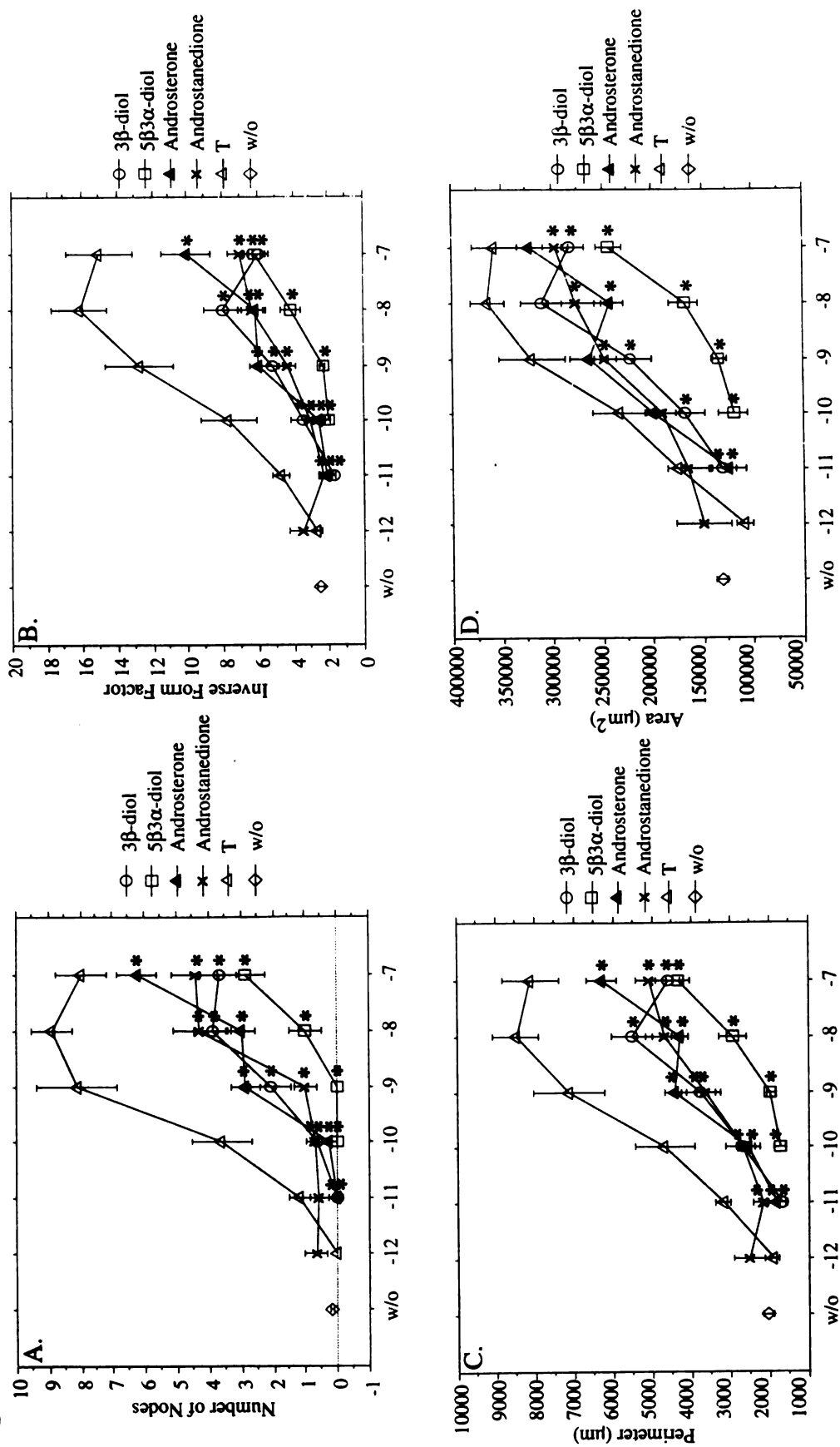
Dose response for APs cultured for 6 days with T, Androstosterone, Androstenedione, 3 β -diol, 5 β 3 α -diol and in the absence of androgen (w/o). Four parameters of epithelial morphogenesis were assessed: A. Number of nodes, B. Inverse form factor, C. Epithelial perimeter, D. Epithelial area. * Indicates significantly different from T at the same dose ($p < .05$).

Count of glands per data point on graph

w/o n=43	10 ⁻¹² M	10 ⁻¹¹ M	10 ⁻¹⁰ M	10 ⁻⁹ M	10 ⁻⁸ M	10 ⁻⁷ M
T	n=6	n=11	n=10	n=10	n=25	n=10
Androstosterone	ND	n=5	n=7	n=7	n=7	n=4
Androstenedione	n=4	n=7	n=10	n=9	n=10	n=9
3 α -diol	ND	n=7	n=8	n=10	n=9	n=13
5 β 3 α -diol	ND	ND	n=3	n=8	n=8	n=8

ND=Not done

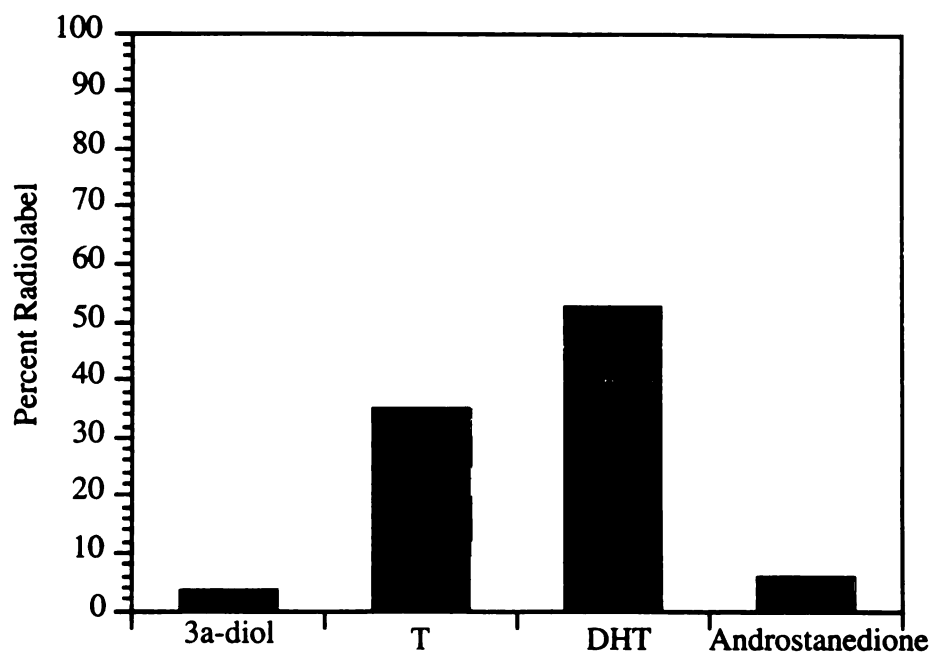
Figure 6



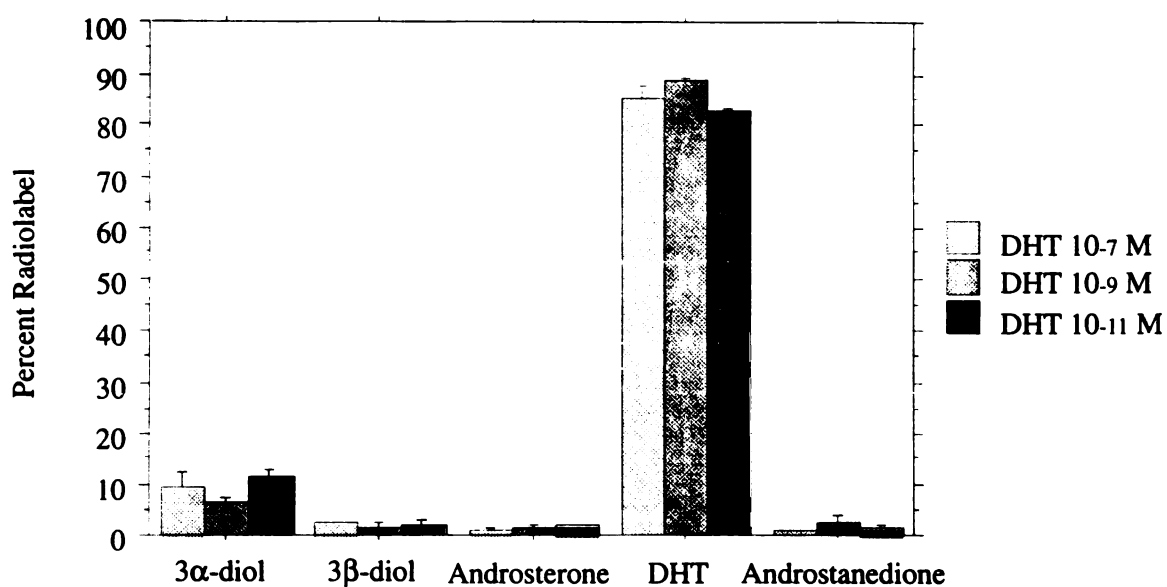
* Different from T at the same concentration

Figure 7

Metabolism of T



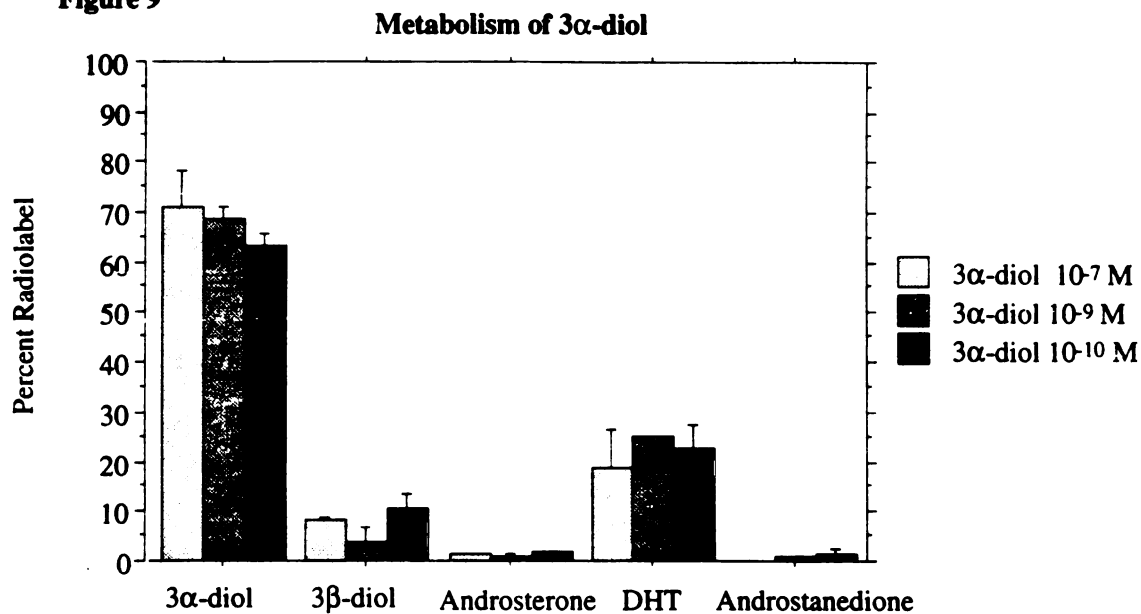
Percent conversion of testosterone into various metabolites by rat APs after 48 hours in culture. Neonatal rat APs were cultured for 48 hours with ^3H -testosterone at 10^{-8} M. The extracted metabolites were analyzed by TLC.

Figure 8**Metabolism of DHT**

Percent conversion of DHT into various metabolites by rat APs after 24 hours in culture. Neonatal rat AP were cultured for 48 hours with ³H-radiolabeled DHT at concentrations of 10⁻⁷ M, 10⁻⁹ M or 10⁻¹⁰ M. The extracted metabolites were analyzed by TLC. Results are represented as mean percent ± standard error, n=3.

Percent Conversion of DHT After 48 Hours of Culture

[DHT]	Metabolites				
	3α-diol	3β-diol	Androsterone	DHT	Androstandione
DHT 10 ⁻⁷ M	9.9±2.6%	2.3±0.2%	1.2±0.2%	85.6±2.4%	1.0±0.1%
DHT 10 ⁻⁹ M	6.6±0.9%	1.7±0.6%	1.4±0.3%	89.0±0.3%	2.4±1.7%
DHT 10 ⁻¹¹ M	11.7±1.2%	2.1±0.8%	1.8±0.3%	82.5±0.5%	1.6±0.2%
Mean of Total	9.4±2.7%	2.0±0.6%	1.5±0.4%	85.7±3.0%	1.7±1.0%

Figure 9

Percent conversion of 3 α -diol into various metabolites by rat APs after 24 hours in culture. Neonatal rat APs were cultured for 48 hours with ³H-radiolabelled 3 α -diol at concentrations of 10⁻⁷ M, 10⁻⁹ M or 10⁻¹⁰ M. The extracted metabolites were analyzed by TLC. Results are represented as mean percent $\pm$ standard error, n=3.

Percent Conversion of 3 α -diol After 48 Hours of Culture

[3 α -diol]	Metabolites				
	3 α -diol	3 β -diol	Androsterone	DHT	Androstandione
3 α -diol 10 ⁻⁷ M	70.8 $\pm$ 7.5%	8.2 $\pm$ 0.3%	1.4 $\pm$ 0.2%	18.9 $\pm$ 7.6%	0.2 $\pm$ 0.2%
3 α -diol 10 ⁻⁹ M	68.6 $\pm$ 2.3%	4.1 $\pm$ 2.7%	1.2 $\pm$ 0.2%	25.1 $\pm$ 0.2%	1.0 $\pm$ 0.2%
3 α -diol 10 ⁻¹¹ M	63.4 $\pm$ 2.2%	10.5 $\pm$ 3.2%	1.7 $\pm$ 0.3%	22.5 $\pm$ 5.2%	1.6 $\pm$ 0.8%
Mean of Total	67.6 $\pm$ 5.2%	7.6 $\pm$ 3.5%	1.4 $\pm$ 0.3%	22.2 $\pm$ 5.3%	0.9 $\pm$ 0.7%

APs Cultured with Growth Factors

Newborn rat APs were cultured for six days either in the presence or absence of T in combination with a variety of growth factors or neutralizing agents to growth factors to assay their effect on ductal branching morphogenesis. The growth factors tested were: EGF, TGF- α , anti-TGF- α , TGF- β 1, TGF- β 2, anti-TGF- β , FGF-7/KGF, anti-KGF, FGF-1/aFGF (acidic FGF), FGF-2/bFGF (basic FGF), and HGF. Ductal branching morphogenesis was measured using four epithelial parameters (node number, inverse form factor, epithelial perimeter and epithelial area) as determined by Prism View software. Ductal branching morphogenesis was effected by EGF, TGF- α , anti-TGF- α , TGF- β 1, TGF- β 2 and FGF-7/KGF. However, anti-TGF- β , anti-KGF, FGF-1/aFGF, FGF-2/bFGF, and HGF did not effect ductal branching morphogenesis.

APs Cultured with EGF

Newborn rat APs cultured with T 10^{-8} M and EGF (1-50 ng/ml) had a distorted pattern of ductal branching. The epithelial ducts were thickened and in many cases the epithelium was difficult to see clearly (Fig. 10). Nodes/explant decreased at higher doses of EGF (Fig. 12A). At 10, 20 and 40 ng/ml EGF plus T 10^{-8} M nodes/explant were 53.1%, 49.4% and 58.9%, respectively, of that in explants grown with T alone. Inverse form factor decreased in all cultures grown with T plus EGF (1-40 ng/ml) (Fig. 12B). Thus, EGF in combination with T decreased the complexity of the epithelial shape. At higher doses of EGF (10, 20 and 40 ng/ml) the inverse of the form factor was 32-37% of that elicited by T alone and was not different from explants grown in the absence of T. Epithelial perimeter decreased in explants cultured with T and EGF (Fig. 12C). Thus, EGF disrupted the normal pattern of T-induced ductal branching. Epithelial area decreased with EGF treatment (1-20 ng/ml), but the decrease was modest being 71-80% of that elicited by T. At the highest dose of EGF (40 ng/ml) the epithelial area was not different from that elicited by T itself (Fig. 12D).

APs cultured with EGF but in the absence of T did not undergo ductal branching morphogenesis (Fig. 11). EGF-treated explants were not different from explants cultured in the absence of T for all parameters measured (node number, inverse form factor, epithelial perimeter or epithelial area) (Fig. 12). Thus, EGF was not able to induce ductal branching in the absence of androgens. Furthermore, EGF distorted and inhibited the pattern of ductal branching in the presence of T.

APs Cultured with TGF- α

Newborn rat APs cultured with T 10^{-8} M plus TGF- α (5-50 ng/ml) also had a distorted pattern of branching morphogenesis similar to that produced by EGF (Fig. 13). Nodes/explant in glands treated with TGF- α plus T 10^{-8} M were not different from that elicited by T (Fig. 15A). However, the inverse form factor decreased in all cultures with T 10^{-8} M plus TGF- α (5-50 ng/ml) (Fig. 15B). In cultures treated with 5, 10 or 20 ng/ml of TGF- α plus T 10^{-8} M, inverse form factor was 70.5%, 67.7% and 57.5%, respectively, of that elicited by T alone. At the highest dose of TGF- α (50 ng/ml) in combination with T 10^{-8} M, inverse of the form factor was reduced to 39.5% of that elicited by T alone and was not different from explants cultured in the absence of androgen. Thus, TGF- α decreased the complexity of epithelial shape and distorted the normal pattern of ductal branching. While the epithelial perimeter decreased in explants cultured with TGF- α (10-50 ng/ml) in combination with T 10^{-8} M (Fig. 15C), the epithelial area was not significantly different from that elicited by T alone (Fig. 15D). Thus, TGF- α distorted the pattern of ductal branching morphogenesis in the presence of T.

APs cultured with TGF- α but in the absence of T did not undergo ductal branching morphogenesis (Fig. 14). With regard to node number, inverse factor and epithelial perimeter, TGF- α treated explants were not different from explants cultured in the absence of T (Fig. 15A, 15B, 15D). However, TGF- α treated explants (20 and 50 ng/ml) had an increase in epithelial area compared to APs cultured in the absence of T (Fig. 15C). In

explants treated with 20 or 50 ng/ml of TGF- α , but in the absence of T, epithelial area was 145% and 151%, respectively, of that observed in explants cultured in the absence of T. Thus, while TGF- α appeared to stimulate epithelial growth, TGF- α was not able to induce ductal branching in the absence of androgens.

APs Cultured with Neutralizing Antiserum to TGF- α

APs were cultured with T 10^{-8} M plus neutralizing antisera to TGF- α . Two such antisera were used. Both were goat anti-rat TGF- α polyclonal antisera. One is available commercially from Biotope (anti-TGF- α I) and the other was made by Dr. R. Coffey (anti-TGF- α II). Both antisera are reported to be specific for TGF- α and do not cross react with EGF (Cheng et al., 1993; Perroteau et al., 1986). Both antisera profoundly inhibited T-induced ductal branching morphogenesis (Fig. 16 and 17), although the efficacy varied between the two antisera. At very high concentrations of both antisera (anti-TGF- α I, 1:50; anti-TGF- α II, 1:250-1:1000) the epithelium was difficult to see through the surrounding mesenchyme in wholemount preparations (Fig. 16 and 17). At a dilution of 1:200 anti-TGF- α I partially inhibited T-induced ductal branching morphogenesis as assessed by decreased in node number, inverse form factor and epithelial perimeter when compared to that elicited by T alone (Fig 19). For TGF- α I at a dilution of 1:200 nodes/explant were 61%, inverse form factor was 58.5% and epithelial perimeter was 70% of that elicited by T alone. At dilutions of 1:100 anti-TGF- α I completely inhibited T-induced ductal branching morphogenesis. Explants cultured with T 10^{-8} M plus a 1:100 dilution of anti-TGF- α I were not different from explants cultured in the absence of T by three of the parameters measured (node number, inverse form factor and epithelial perimeter) (Fig. 19). At this concentration of anti-TGF- α I the epithelial area was 68.1% of that elicited by T alone. Thus, at a 1:100 dilution anti-TGF- α I profoundly inhibited T-induced ductal branching morphogenesis and partially inhibited epithelial growth as

assessed by epithelial area. At a 1:50 dilution anti-TGF- α I also completely inhibited ductal branching morphogenesis as measured by all four parameters (Fig. 19).

Anti-TGF- α II also inhibited T-induced branching morphogenesis at dilutions of 1:250 to 1:16,000. (Fig. 19). Anti-TGF- α II at a dilution of 1:32,000 did not inhibit T-induced ductal branching morphogenesis. Explants treated with a 1:32,000 dilution of anti-TGF- α II were not different from explants cultured with T alone (Fig. 19) as assessed by node number, inverse form factor, epithelial perimeter and epithelial area (Fig. 19).

Inhibition of T-induced ductal branching morphogenesis by anti-TGF- α (I and II) was dose-dependent and reversible. Following 4-6 days of culture with anti-TGF- α branching morphogenesis resumed when explants were cultured for an additional 4 days with T 10^{-8} M but in the absence of antisera (Fig. 18). Therefore, inhibition of ductal branching with anti-TGF- α was not due to toxicity.

Thus, both neutralizing antisera to TGF- α blocked ductal branching. Inhibition of prostatic development with anti-TGF- α was reversible and not due to toxicity. These studies suggest that TGF- α is produced by the developing rat prostate and blocking the activity of endogenous TGF- α inhibits ductal branching. Thus, TGF- α is important in ductal branching morphogenesis in the developing rat prostate.

APs cultured with TGF- β 1

TGF- β 1 profoundly inhibited T-induced ductal branching morphogenesis in cultured newborn rat APs (Fig. 20A, 20C, 20E). In TGF- β 1 treated explants the epithelium was difficult to see clearly through the surrounding mesenchyme. At 20 ng/ml, TGF- β 1 disrupted normal T-induced ductal branching as determined by a decrease in node number, inverse form factor and epithelial perimeter compared to explants grown with T alone (Fig. 21). In APs cultured with 20 ng/ml TGF- β 1 plus T 10^{-8} M, nodes/explant, inverse form factor and epithelial perimeter were reduced to 55.5%, 42.2% and 58.1%, respectively, of that elicited by T alone. The epithelial area of APs treated with T 10^{-8} M

plus 20 ng/ml of TGF- β 1 was not different those treated with T alone, suggesting that TGF- β 1 at 20 ng/ml did not inhibit epithelial growth. Nodes/explant, inverse form factor and epithelial perimeter were not different in APs cultured with 50 ng/ml TGF- β 1 plus T 10^{-8} M versus explants cultured in the absence of T, indicating that TGF- β 1 at 50 ng/ml completely inhibited T-induced ductal branching. In contrast, in these TGF- β 1 (50 ng/ml) treated cultures epithelial area was 64.9% of that elicited by T. However, the T plus TGF- β cultures were still significantly larger (196%) than glands grown in the absence of T (Fig. 21). Thus, TGF- β 1 was able to completely inhibit ductal branching morphogenesis as determined by node number, inverse form factor and epithelial perimeter but had only a graded effect on epithelial area.

APs cultured with TGF- β 1 (20 or 50 ng/ml) but in the absence of T did not undergo ductal branching morphogenesis (Fig. 20B, 20D, 20F), nor were nodes/explant, inverse form factor, epithelial perimeter and epithelial area different from those cultured in the absence of T (Fig. 21). Significantly, in the absence of T, TGF- β 1 did not elicit further reduction in any of the parameters measured.

APs Cultured with TGF- β 2

TGF- β 2 profoundly inhibited T-induced ductal branching morphogenesis similar to the inhibition observed with TGF- β 1 (Fig. 22). At low concentrations (2.5-5 ng/ml), TGF- β 2 affected the pattern of T-induced ductal branching. At 2.5 ng/ml of TGF- β 2, the pattern of T-induced ductal branching morphogenesis was distorted as reflected by a decrease in nodes/explant (37.6% of that elicited by T alone), inverse form factor (55.6% of that elicited by T alone) and epithelial perimeter (56% of that elicited by T alone). However, in cultures treated with 2.5 ng/ml of TGF- β 2 and T 10^{-8} M, epithelial area was not different from cultures treated with T 10^{-8} M alone (Fig. 23). Likewise, 5 ng/ml of TGF- β 2 distorted the pattern of T-induced ductal branching as reflected by a decrease in nodes/explant (64.5% of that elicited by T alone), inverse form factor (75.5% of that

elicited by T alone) and epithelial perimeter (71% of that elicited by T alone) while epithelial area was not different from cultures treated with T 10^{-8} M (Fig. 23). At 10 ng/ml of TGF- β 2 plus T 10^{-8} M nodes/explant and inverse form factor were not different from explants cultured in the absence of T, while epithelial perimeter was 47% of that elicited by T. Epithelial area was not different from that elicited by T alone (Fig. 23). Thus, T-induced ductal branching morphogenesis was profoundly inhibited by TGF- β 2. Following culture with T 10^{-8} M plus TGF- β 2, explants continued to grow but did not resume ductal branching when cultured for an additional 4 days without TGF- β 2, thus, indicating that TGF- β 2 treatment is not toxic.

Qualitatively TGF- β 1 and TGF- β 2 inhibited T-induced ductal branching morphogenesis in the developing AP in a similar manner. TGF- β induces apoptosis in the adult prostate (Martikainen et al., 1990). In the present experiments TGF- β treatment probably did not induce apoptosis in the developing prostate as indicated by the comparable epithelial areas of glands cultured in the absence of T plus or minus TGF- β 1, as well as, the comparable epithelial areas of glands cultured with T 10^{-8} M plus or minus TGF- β 2 (2.5-40 ng/ml). Thus, unlike the adult prostate, the developing gland does not appear to undergo massive apoptosis in response to TGF- β either in the presence or absence of T.

APs Cultured with anti-TGF- β

APs were cultured with or without T plus an antibody to TGF- β (anti-TGF- β). This polyclonal antisera was a gift from Dr. H. Moses. Anti-TGF- β recognizes all three forms of TGF- β (TGF- β 1, - β 2 and - β 3) and blocks the effects of TGF- β . Anti-TGF- β did not effect T-induced ductal branching morphogenesis as assessed by node number, inverse form factor, epithelial perimeter and epithelial area (Fig. 24 and 25). Nor did anti-TGF- β have an affect on APs cultured in the absence of T (Fig. 24 and 25). It is not known if this antibody cross reacts with rodent. Therefore, the negative results obtained in these experiments could be due to lack of cross-reactivity of the antibody with rat TGF- β s.

Alternatively, if TGF- β is not involved in ductal branching morphogenesis, blocking its activity would not have an effect. It has been demonstrated that the developing AP can respond to exogenous TGF- β 1 and TGF- β 2. However, this does not indicate that TGF- β is involved in ductal branching of the developing prostate.

APs Cultured with FGF-7/KGF

APs were cultured either with or without T 10^{-8} M plus FGF-7/KGF (0.5-500 ng/ml). APs cultured with T 10^{-8} M plus high doses of FGF-7/KGF (50 or 500 ng/ml) had a distorted pattern of ductal branching with decreased ductal branching and thickened epithelial ducts (Fig. 26). In APs cultured with T 10^{-8} M plus 50 or 500 ng/ml of FGF-7/KGF, nodes/explant and inverse form factor was not different from APs cultured in the absence of T (Fig 28). Epithelial perimeter was decreased in cultures treated with T 10^{-8} M plus either 50 or 500 ng/ml of FGF-7/KGF (59.8% and 67.1% of that elicited by T alone, respectively). However, epithelial area was greater in explants treated with T 10^{-8} M plus either 50 or 500 ng/ml of FGF-7/KGF relative to explants cultured with T alone (143% and 253% of elicited by T, respectively) (Fig. 28). Thus, FGF-7/KGF induced epithelial growth but disrupted normal ductal branching when given in combination with T.

APs cultured with FGF-7/KGF but in the absence of T did not undergo ductal branching morphogenesis (Fig. 27), but FGF-7/KGF at 100 or 500 ng/ml in the absence of T increased epithelial area by 150% and 233% relative to APs grown in the absence of T. However, nodes/explant, inverse form factor and epithelial perimeter of APs grown with FGF-7/KGF were not different from APs grown in the absence of T. Even though FGF-7/KGF in the absence of T increased the amount of epithelium, FGF-7/KGF by itself did not have a morphogenetic effect on the developing rat AP and was not able to induce ductal branching morphogenesis.

APs Cultured with anti-KGF

APs were cultured with T 10^{-8} M and anti-KGF. Two anti-KGF compounds were used in these studies, 1G4 antibody and 412 peptide. 1G4 is a neutralizing monoclonal antibody to human FGF-7/KGF (Rubin et al., 1989), and 412 is a synthetic peptide based on the ligand binding domain of FGFR2 that binds FGF-7/KGF. Thus, 412 competes with the KGFR for endogenous FGF-7/KGF (Bottaro et al., 1993). Both anti-KGFs were gifts from Dr. J. Rubin (NIH). Neither anti-KGF affected T-induced ductal branching morphogenesis of the developing AP (Fig. 29). APs cultured with T 10^{-8} M plus 1G4 at 6 or 30 μ g/ml were not different from explants cultured with T alone as assessed by nodes/explant, inverse form factor, epithelial perimeter and epithelial area (Fig. 30). Likewise, 50 or 250 μ g/ml of 412 had no effect on T-induced nodes/explant, inverse form factor or epithelial perimeter. However, at 50 or 250 μ g/ml 412 did decrease epithelial area to 70.6% and 71.1% of that elicited by T, respectively. Thus, anti-KGF 412 slightly inhibited epithelial growth, but did not effect T-induced ductal branching morphogenesis.

As indicated below, both of these anti-KGF agents inhibited T-induced ductal branching morphogenesis in the developing rat VP at comparable levels. Additionally, KGF induced ductal branching morphogenesis in the absence of T in the developing rat VP. (Data will be presented later in this thesis). Therefore, the negative results obtained with the anti-KGF agents in the rat AP is not likely to be due to failure of the agents to neutralize FGF-7/KGF. Thus, this data indicates that FGF-7/KGF does not play a role in ductal branching morphogenesis of the developing newborn rat AP. Further investigation is needed to determine the possible role of FGF-7/KGF in ductal branching of older more highly branched APs.

APs Cultured with FGF-1/aFGF

APs were cultured either with or without T 10^{-8} M in combination with FGF-1/aFGF (acidic FGF) (3 or 30 ng/ml). T-induced ductal branching morphogenesis

was not affected by FGF-1/aFGF (Fig. 31A, 31C, 31E and 32). In cultures treated with T 10^{-8} M plus FGF-1/aFGF (3 or 30 ng/ml), nodes/explant, inverse form factor, epithelial perimeter or epithelial area were not different from values observed in cultures grown with T alone (Fig. 32).

APs cultured in the absence of T but with FGF-1/aFGF (3 or 30 ng/ml) did not undergo ductal branching morphogenesis (Fig. 31B, 31D, 31F). All parameters were similar in cultures treated in the absence of T either with or without FGF-1/aFGF (Fig. 32). Thus, FGF-1/aFGF did not have an observable effect on ductal branching morphogenesis, suggesting that FGF-1/aFGF is not involved in ductal branching morphogenesis of the developing 0 day rat AP.

APs Cultured with FGF-2/bFGF

APs were cultured either with or without T 10^{-8} M in combination with FGF-2/bFGF (basic FGF) (3 or 30 ng/ml). T-induced ductal branching morphogenesis was not affected by FGF-2/bFGF (Fig. 33A, 33C, 33E and 34). However, at 30 ng/ml of FGF-2/bFGF plus T 10^{-8} M the mesenchymal compartment appeared to be increased by visual observation. In these cultures the epithelium did not fill the entire mesenchymal compartment (Fig. 33E). This did not affect ductal branching, since in cultures treated with T 10^{-8} M plus FGF-2/bFGF (3 or 30 ng/ml) nodes/explant, inverse form factor, epithelial perimeter and epithelial area were comparable to that of cultures treated with T alone (Fig. 34).

APs cultured with FGF-2/bFGF (3 or 30 ng/ml) in the absence of T did not undergo ductal branching morphogenesis (Fig. 33B, 33D, 33E). None of the parameters measured were significantly different in cultures treated with or without FGF-2/bFGF in the absence of T (Fig. 34). FGF-2/bFGF did not have an observable effect on ductal branching morphogenesis on the developing rat AP, even though the mesenchyme appears to increase in response to exogenous FGF-2/bFGF.

APs Cultured with HGF

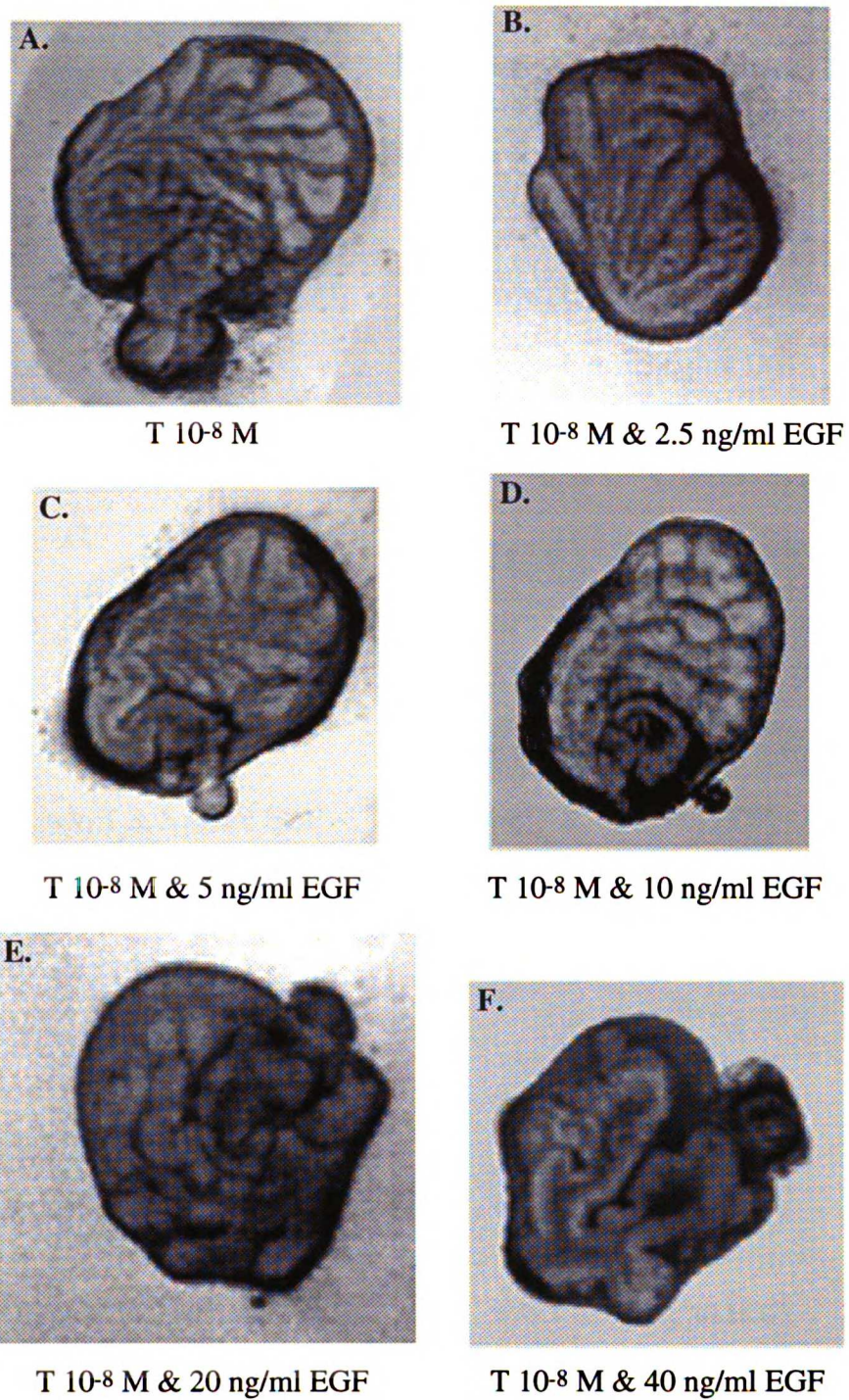
APs were cultured either with or without $T\ 10^{-8}\ M$ plus HGF (1.8, 2.5, or 25 ng/ml). HGF did not effect T-induced ductal branching morphogenesis (Fig. 35A, 35C, 35E). APs cultured with $T\ 10^{-8}\ M$ plus HGF (1.8, 2.5, or 25 ng/ml) were not different from those cultured with T alone as assayed by nodes/explant, inverse form factor, epithelial perimeter and epithelial area (Fig. 37). In the absence of T, HGF did not induce ductal branching morphogenesis (Fig. 35B, 35D, 35F), as nodes/explant, inverse form factor, epithelial perimeter and epithelial area were not different from explants cultured in the absence of T (Fig. 37). Thus, HGF did not have an observable effect on ductal branching morphogenesis at the concentrations of HGF used in these experiments, suggesting that HGF is not involved in ductal branching morphogenesis of the developing 0 day AP.

Summary

Exogenous EGF and TGF- α distort the pattern of T-induced ductal branching in 0 day rat APs, indicating that the APs at this stage of development can respond to both EGF and TGF- α . TGF- α is also implicated as a molecule involved in ductal branching in the developing rat AP, because blocking endogenous TGF- α with neutralizing anti-bodies inhibited ductal branching morphogenesis. The developing rat AP can respond to exogenous TGF- β 1 and TGF- β 2, which distorted ductal branching at low concentrations of TGF- β and inhibited ductal branching morphogenesis at high concentrations. However, it is not known if TGF- β participates in ductal branching of the developing rat AP *in vivo*. Anti-TGF- β had no effect on T-induced ductal branching, but the cross-reactivity with rat TGF- β was not known for the antibody. The developing rat AP was able to respond to high concentrations of exogenous FGF-7/KGF with an increase in epithelial area and distortion of T-induced ductal branching. However, FGF-7/KGF could not induce ductal branching in the absence of T. Furthermore, anti-KGF did not disrupt T-induced ductal

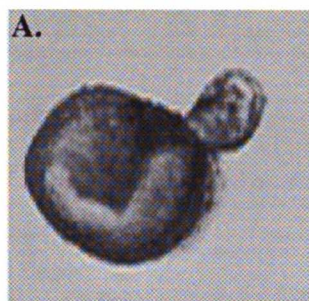
branching morphogenesis. Thus, while the developing 0 day rat AP can respond to exogenous FGF-7/KGF at high concentrations, FGF-7/KGF does not seem to play an important role in ductal branching morphogenesis of the developing rat AP. While, FGF-2/bFGF appears to increase the mesenchymal compartment of the developing rat AP, FGF-2/bFGF is not a key regulator of ductal branching in the developing AP since exogenous FGF-2/bFGF did not effect T-induced ductal branching, nor did it induce ductal branching in the absence of T. Exogenous FGF-1/aFGF and HGF did not affect T-induced ductal branching, nor did either induce ductal branching in the absence of T. Thus, FGF-1/aFGF and HGF do not appear to be involved in ductal branching in the developing 0 day rat AP.

Figure 10



Representative images of neonatal rat APs cultured 6 days with T 10^{-8} M (A.), or with T 10^{-8} M plus EGF (B.-F.).

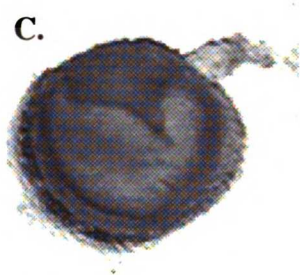
Figure 11



w/o T



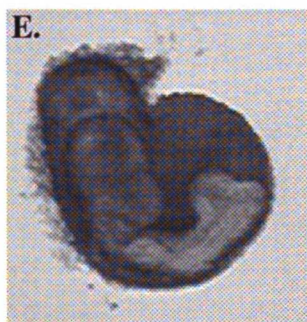
w/o T & 2.5 ng/ml EGF



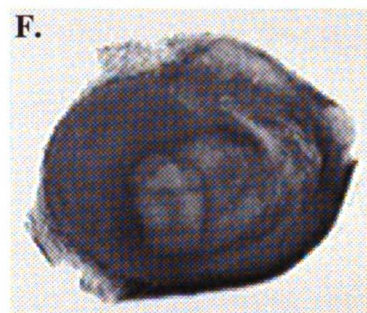
w/o T & 5 ng/ml EGF



w/o T & 10 ng/ml EGF



w/o T & 20 ng/ml EGF



w/o T & 40 ng/ml EGF

————— 1000 μ m

Representative images of neonatal rat APs cultured 6 days in the absence of T (A.) or with EGF (B.-F.) plus in the absence of T.

Figure 12

Newborn rat APs cultured for 6 days with T 10^{-8} M plus or minus EGF (1-40 ng/ml), or in the absence of T plus or minus EGF (1-40 ng/ml). Four parameters of epithelial morphogenesis were assessed: A. Node number, B. Inverse form factor, C. Epithelial perimeter, and D. Epithelial area.

* Indicates significantly different from APs cultured with T 10^{-8} M (positive control) and Δ indicates significantly different from APs cultured in the absence of T (negative control) ($p < .05$; two-tailed Dunnett test).

Figure 12

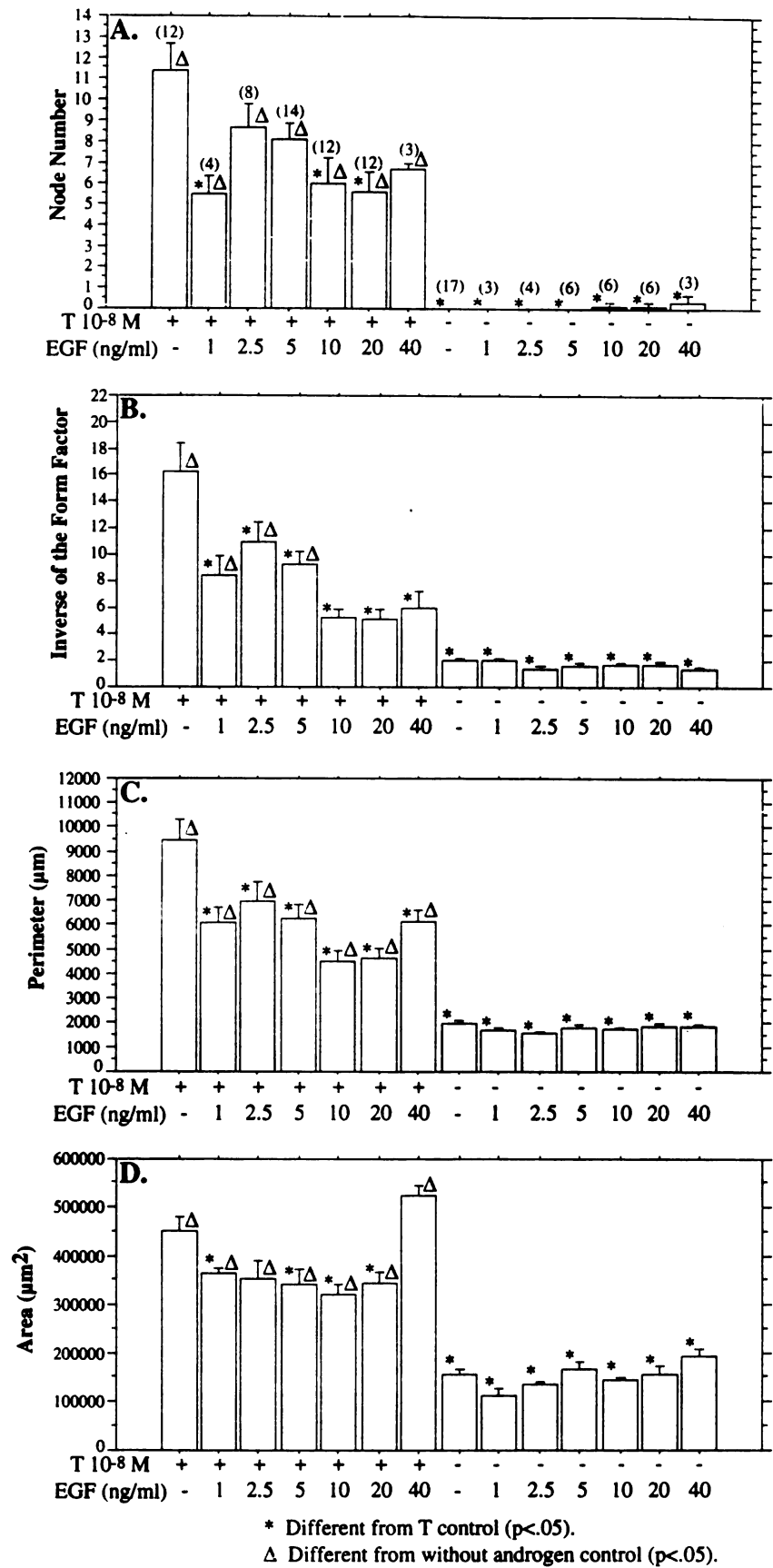
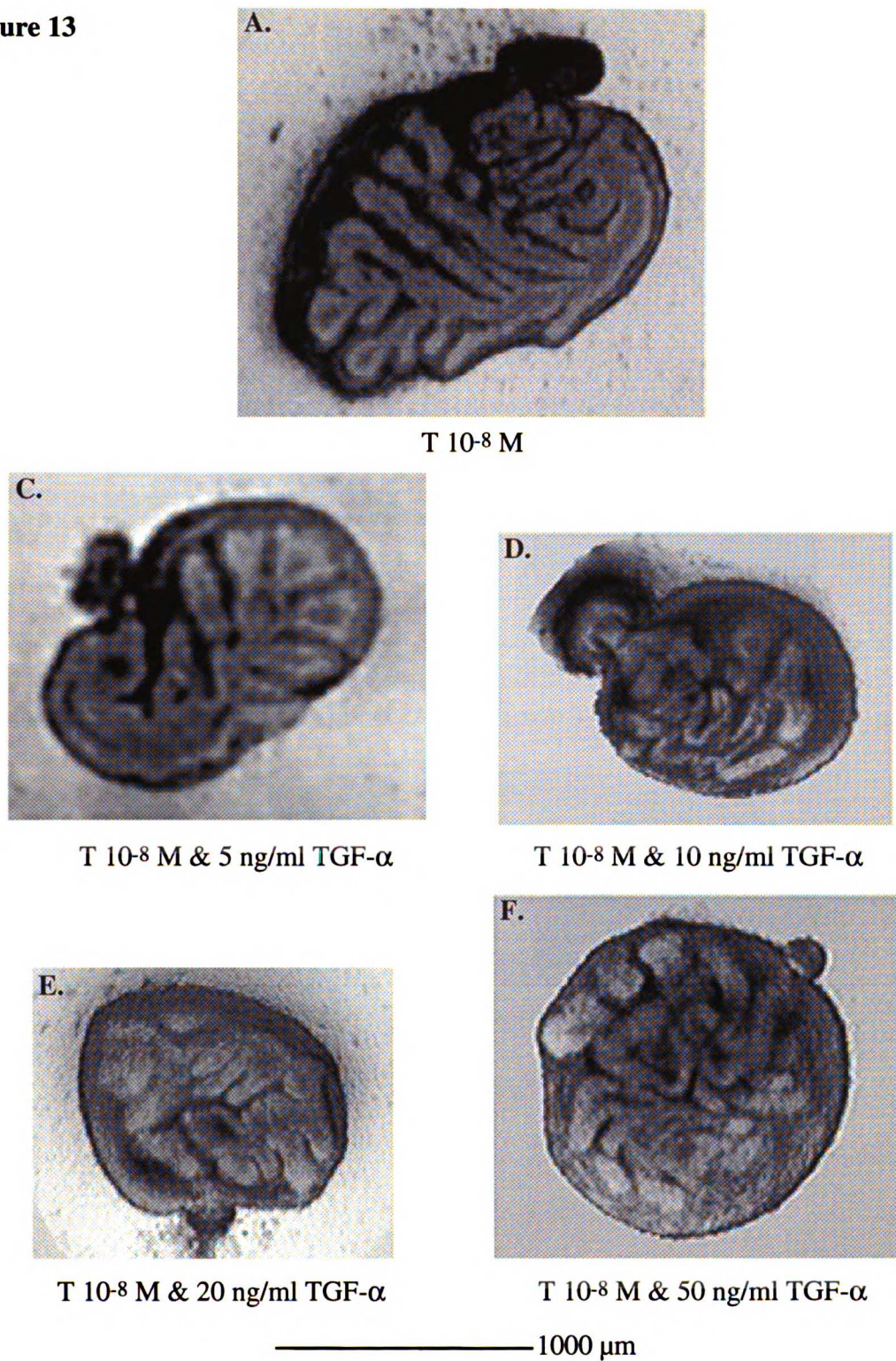
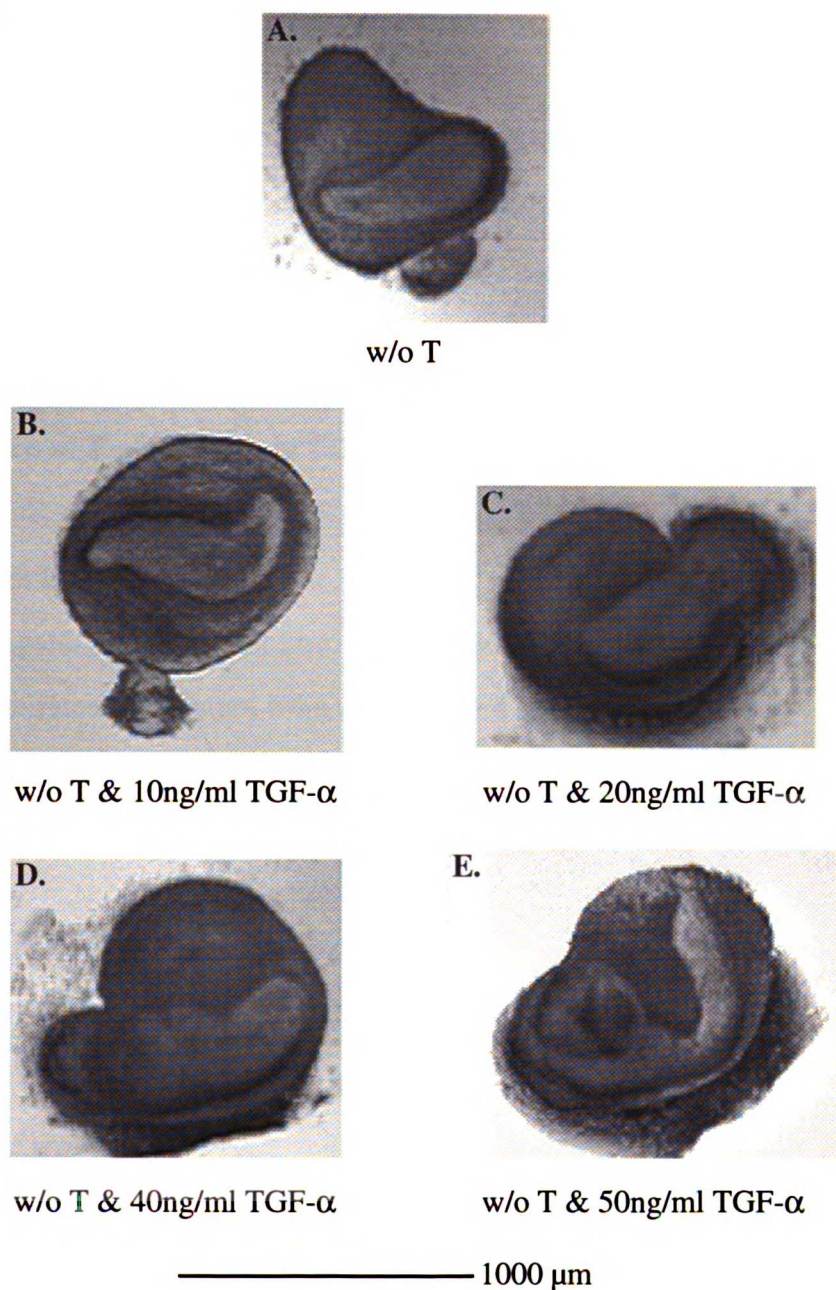


Figure 13



Representative images of neonatal rat APs cultured 6 days with T 10⁻⁸ M (A.), or with T 10⁻⁸ M plus TGF- α (B.- F.).

Figure 14



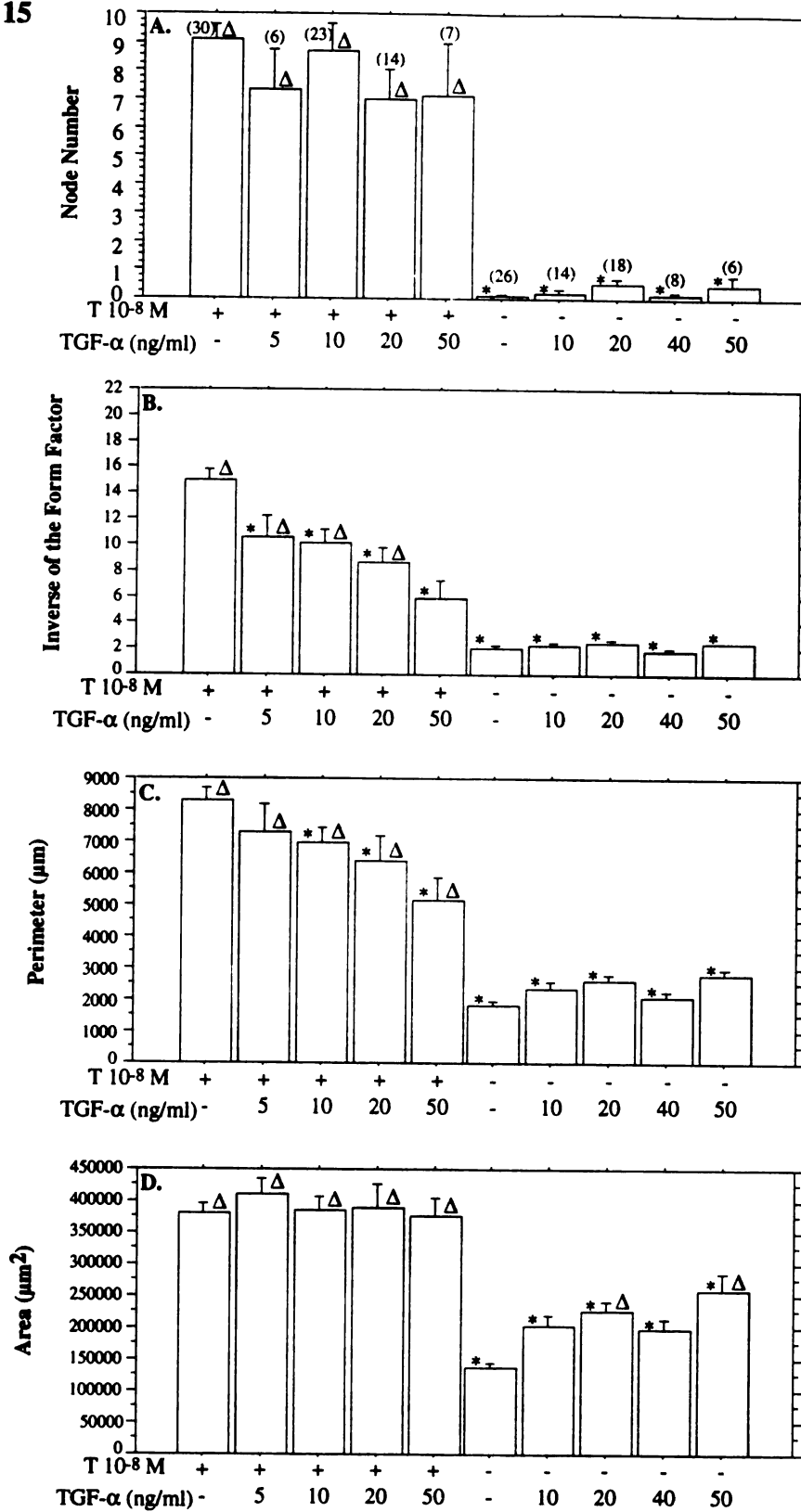
Representative images of neonatal rat APs cultured 6 days in the absence of androgen (A.) or with TGF- α plus in the absence of androgen (B.-E.).

Figure 15

Newborn rat APs cultured for 6 days with T 10^{-8} M plus or minus TGF- α (5-50 ng/ml), or in the absence of T plus or minus TGF- α (10-50 ng/ml). Four parameters of epithelial morphogenesis were assessed: A. Node number, B. Inverse form factor, C. Epithelial perimeter, and D. Epithelial area.

* Indicates significantly different from APs cultured with T 10^{-8} M (positive control) and Δ indicates significantly different from APs cultured in the absence of T (negative control) ($p < .05$; two-tailed Dunnett test).

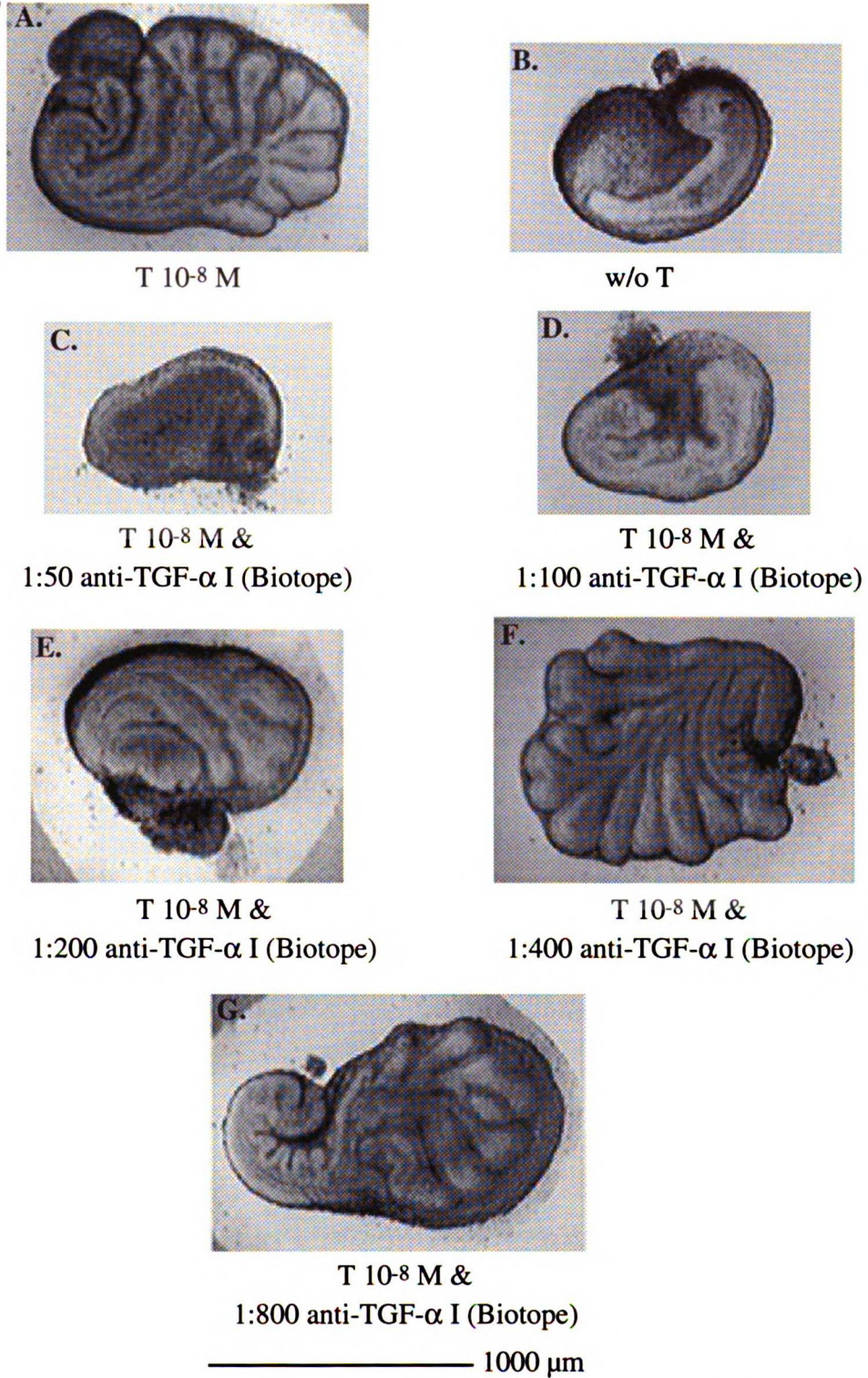
Figure 15



* Different from T control (p<.05).

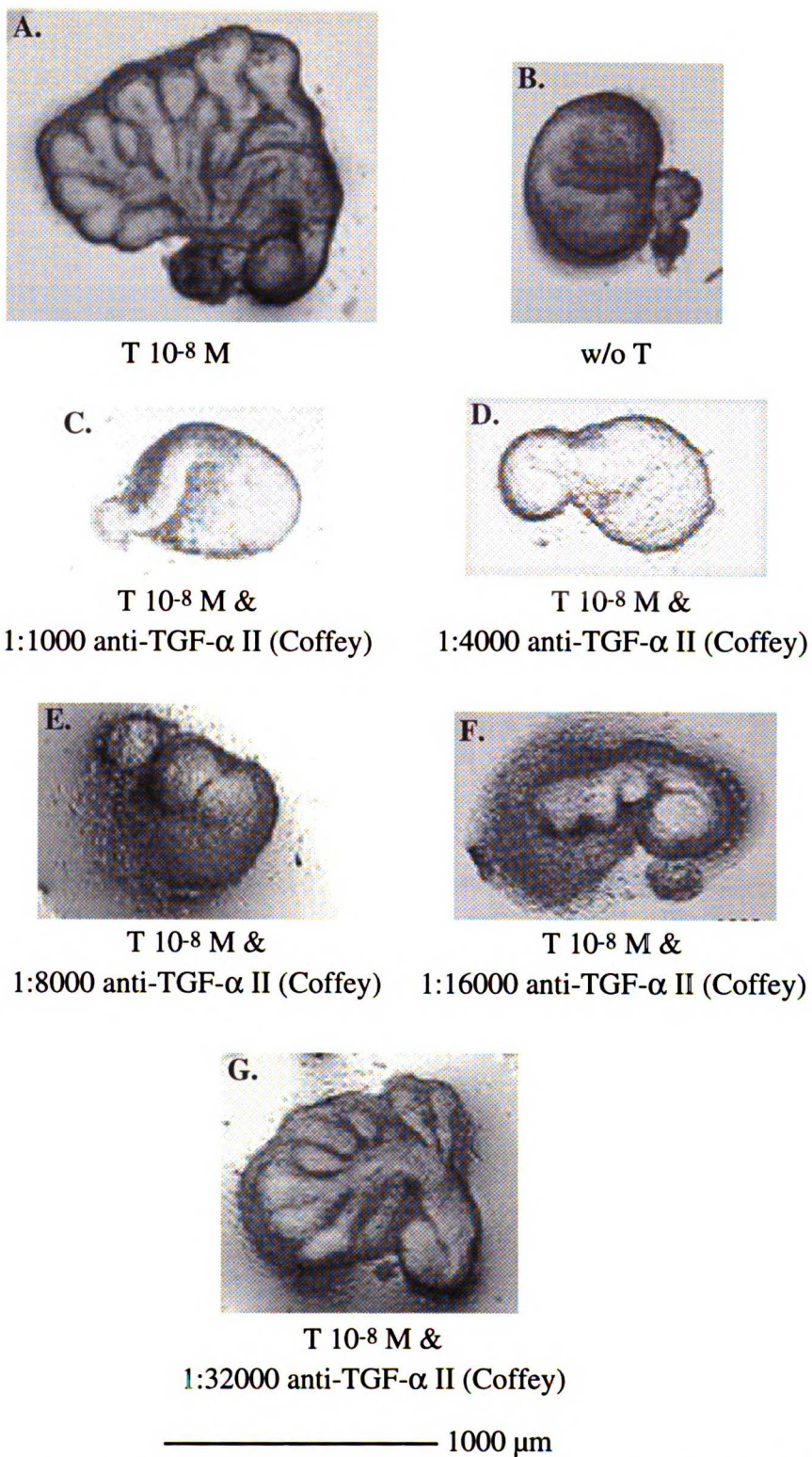
Δ Different from without androgen control (p<.05).

Figure 16



Representative images of neonatal rat APs cultured 6 days with T 10^{-8} M (A.), in the absence of T (B.) or with T 10^{-8} M plus anti-TGF- α I from Biotope (C.-G.) (Dilutions of 1:50 to 1:800).

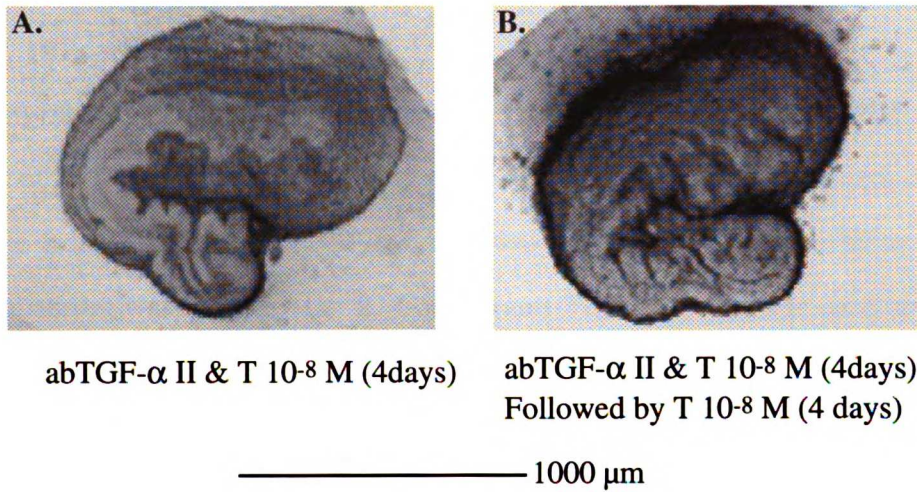
Figure 17



Representative images of neonatal rat APs cultured 6 days with T 10^{-8} M (A.), in the absence of T (B.), or with T 10^{-8} M plus anti-TGF- α II from Dr. Coffey (C.-G.) (Dilutions of 1:1000 to 1:32000).

Figure 18

abTGF- α Recovery



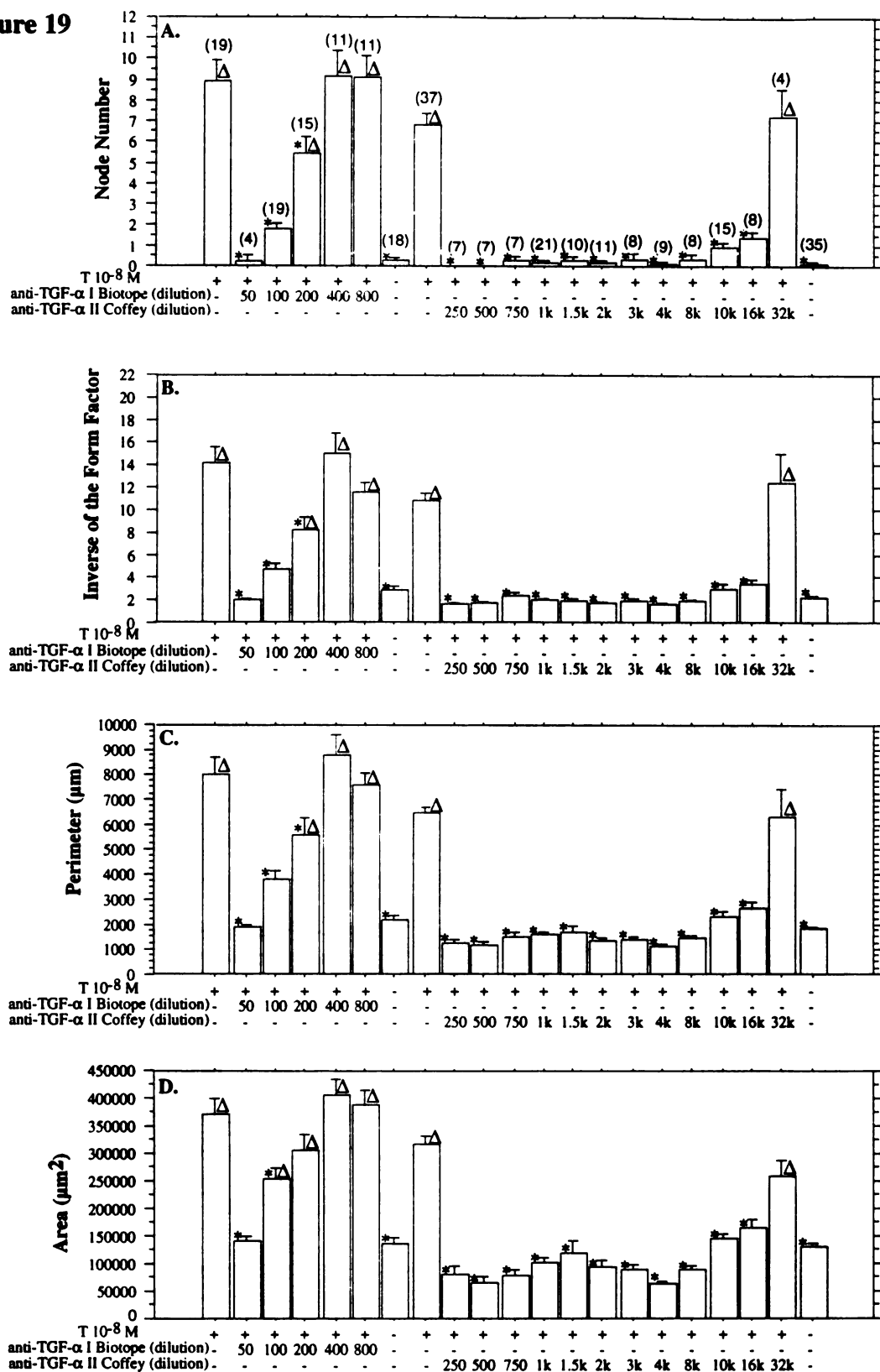
(A.) Neonatal rat AP cultured 4 days with anti-TGF- α II (1:10,000) plus T 10^{-8} M. Ductal branching morphogenesis was inhibited. (B.) The same gland cultured an additional 4 days with T 10^{-8} M alone. Ductal branching morphogenesis resumed when anti-TGF- α II was removed.

Figure 19

Newborn rat APs cultured for 6 days with T 10^{-8} M plus or minus anti-TGF- α , or in the absence of T. Two goat anti-rat TGF- α polyclonal neutralizing antisera were used. One is available from Biotope (anti-TGF- α I) and was used at dilutions of 1:50 to 1:800. The other antiserum was made by Dr. R. Coffey (anti-TGF- α II) and was used at dilutions of 1:250 to 1:32000. Four parameters of epithelial morphogenesis were assessed: A. Node number, B. Inverse form factor, C. Epithelial perimeter, and D. Epithelial area.

* Indicates significantly different from APs cultured with T 10^{-8} M (positive control) and Δ indicates significantly different from APs cultured in the absence of T (negative control) ($p < .05$; two-tailed Dunnett test).

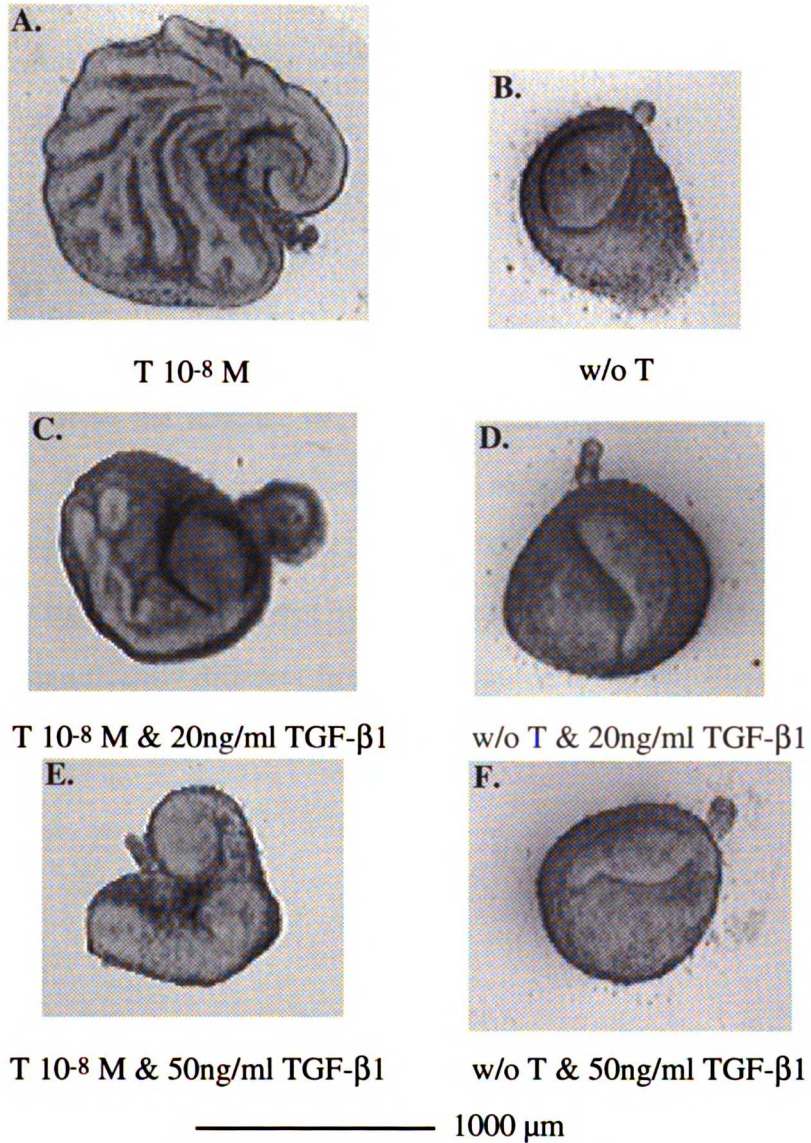
Figure 19



* Different from T control (p<.05).

Δ Different from without androgen control (p<.05).

Figure 20



Representative images of neonatal rat APs cultured 6 days with T 10^{-8} M (A., C., D.) or in the absence of T (B., D., F.) plus 20ng/ml TGF- β 1 (C. and D.) or 50ng/ml TGF- β 1 (E. and F.).

Figure 21

Newborn rat APs cultured for 6 days with T 10^{-8} M plus or minus TGF- β 1 (20 and 50 ng/ml), or in the absence of T plus or minus TGF- β 1 (20 and 50 ng/ml). Four parameters of epithelial morphogenesis were assessed: A. Node number, B. Inverse form factor, C. Epithelial perimeter, and D. Epithelial area.

* Indicates significantly different from APs cultured with T 10^{-8} M (positive control) and Δ indicates significantly different from APs cultured in the absence of T (negative control) ($p < .05$; two-tailed Dunnett test).

Figure 21

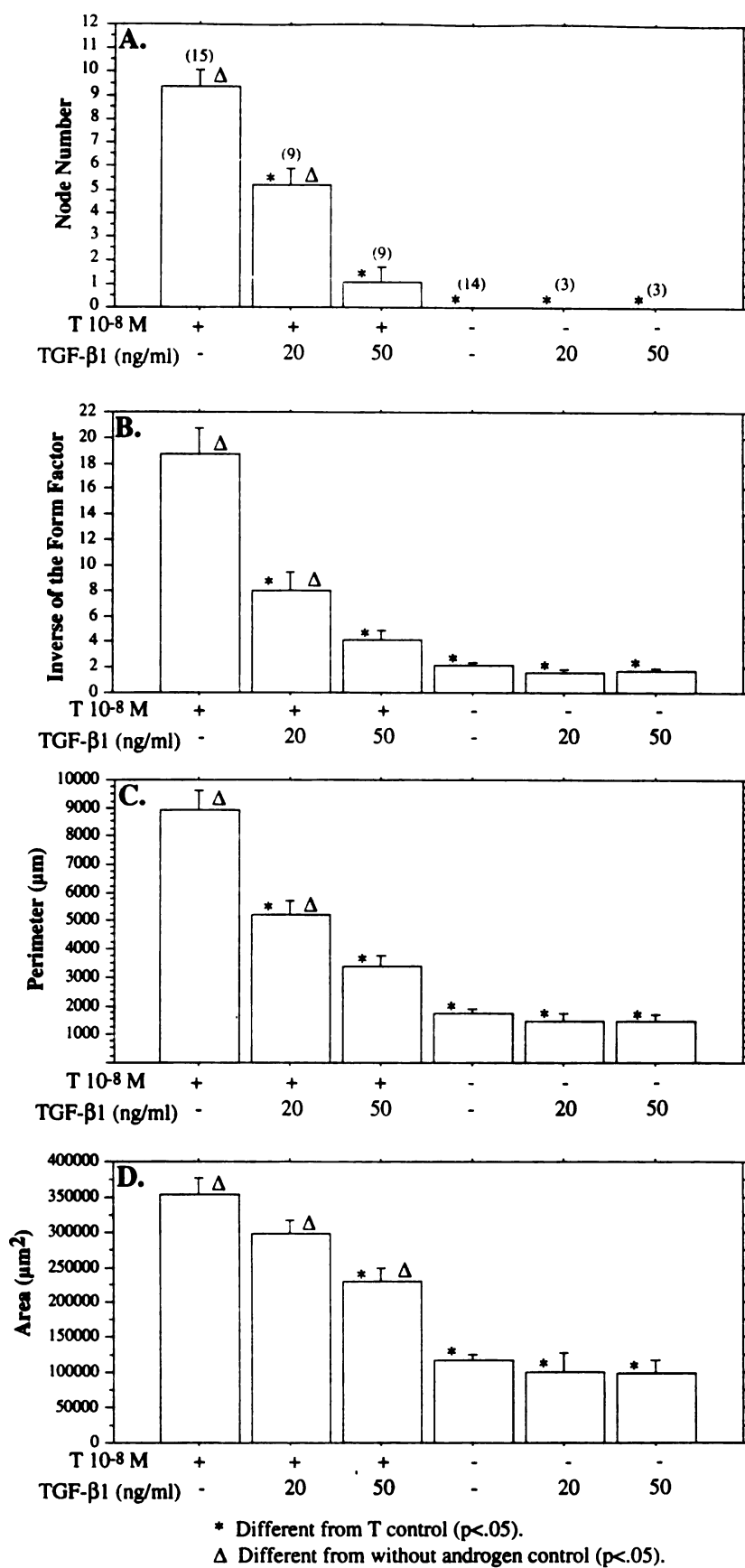
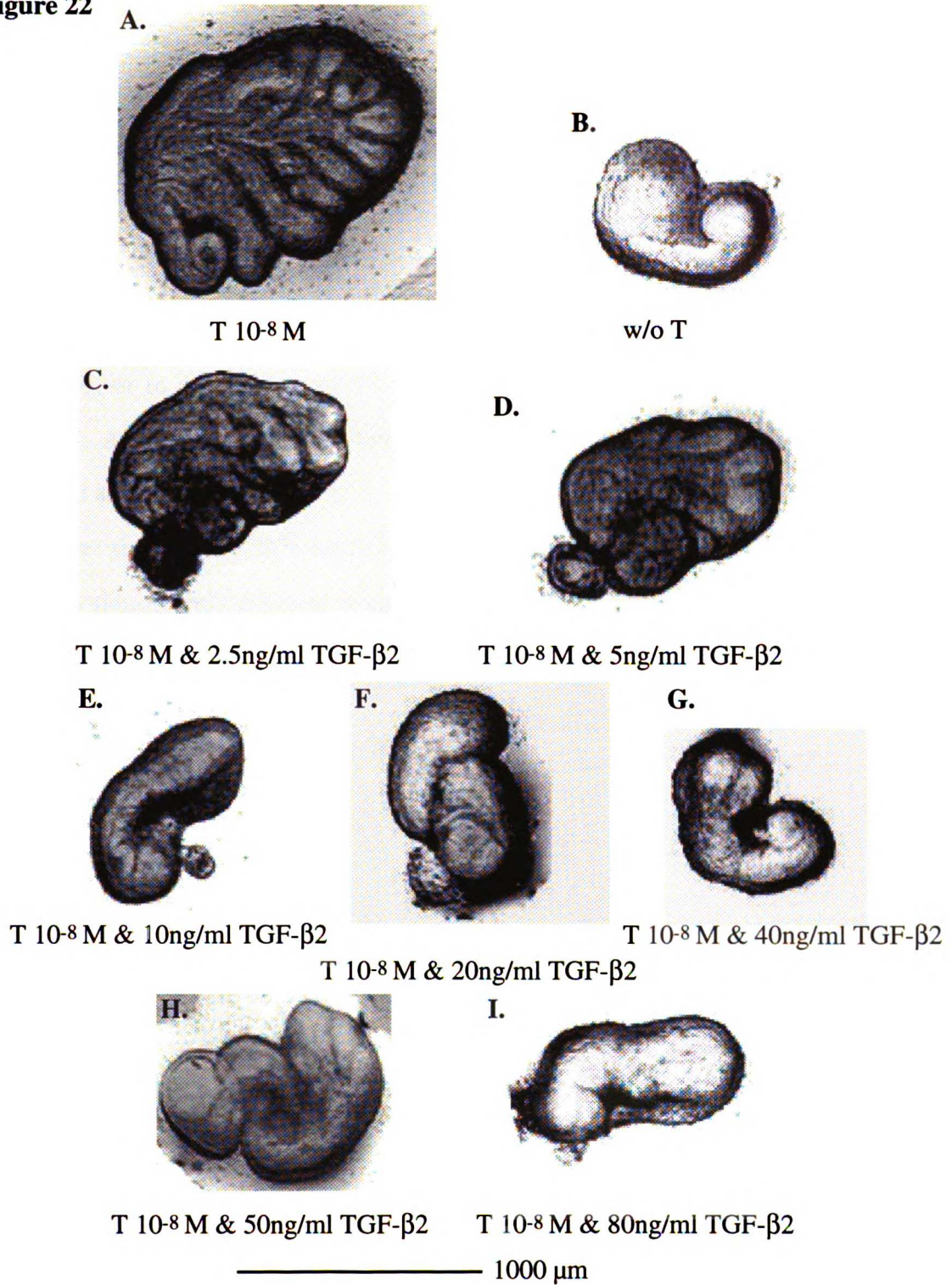


Figure 22



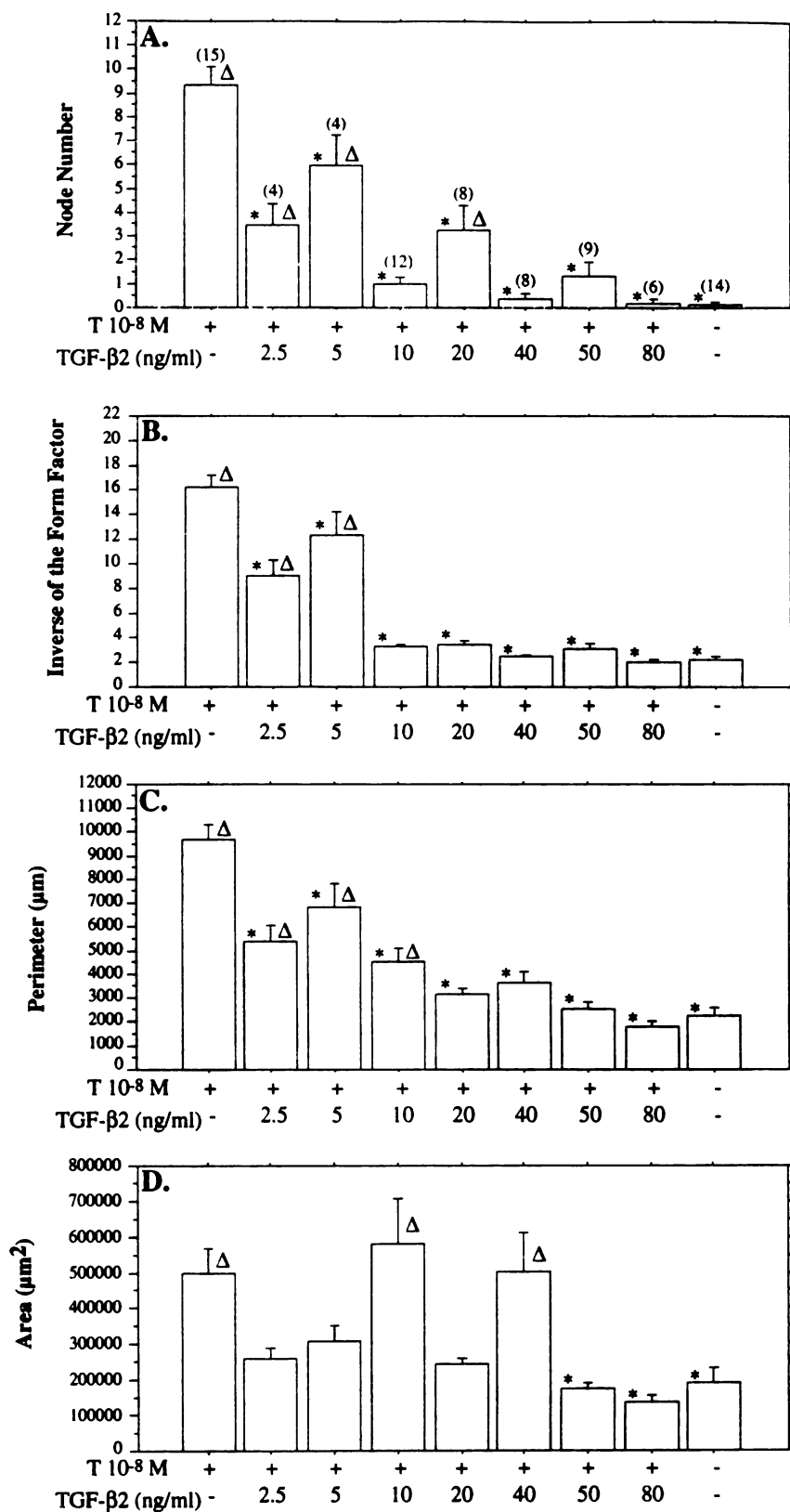
Representative images of neonatal rat APs cultured 6 days with T 10^{-8} M (A.), in the absence of T (B.), or with TGF- β 2 plus T 10^{-8} M (C.-I.).

Figure 23

Newborn rat APs cultured for 6 days with T 10^{-8} M plus or minus TGF- β 2 (2.5-80 ng/ml), or in the absence of T. Four parameters of epithelial morphogenesis were assessed: A. Node number, B. Inverse form factor, C. Epithelial perimeter, and D. Epithelial area.

* Indicates significantly different from APs cultured with T 10^{-8} M (positive control) and Δ indicates significantly different from APs cultured in the absence of T (negative control) ($p < .05$; two-tailed Dunnett test).

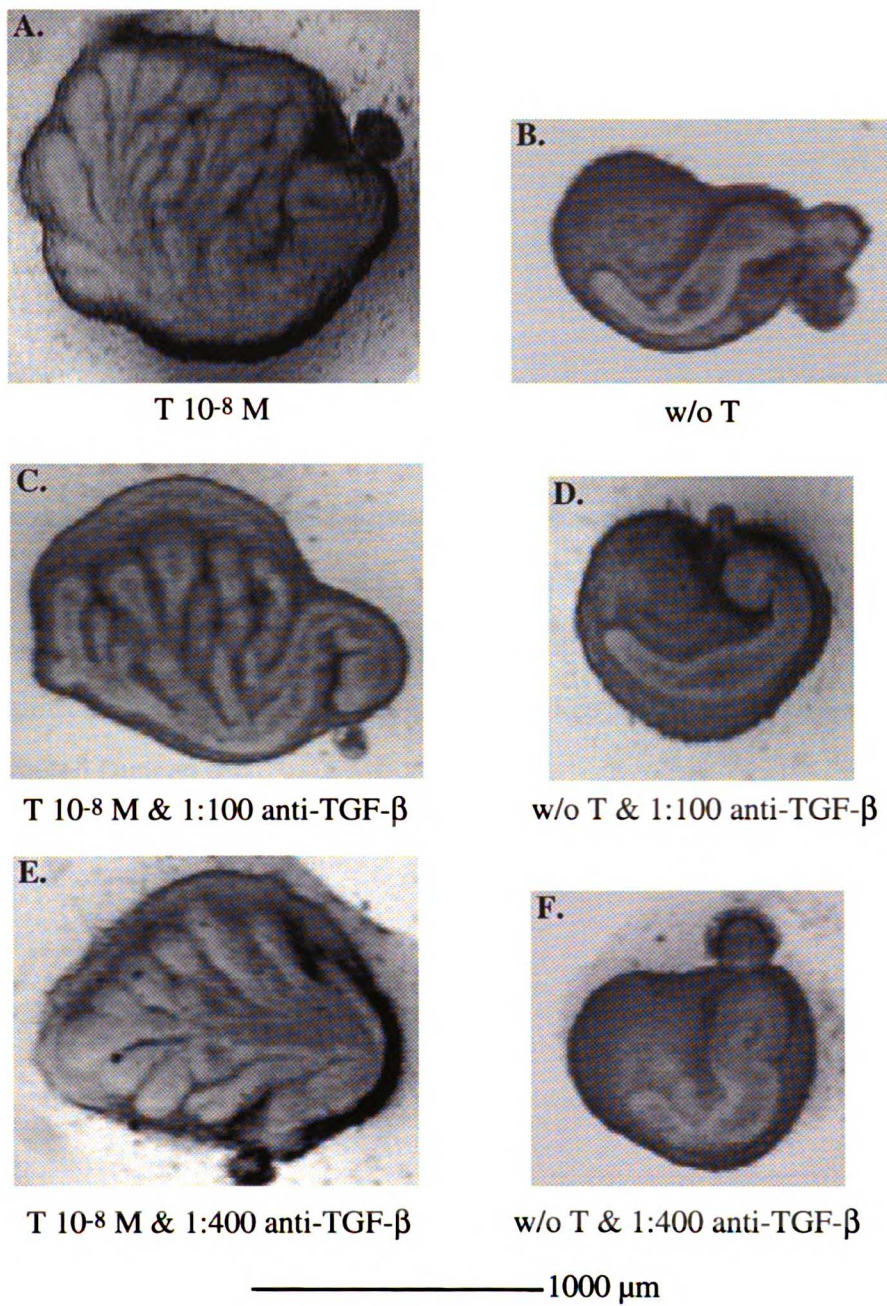
Figure 23



* Different from T control (p<.05).

Δ Different from without androgen control (p<.05).

Figure 24



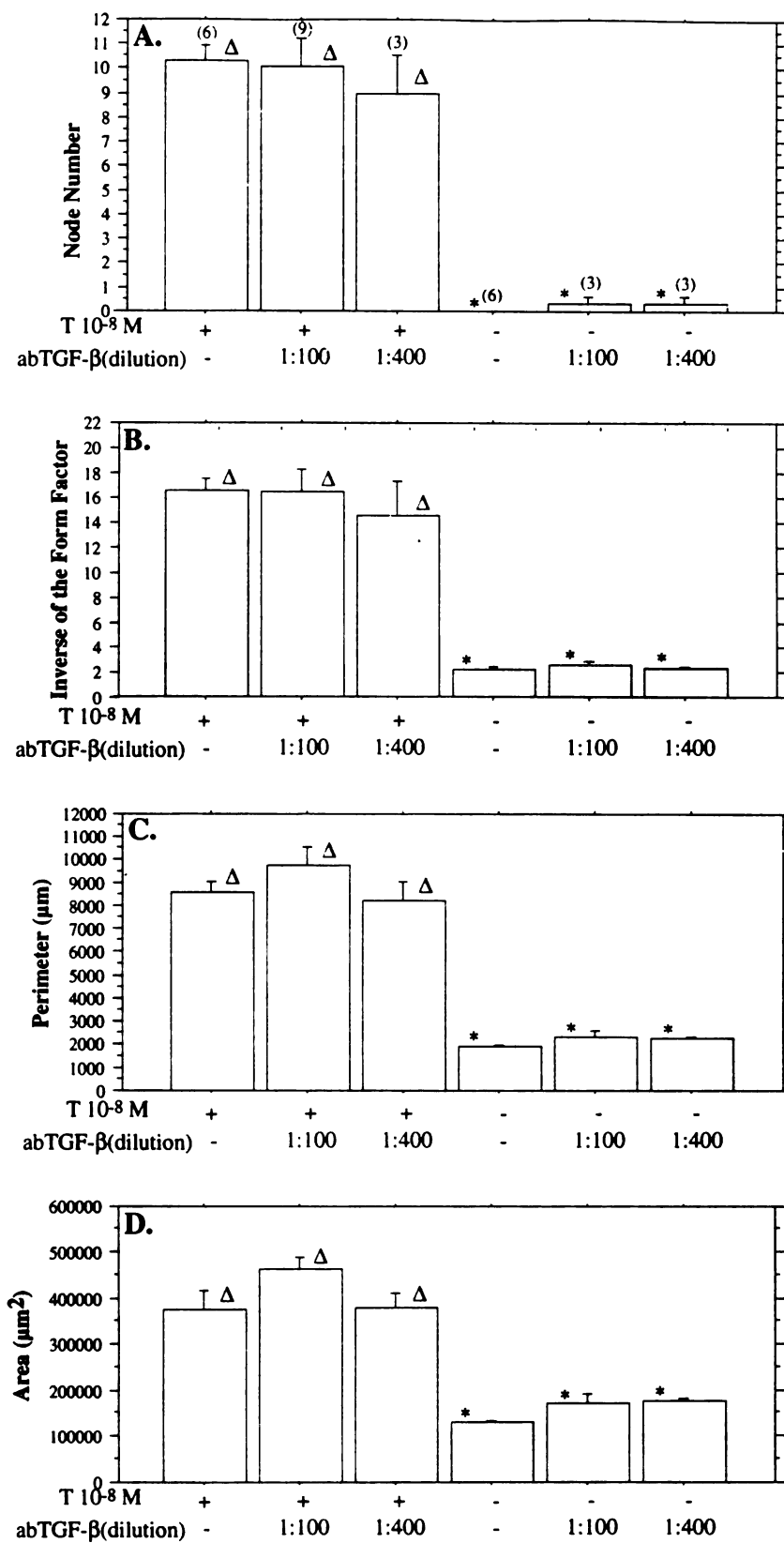
Representative images of neonatal rat APs cultured 6 days with $T 10^{-8} M$ (A., C., E.) or in the absence of T (B., D., F.) plus with anti-TGF- β (C.- F.).

Figure 25

Newborn rat APs cultured for 6 days with T 10^{-8} M plus or minus anti-TGF- β (1:100 or 1:400 dilution), or in the absence of T plus or minus anti-TGF- β (1:100 or 1:400 dilution). Four parameters of epithelial morphogenesis were assessed: A. Node number, B. Inverse form factor, C. Epithelial perimeter, and D. Epithelial area.

* Indicates significantly different from APs cultured with T 10^{-8} M (positive control) and Δ indicates significantly different from APs cultured in the absence of T (negative control) ($p < .05$; two-tailed Dunnett test).

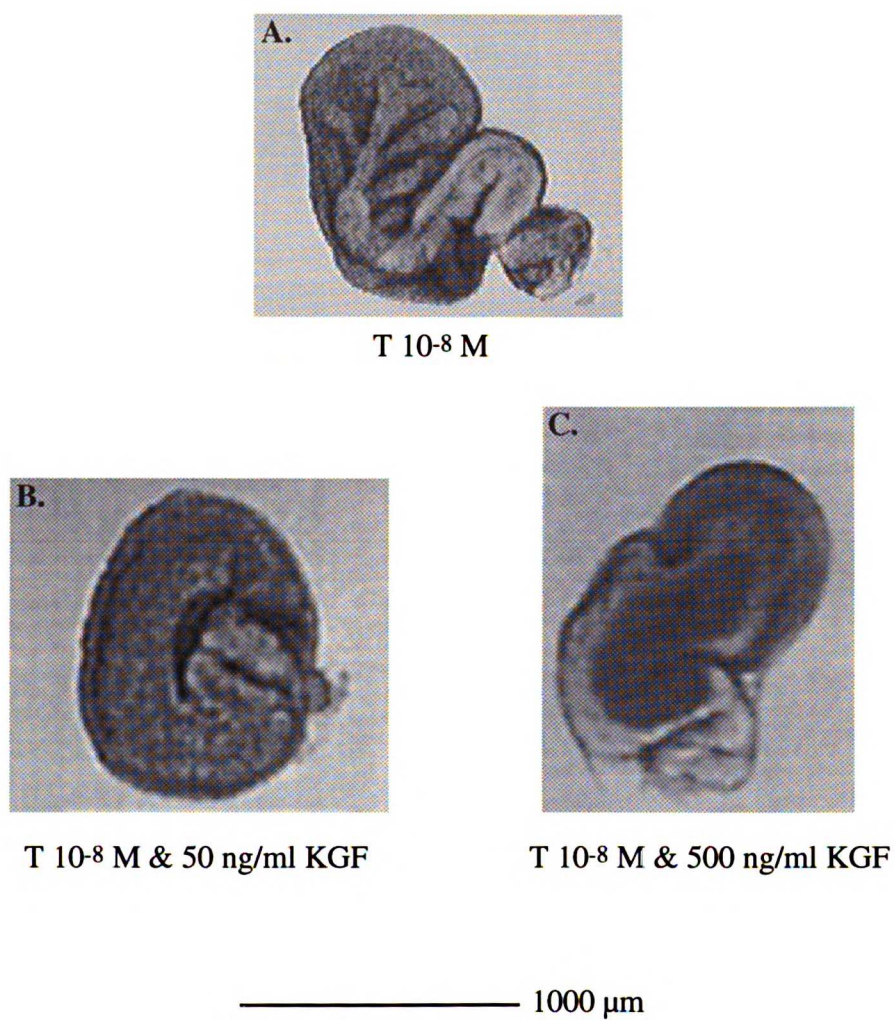
Figure 25



* Different from T control (p<.05).

Δ Different from without androgen control (p<.05).

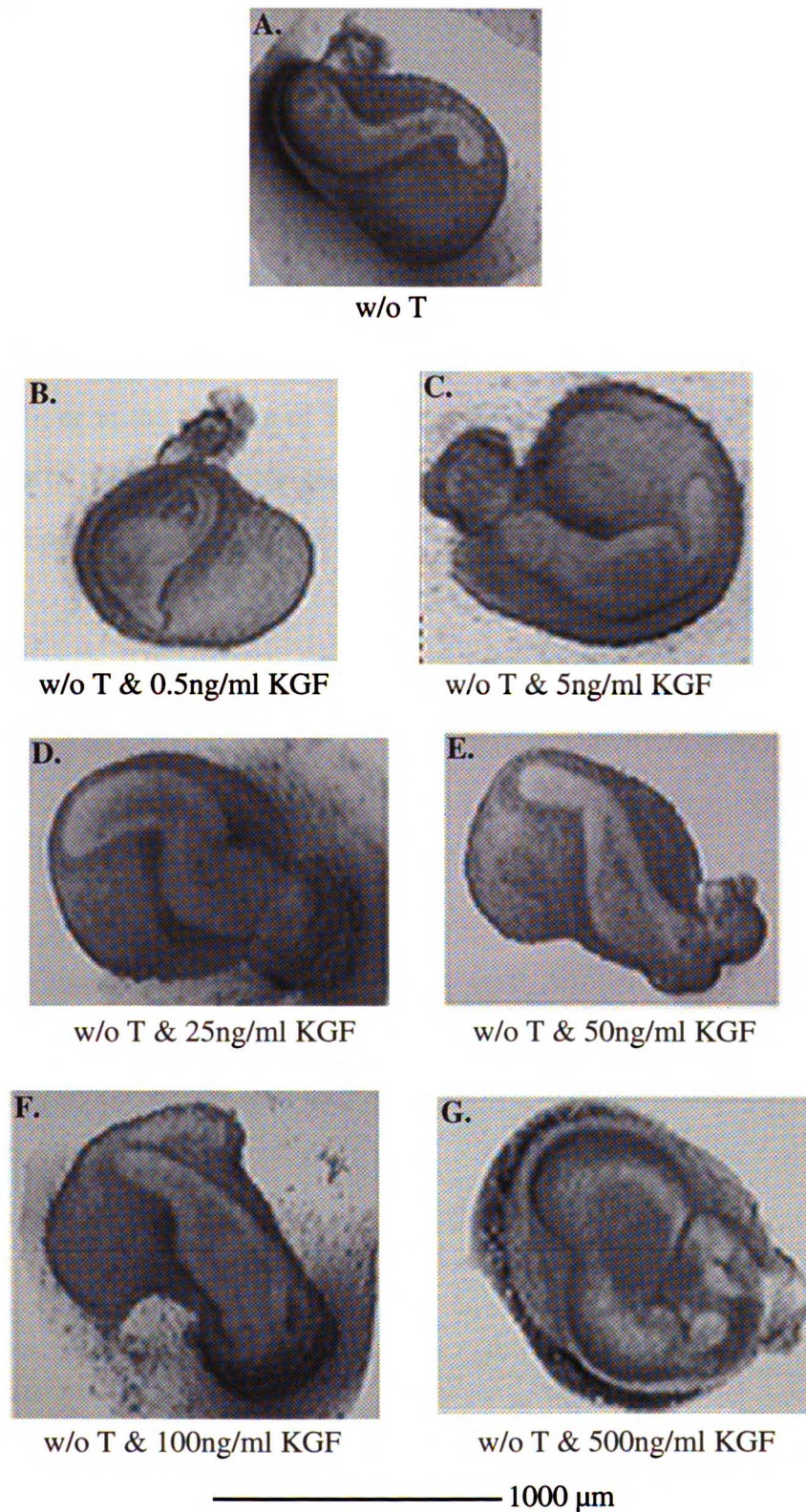
Figure 26



Representative images of neonatal rat APs cultured 6 days with T 10^{-8} M (A.) or with KGF plus T 10^{-8} M (B. and C.).

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Figure 27



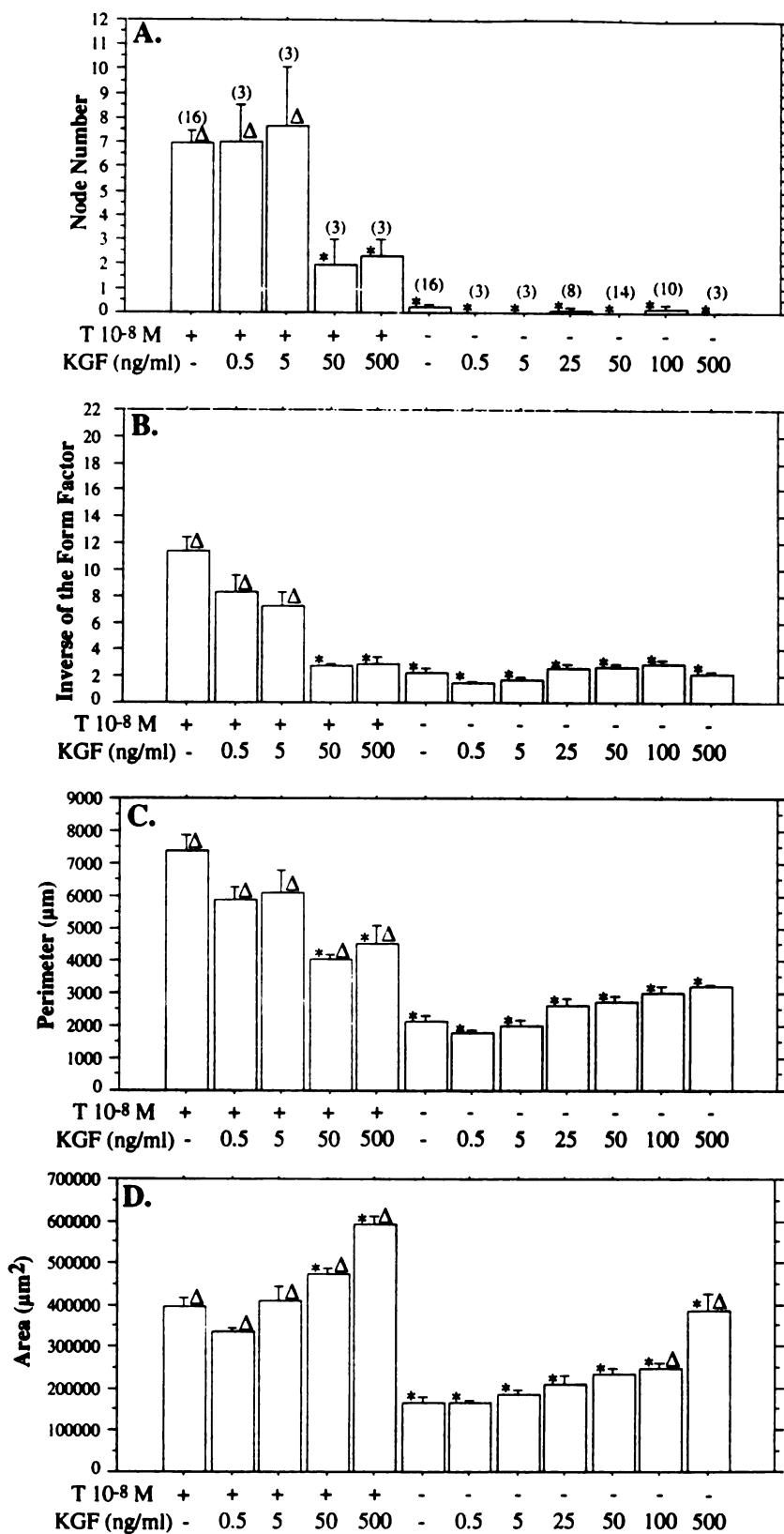
Representative images of neonatal rat APs cultured 6 days in the absence of T (A.) or with KGF plus in the absence of T (B.-G.).

Figure 28

Newborn rat APs cultured for 6 days with T 10^{-8} plus or minus FGF-7 /KGF (0.5 - 500 ng/ml), or in the absence of T plus or minus FGF-7/KGF (0.5 - 500 ng/ml). Four parameters of epithelial morphogenesis were assessed: A. Node number, B. Inverse form factor, C. Epithelial perimeter, and D. Epithelial area.

* Indicates significantly different from APs cultured with T 10^{-8} M (positive control) and Δ indicates significantly different from APs cultured in the absence of T (negative control) ($p < .05$; two-tailed Dunnett test).

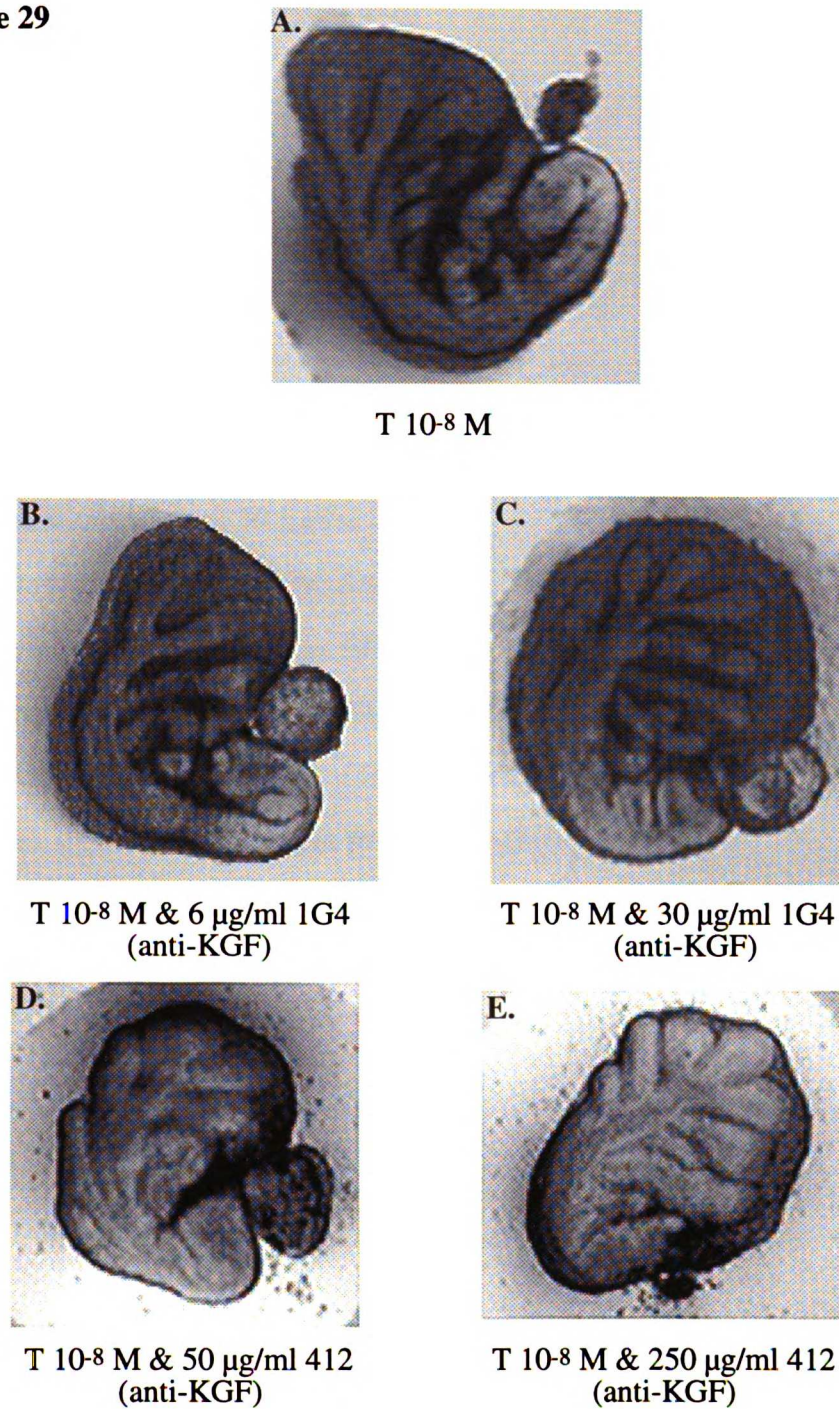
Figure 28



* Different from T control ($p < .05$).

Δ Different from without androgen control ($p < .05$).

Figure 29



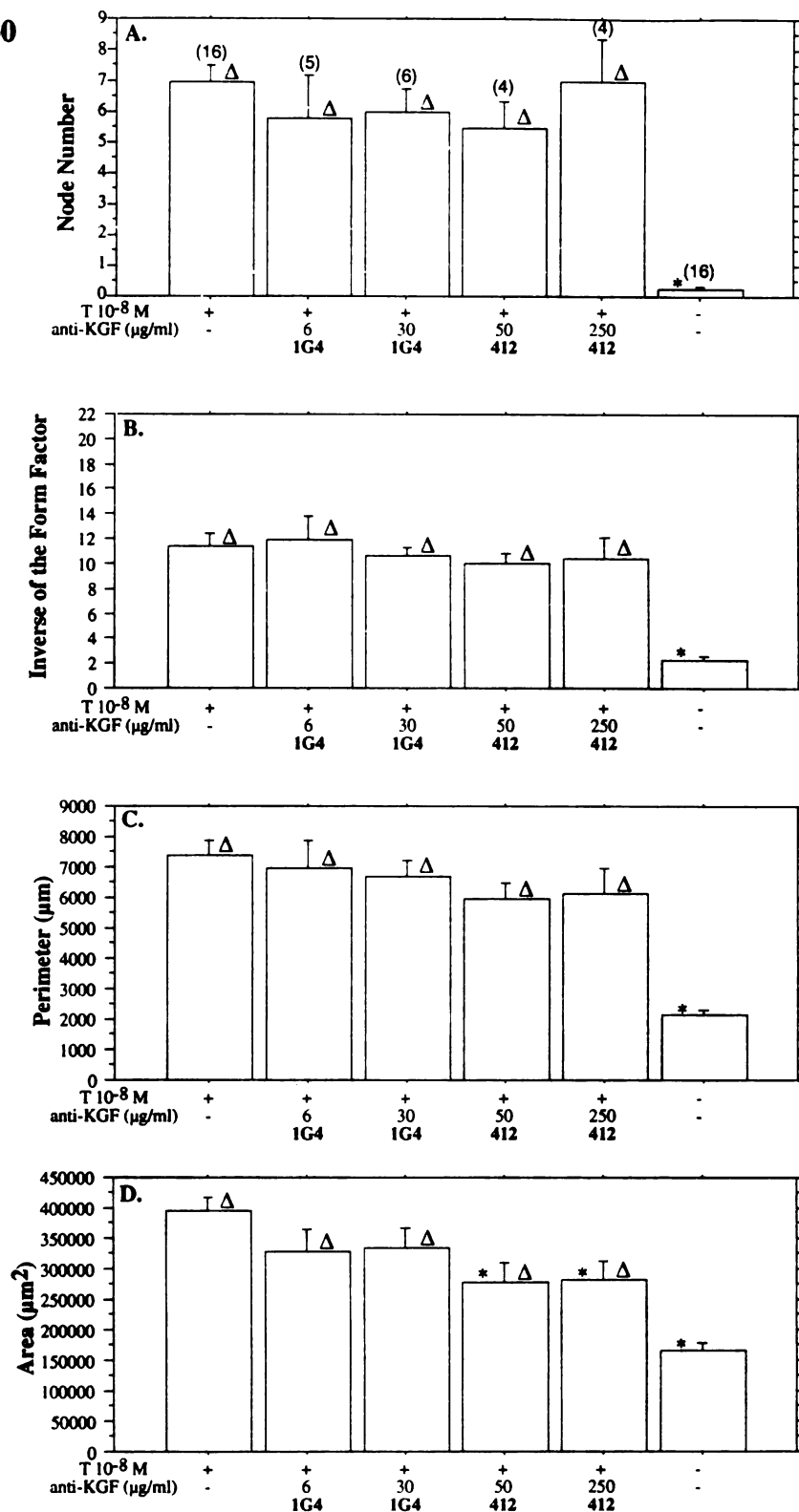
Representative images of neonatal rat APs cultured 6 days with T 10⁻⁸ M (A.), or T 10⁻⁸ M plus anti-KGF (1G4: B., C., or 412: D., E.).

Figure 30

Newborn rat APs cultured for 6 days with T 10^{-8} M plus or minus anti-KGF (anti-FGF-7), or in the absence of T. 1G4 is a neutralizing monoclonal antibody to KGF and was used at concentrations of 6 or 30 $\mu\text{g/ml}$. 412 is a synthetic peptide which binds KGF. Thus, competes with the KGFR for endogenous KGF. The 412 peptide was used at 50 or 250 $\mu\text{g/ml}$. Four parameters of epithelial morphogenesis were assessed: A. Node number, B. Inverse form factor, C. Epithelial perimeter, and D. Epithelial area.

* Indicates significantly different from APs cultured with T 10^{-8} M (positive control) and Δ indicates significantly different from APs cultured in the absence of T (negative control) ($p < .05$; two-tailed Dunnett test).

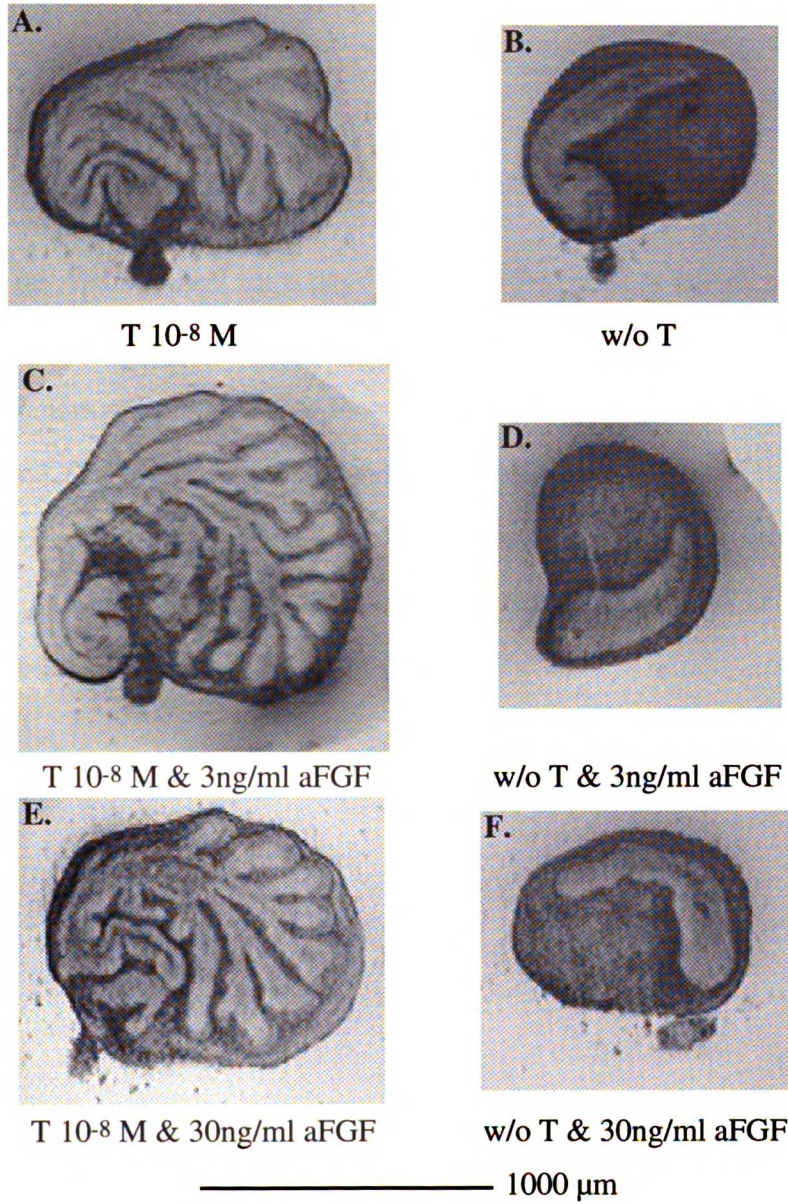
Figure 30



* Different from T control (p<.05).

Δ Different from without androgen control (p<.05).

Figure 31



Representative images of neonatal rat APs cultured 6 days with $T 10^{-8} M$ (A., C., E.) or in the absence of T (B., D., F.) plus 3 ng/ml aFGF (C. and D.) or 30 ng/ml aFGF (E. and F.).

Figure 32

Newborn rat APs cultured for 6 days with T 10^{-8} M plus or minus acidic FGF (aFGF or FGF-1) (3 and 30 ng/ml), or in the absence of T plus or minus aFGF (3 and 30 ng/ml). Four parameters of epithelial morphogenesis were assessed: A. Node number, B. Inverse form factor, C. Epithelial perimeter, and D. Epithelial area.

* Indicates significantly different from APs cultured with T 10^{-8} M (positive control) and Δ indicates significantly different from APs cultured in the absence of T (negative control) ($p < .05$; two-tailed Dunnett test).

Figure 32

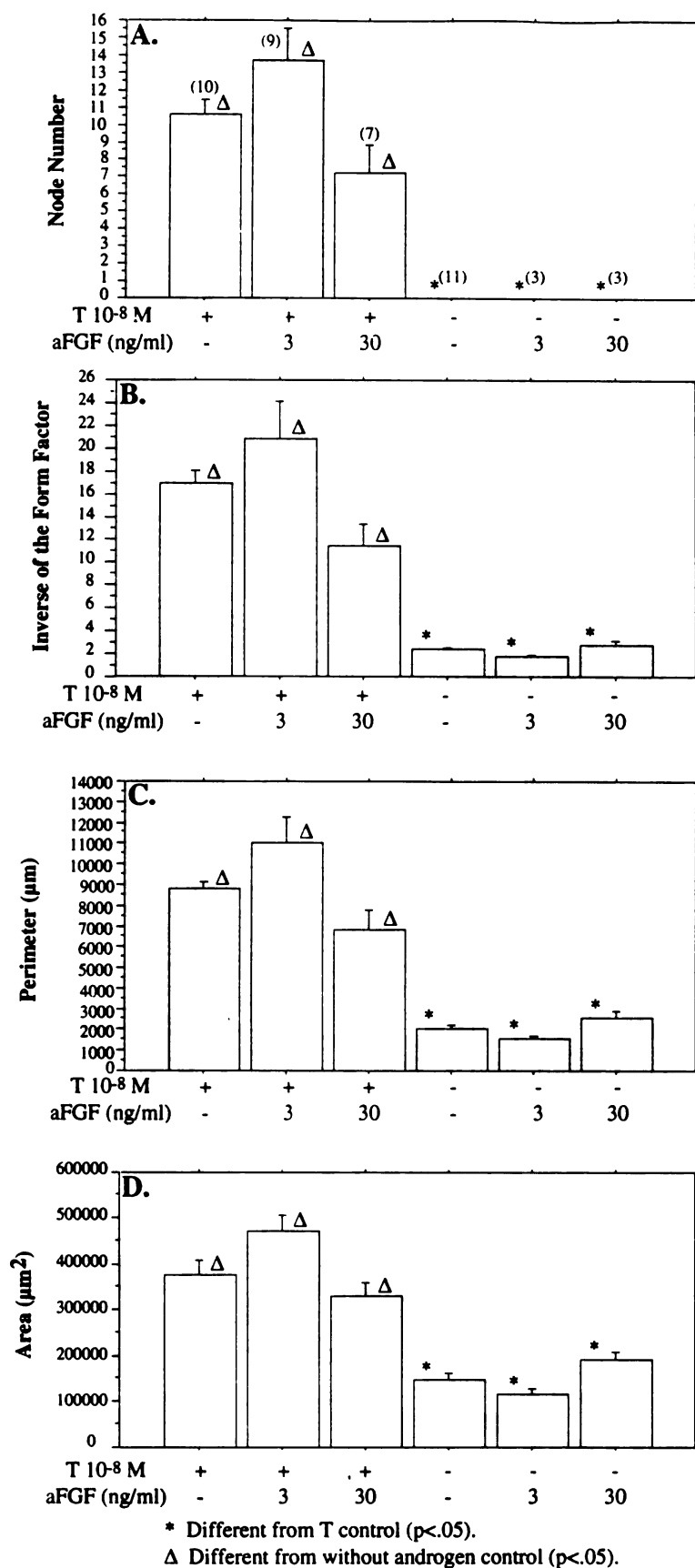
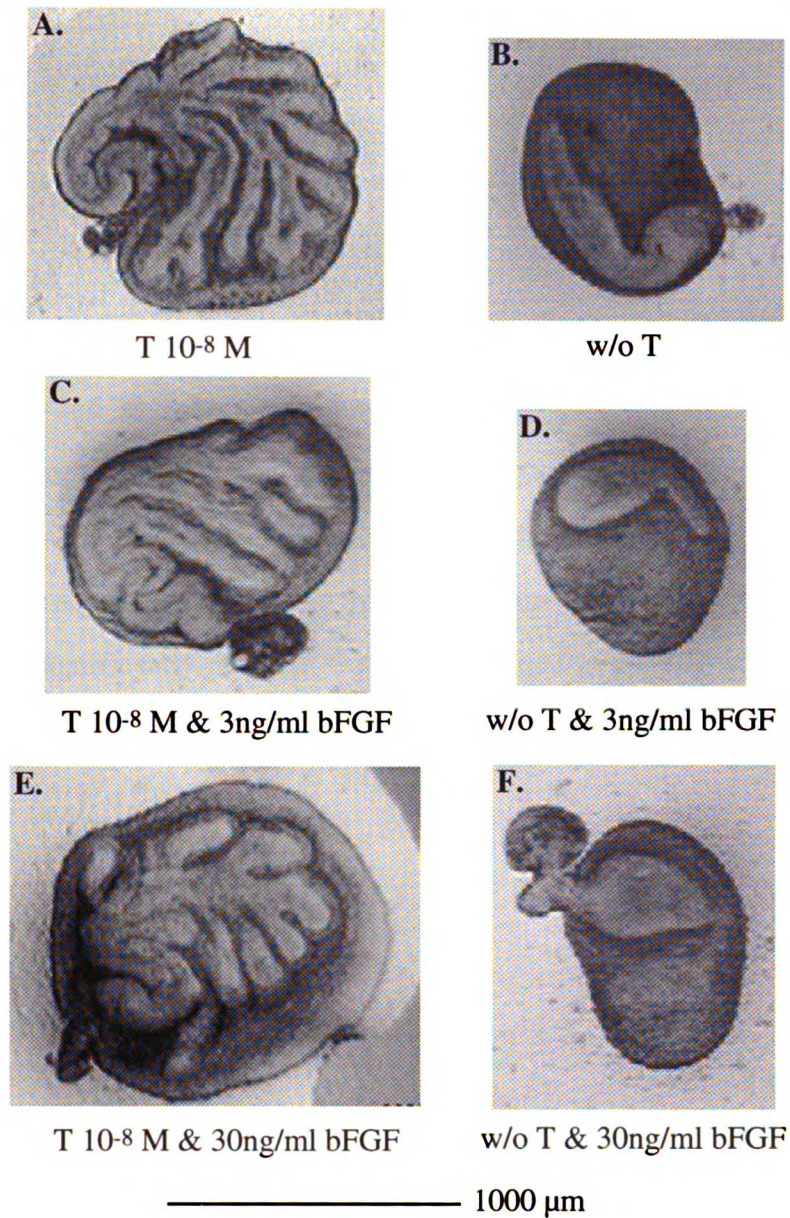


Figure 33



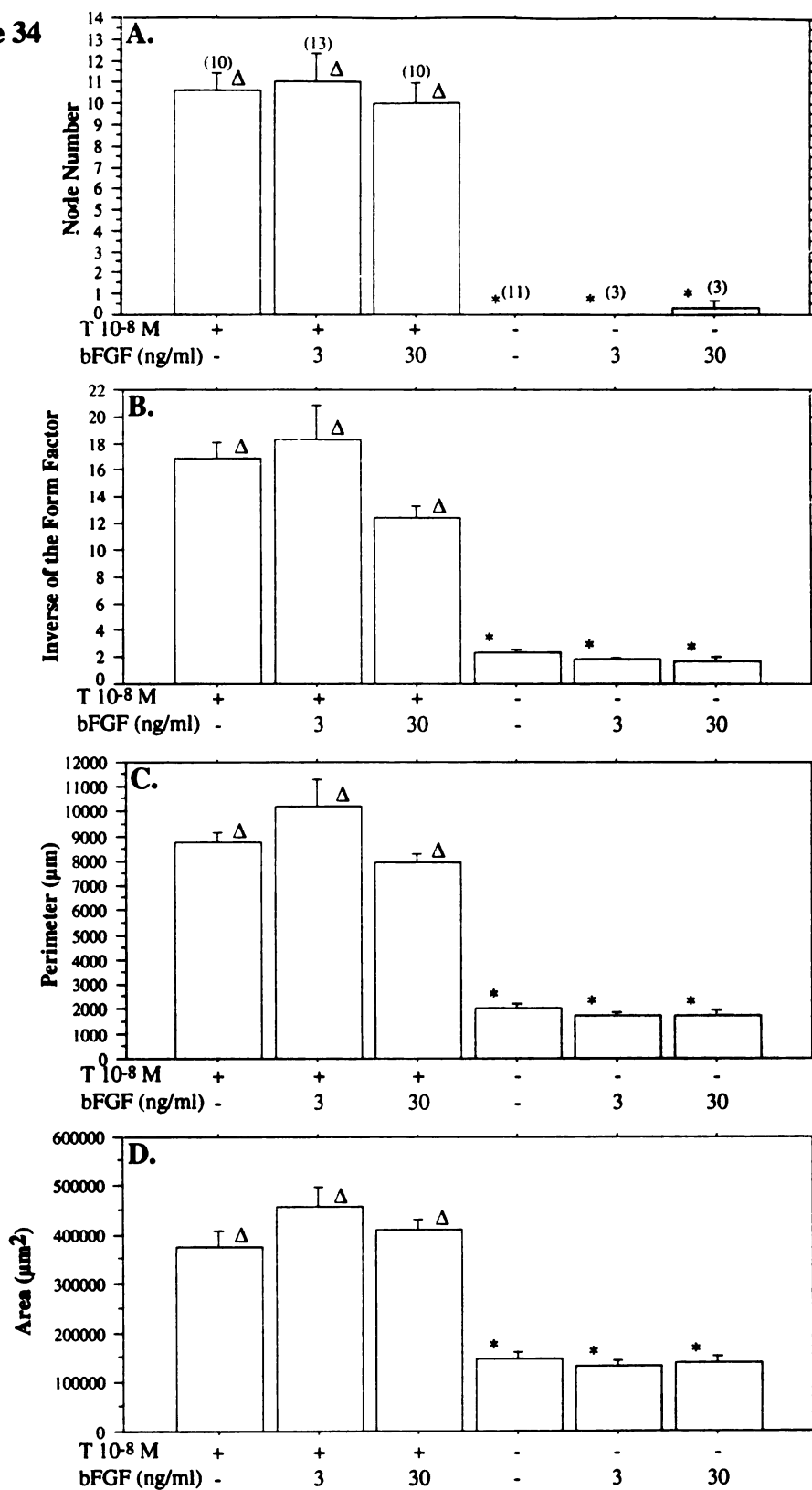
Representative images of neonatal rat APs cultured 6 days with $T 10^{-8} M$ (A., C., E.) or in the absence of T (B., D., F.) plus 3 ng/ml FGF-2/bFGF (C. and D.) or 30 ng/ml FGF-2/bFGF (E. and F.).

Figure 34

Newborn rat APs cultured for 6 days with T 10^{-8} M plus or minus basic FGF (bFGF or FGF-2) (3 and 30 ng/ml), or in the absence of T plus or minus bFGF (3 and 30 ng/ml). Four parameters of epithelial morphogenesis were assessed: A. Node number, B. Inverse form factor, C. Epithelial perimeter, and D. Epithelial area.

* Indicates significantly different from APs cultured with T 10^{-8} M (positive control) and Δ indicates significantly different from APs cultured in the absence of T (negative control) ($p < .05$; two-tailed Dunnett test).

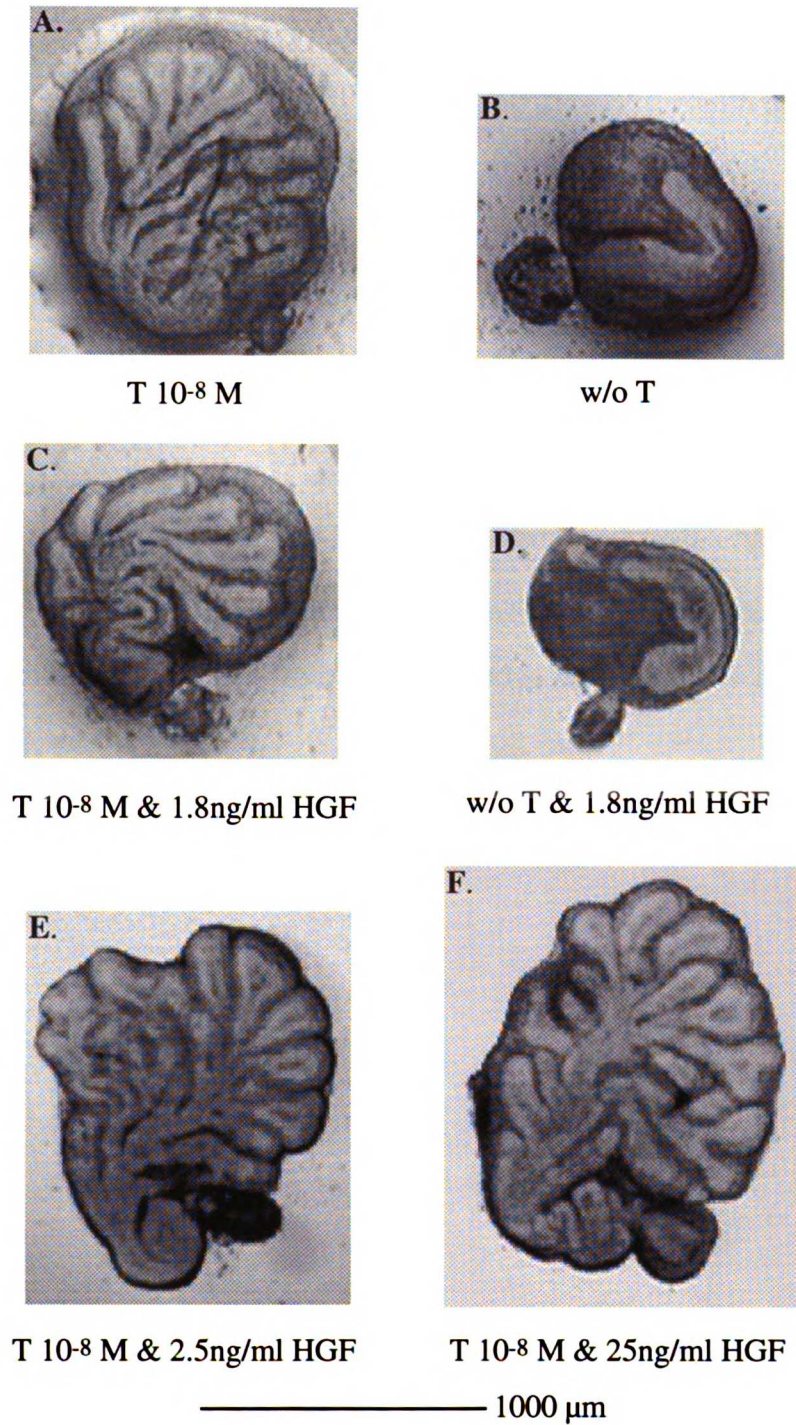
Figure 34



* Different from T control (p<.05).

Δ Different from without androgen control (p<.05).

Figure 35



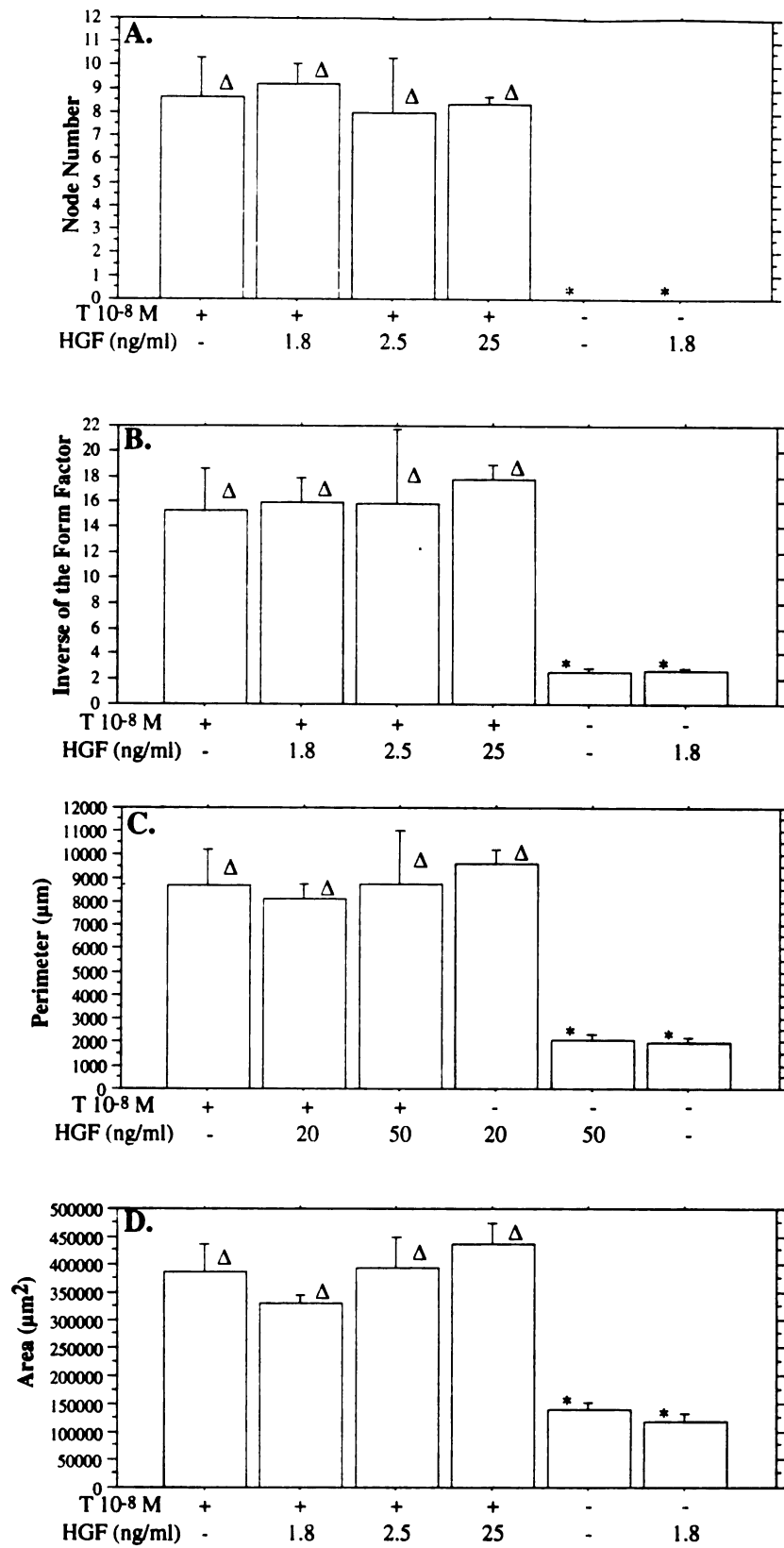
Representative images of neonatal rat APs cultured 6 days with T 10^{-8} M (A.), in the absence of T (B.), HGF plus in the absence of T (D.), or HGF plus T 10^{-8} M (C., E., F.).

Figure 36

Newborn rat APs cultured for 6 days with T 10^{-8} M plus or minus HGF (1.8-25 ng/ml), or in the absence of T plus or minus HGF (1.8 ng/ml). Four parameters of epithelial morphogenesis were assessed: A. Node number, B. Inverse form factor, C. Epithelial perimeter, and D. Epithelial area.

* Indicates significantly different from APs cultured with T 10^{-8} M (positive control) and Δ indicates significantly different from APs cultured in the absence of T (negative control) ($p < .05$; two-tailed Dunnett test).

Figure 36



* Different from T control.

Δ Different from without androgen control.

VPs Cultured with Growth Factors

Overview

Newborn rat VPs were cultured for six days either with or without T in combination with a variety of growth factors or neutralizing agents to growth factors to assay their effect on ductal branching morphogenesis. The factors tested were T, EGF, TGF- α , anti-TGF- α II, KGF and anti-KGF. Ductal branching morphogenesis was androgen-dependent and ductal branching was induced by T in a dose-dependent manner. EGF distorted and inhibited T-induced ductal branching. Neither EGF nor TGF- α induced ductal branching morphogenesis in the absence of T. Anti-TGF- α inhibited T-induced ductal branching morphogenesis in the VP. Surprisingly, KGF induced ductal branching morphogenesis in the absence of T. Moreover, anti-KGF inhibited T-induced branching morphogenesis. Anti-TGF- α also inhibited KGF-induced ductal branching morphogenesis. Thus, KGF and TGF- α are mediators of androgen-induced ductal branching morphogenesis in the developing rat VP. TGF- α may be a downstream mediator of KGF effects in ductal branching morphogenesis of VP.

General Observations

At birth rat VPs were composed of 2-3 main ducts with several branch points surrounded by mesenchyme (Fig. 37A). VP are developmentally more advanced than the AP from the same newborn rat. At birth the VP have some branching, while the AP is unbranched at this time. VPs from 6 day old rats were substantially larger than 0 day VPs and had a highly branched ductal tree (Fig. 37B). Androgen-dependent development of the VP in culture mimicked the development of the gland *in vivo* (Fig. 37). VPs cultured in the absence of T underwent some ductal branching, but the degree of ductal branching was substantially less than glands grown 6 days *in vivo* or cultured 6 days with T 10^{-8} M (Fig. 37C). Moreover, there was a substantial amount of growth during 6 days of culture

even in the absence of T. As expected, ductal branching morphogenesis was androgen-dependent in a dose dependent manner. VPs cultured in the absence of T or in the presence of T at either 10^{-11} M or 10^{-10} M underwent minimal ductal branching (Fig. 37C-E). In contrast, the epithelium of VPs cultured with T 10^{-9} M, T 10^{-8} M or T 10^{-7} M developed into highly branched ductal trees, similar in complexity to glands *in situ* at 6 days (Fig. 37F-H). However, the pattern of T-induced ductal branching in culture was qualitatively different from that *in vivo*. The epithelial ducts of cultured glands were thicker and the main ducts were not as easily identified. Additionally, during the 6 days of culture the epithelium completely filled the surrounding mesenchyme. The culture system biases the VP to grow 2-dimensionally, while *in vivo* the growth of the VP is very 3-dimensional. This could, in part, account for some of the differences observed in the pattern of ductal branching between VPs grown *in vitro* versus *in vivo*. Despite these qualitative differences in development of VPs *in vivo* versus *in vitro*, dramatic morphogenetic changes could be elicited by T in newborn VPs during a 6 day culture period. Furthermore, the developmental process observed *in vitro* was comparable to that *in vivo*.

VPs Cultured with EGF

Newborn rat VPs were cultured 6 days with T 10^{-8} M plus EGF (2.5-40 ng/ml). VPs cultured with high doses of EGF (10, 20 and 40 ng/ml) plus T 10^{-8} M had less ductal branching than VPs cultured with T alone (Fig. 38). In many cases the epithelium was difficult to see clearly through the mesenchyme, suggesting that EGF may have effected the mesenchyme. Thus, as observed in the developing AP, the developing rat VP was also inhibited by exogenous EGF. However, it is not known if EGF is actually involved in ductal branching *in vivo* in the developing VP.

VPs Cultured with FGF-7/KGF and Anti-KGF

Surprisingly, KGF (100 ng/ml) induced ductal branching morphogenesis in the absence of T in newborn rat VPs during 6 days of culture (Fig. 39C). VPs cultured 6 days with KGF (100 ng/ml) but in the absence of T were comparable in size and complexity to VPs cultured with T 10^{-8} M. A neutralizing antibody to KGF (1G4) at 100 μ g/ml inhibited T-induced ductal branching morphogenesis (Fig. 39D). Thus, while exogenous KGF did not have a morphogenetic effect on the developing rat AP in the absence of T, in the developing rat VP exogenous KGF induced ductal branching in the absence of T. The importance of KGF as a mediator of T during prostatic ductal branching is further supported by use of anti-KGF which completely inhibited T-induced ductal branching. Thus, KGF has been identified as a mediator of mesenchymal-epithelial interactions in T-dependent development of the rat VP.

VPs cultured with TGF- α and Anti-TGF- α

Newborn rat VPs were cultured 6 days with TGF- α (5-40 ng/ml) in the absence of T. TGF- α did not induce ductal branching morphogenesis in the absence of T (Fig. 40). The epithelium was difficult to see through the mesenchyme in the TGF- α treated cultures, suggesting that TGF- α may have effected the mesenchyme (Fig. 40). Newborn rat VPs were cultured 6 days with T 10^{-8} M plus neutralizing antisera to TGF- α II (anti-TGF- α) made by Dr. R. Coffey (dilution of 1:10000). This antisera was previously shown to inhibit T-induced ductal branching morphogenesis in the developing rat AP (previous section). Anti-TGF- α II profoundly inhibited T-induced ductal branching morphogenesis (Fig. 41). The ductal tips of VPs cultured with T 10^{-8} M plus anti-TGF- α II had a "grape cluster-like" appearance, suggesting that the epithelium was trying to branch but was not capable of ductal outgrowth. Consequently, ductal branching morphogenesis was inhibited. In the developing salivary gland it had previously been shown that epithelial growth is not needed for ductal branching. Tunicamycin treatment halts epithelial

expansion but does not inhibit ductal branching (Spooner et al., 1989). TGF- α could be acting as a mitogenic agent in the developing rat VP. Therefore, blocking the activity of endogenous TGF- α with the neutralizing sera may have inhibited ductal outgrowth resulting in the observed inhibition of ductal branching morphogenesis. Since the ductal tips are the site of the greatest epithelial growth, this could account for the "grape cluster-like" appearance of the ductal tips.

Newborn VPs were also cultured with KGF (100 ng/ml) plus anti-TGF- α II to determine if TGF- α was involved in KGF-induced ductal branching. Anti-TGF- α II also profoundly inhibited KGF-induced ductal branching morphogenesis (Fig. 42), suggesting that TGF- α is involved in the proliferative response of KGF. TGF- α has been proposed to mediate the mitogenic response of KGF-induced proliferation of keratocytes in culture (Dlugosz et al., 1994). Thus, TGF- α may be an important mediator of both T-induced and KGF-induced ductal branching morphogenesis in the developing rat VP and may be a key modulator of epithelial proliferation.

RT-PCR Analysis

Since organ culture experiments using rat AP and VP indicated that TGF- α and KGF were involved in T-induced ductal branching morphogenesis of the developing prostate, RT-PCR analysis was performed to determine if these growth factors and their receptors were expressed during development of the rat prostate. RT-PCR analysis for the detection of glyceraldehyde phosphate dehydrogenase (GAP) (Fig. 43A), TGF- α (Fig. 43B), KGF (Fig. 43C) and KGFR (Fig. 43D) transcripts was performed on seminal vesicle (SV), APs and VPs cultured 6 days with and without T 10^{-8} M (Fig. 43). Rat 14 day embryonic tissue (emb) and 3T3 (fibroblastic cell line) were used as controls. As a negative control PCR reactions were run without cDNA template (no RT). GAP primers yielded a product that was 244 base pairs (bp) in length. All the tissue samples were positive for GAP indicating that the reversed transcribed RNAs from all of the samples

were adequate for RT-PCR analysis (Fig. 43A). TGF- α primers produced a 370 bp product. SVs, APs and VPs cultured with and without T 10^{-8} M were positive for TGF- α (Fig. 43B). KGF primers yielded a 423 bp product. SVs, APs and VPs cultured with and without T 10^{-8} M were positive for KGF (Fig. 43C). 3T3 cells were positive for KGF. KGFR primers yielded a 407 bp product. SVs, APs and VPs cultured with and without T 10^{-8} M were positive for KGFR (Fig. 43D). 14 day rat embryonic tissue was positive for KGFR, but as expected the fibroblastic cell line, 3T3, was negative for KGFR. Hence, cultured SVs, APs and VPs expressed TGF- α , KGF and KGFR. However, regulation of TGF- α , KGF and KGFR expression by T could not be determined, since transcripts were detected in organs cultured both with and without T. It is possible that the expression of TGF- α , KGF and KGFR transcripts were regulated by T but expression was not completely turned off. Therefore, RNase protection assays were performed to determine if TGF- α , KGF, and their receptors were regulated by T.

RNase Protection Assay for TGF- α , EGFR, FGF-7/KGF and KGFR

RNase protection assays were performed in collaboration with Dr. Axel Thomson in our laboratory on RNA isolated from newborn rat APs, SVs and VPs cultured for 6 days with or without T 10^{-8} M to determine the expression pattern of TGF- α , KGF and their receptors. Actin was used as a standard control to normalize for the amount of RNA loaded in each lane. RNA isolated from Dunning tumor epithelial and stromal cell lines (a rat prostatic tumor) was used as a tissue control for all RNase protection assays. An epithelial specific cytokeratin (CK19) was used to estimate of the relative amount of epithelium in the different glands under the various treatment conditions. CK19 was expressed in Dunning epithelium but not in Dunning stroma, confirming its epithelial specificity. Treatment with T did not change CK19 expression in APs and VPs when differences in RNA loading were taken into account (Fig. 44). Treatment with T increased CK19 expression in SV, suggesting that the epithelial to mesenchymal ratio increased.

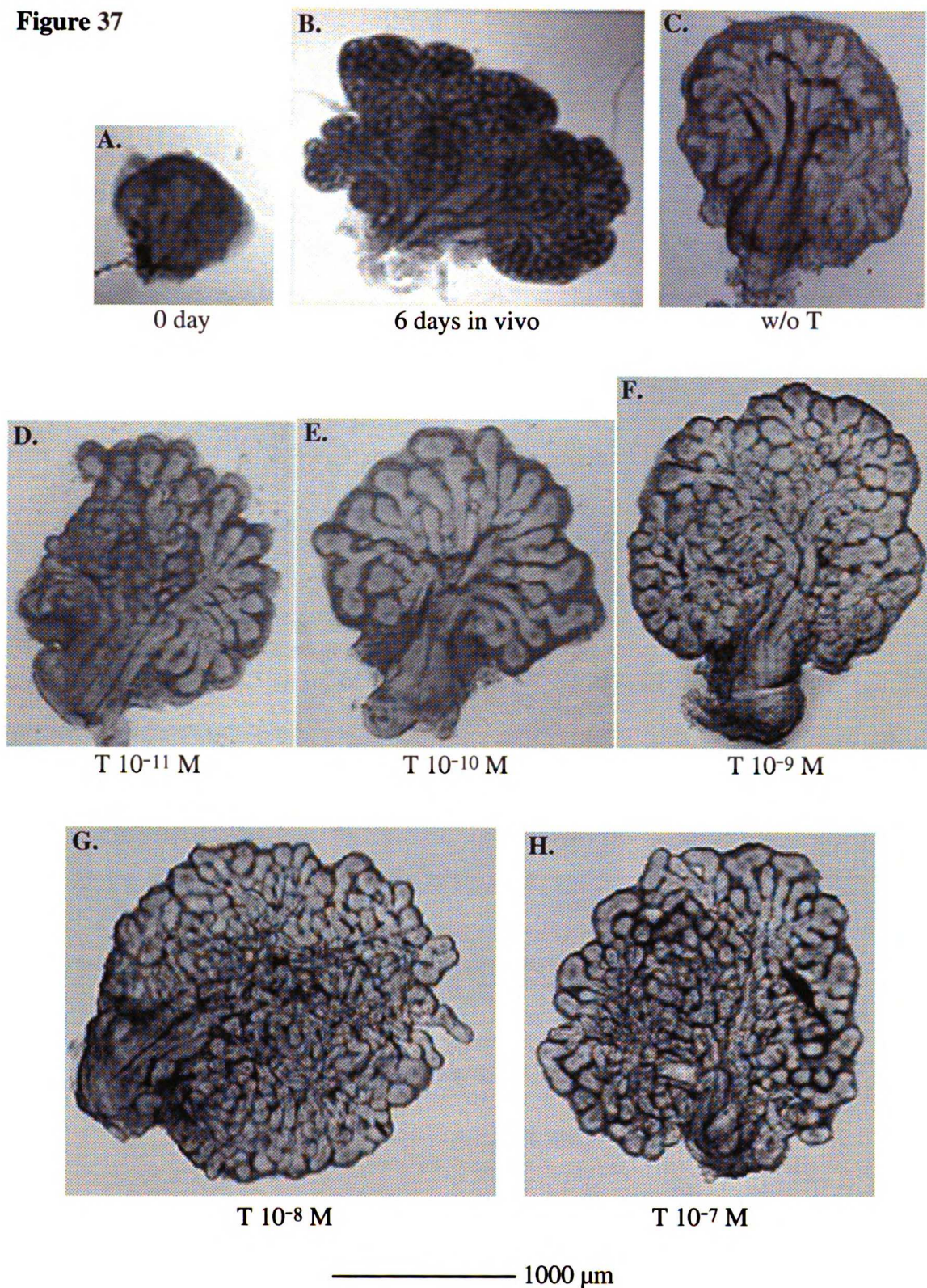
This finding is important to keep in mind when evaluating changes in expression levels of the growth factors and their receptors because an increase in expression in the SV could be due in part to an increase in the epithelial to mesenchymal ratio. This is especially true for factors that are expressed primarily by the epithelium. Treatment with KGF did not change CK19 expression in any of the glands, suggesting that KGF treatment did not change the epithelial to mesenchymal ratio.

Treatment with T or KGF did not change TGF- α expression in APs and VPs (Fig. 45). Thus, TGF- α expression is not androgen regulated, nor was it regulated by KGF. In the SV, treatment with T increased TGF- α expression. However, this increase could have been due to the increase in the epithelial/mesenchymal ratio and not to an actual increase in the expression of TGF- α by individual cells. TGF- α was expressed at high levels by Dunning epithelium and at low levels in Dunning stroma, suggesting that the observed TGF- α expression was primarily in the epithelium of the cultured glands. EGFR was expressed by both Dunning epithelial and mesenchymal cells. The level of EGFR expression did not change in either APs or VPs cultured with the different treatments (Fig. 45). Thus, EGFR expression was not regulated by either androgens or KGF. The increase in EGFR expression in SVs treated with T could have been a result of the increased epithelial to mesenchymal ratio. Thus, neither TGF- α nor its receptor, EGFR, was regulated by either T or KGF in the developing rat AP, SV or VP (Fig. 45).

As expected, KGF was expressed exclusively in Dunning stroma and not in the Dunning epithelium. Unexpectedly, KGF expression decreased with T treatment in APs, SVs and VPs. This decrease in expression of KGF could not be accounted for by a change in epithelial to mesenchymal ratio as measured by CK19 expression in any of the glands. However, it is possible that KGF expression is not homogenous throughout the developing gland but rather has a localized pattern of expression. If KGF is expressed just at the tips of the developing gland, this could account for the apparent decrease in KGF expression. Further studies are needed to determine the localization of KGF expression.

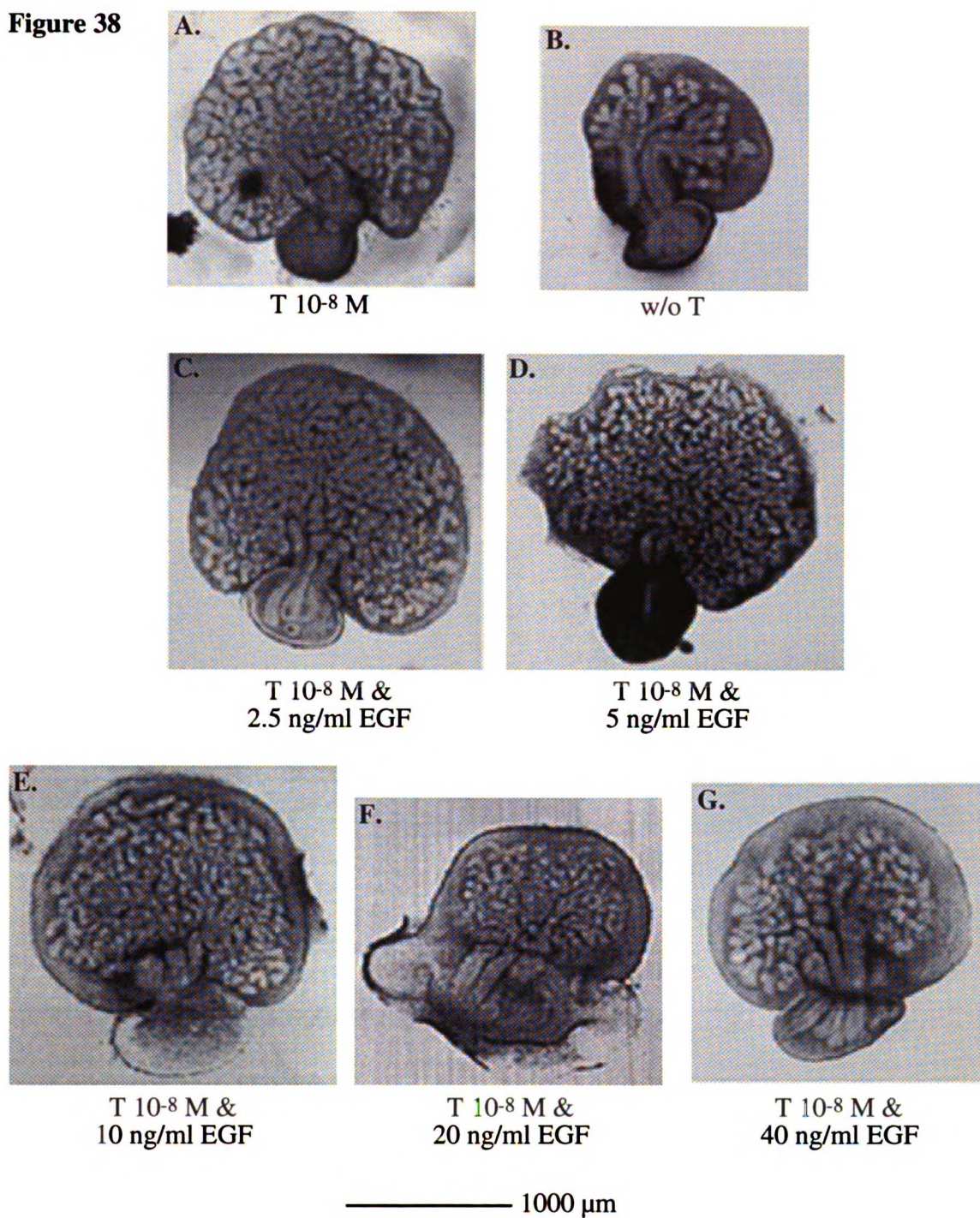
KGFR did not change in APs and VPs cultured either with T or KGF. The increase in KGFR in SVs treated with T could have been due to the change in epithelial to mesenchymal ratio. The probe used in the KGFR RNase protection assay was specific for exon IIIb of the FGFR2. However, while this probe specifically detects the portion of FGFR2 required for KGF ligand binding, it does not give any information on other regions of the receptor. Recently, it has been reported that the FGFR1 gene can be alternatively spliced in several regions of the gene. One spliced variant in particular lacks an internal signaling domain. While such a splice variant has not yet been reported for the FGFR2 gene, the highly conserved structural arrangement of the FGFR1 and FGFR2 genes suggests that such a form exists for FGFR2 (Johnson et al., 1991). This raises the possibility that while exon IIIb of the FGFR2 is expressed by developing APs, VPs and SVs, the functional capability of the FGFR2-IIIb/KGFR may not be equivalent in glands cultured with T or in the absence of T, or even between the different glands themselves under the same treatments. Further research is needed in order to determine which internal and external domains of the KGFR are expressed in the different developing glands and under the various conditions used in these experiments. Thus, in cultured APs, SVs and VPs expression of KGFR was not regulated by T, as measured by expression of the IIIb exon. Nor was KGFR expression regulated by its ligand, KGF. However, regulation of KGFR splicing was not determined. In summary, in the developing rat AP and VP expression of TGF- α , EGFR and KGFR was not regulated by either T or KGF. Additionally, KGF expression in the developing glands appears to have been negatively regulated by T. However, local regulation of KGF was not determined in these experiments.

Figure 37



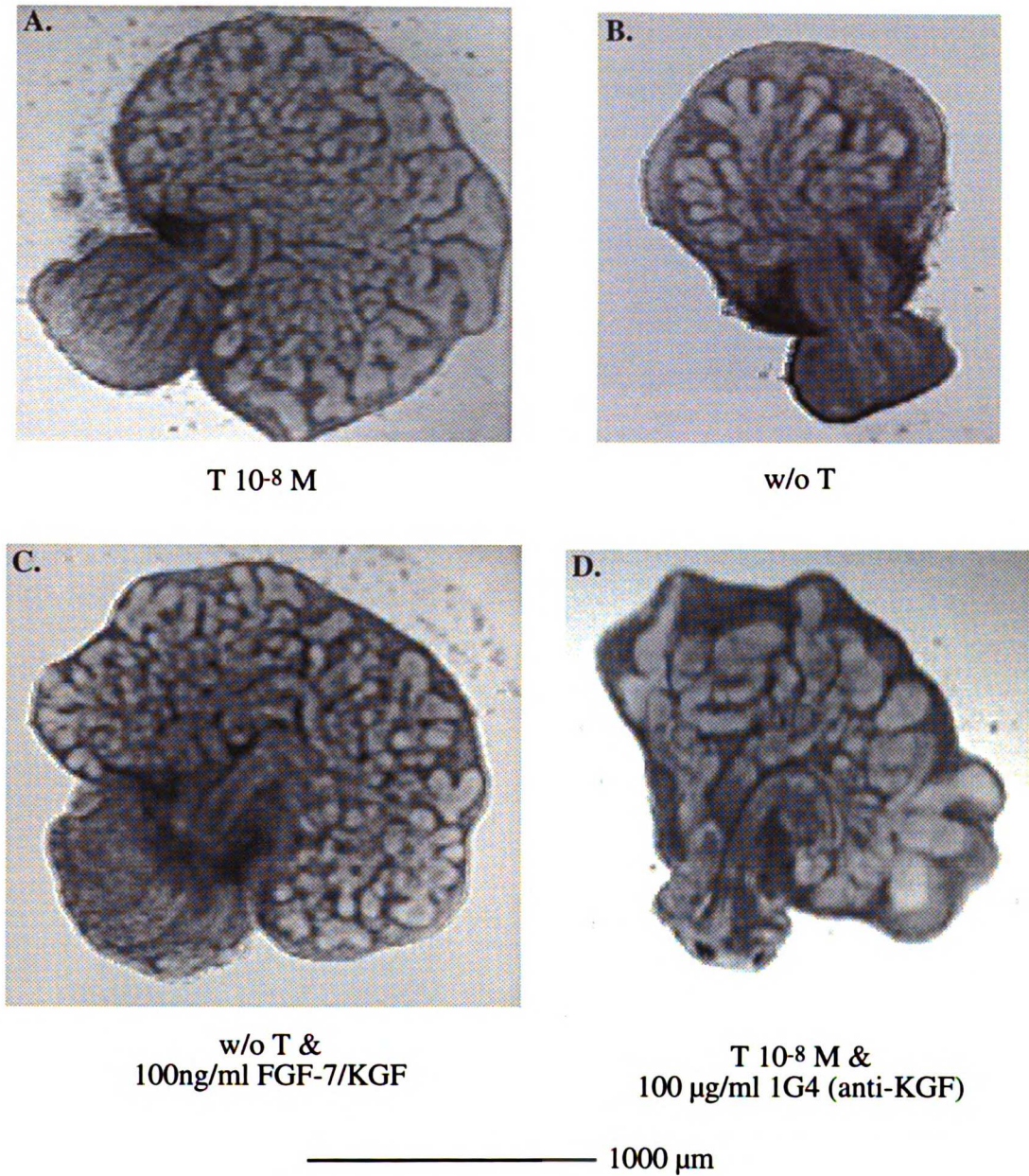
Representative images of 0 day rat VP (A.), 6 day old rat VP (B.) and newborn rat VPs cultured 6 days in the absence of T (C.) or with T 10^{-11} M-T 10^{-7} M (D.-H.).

Figure 38



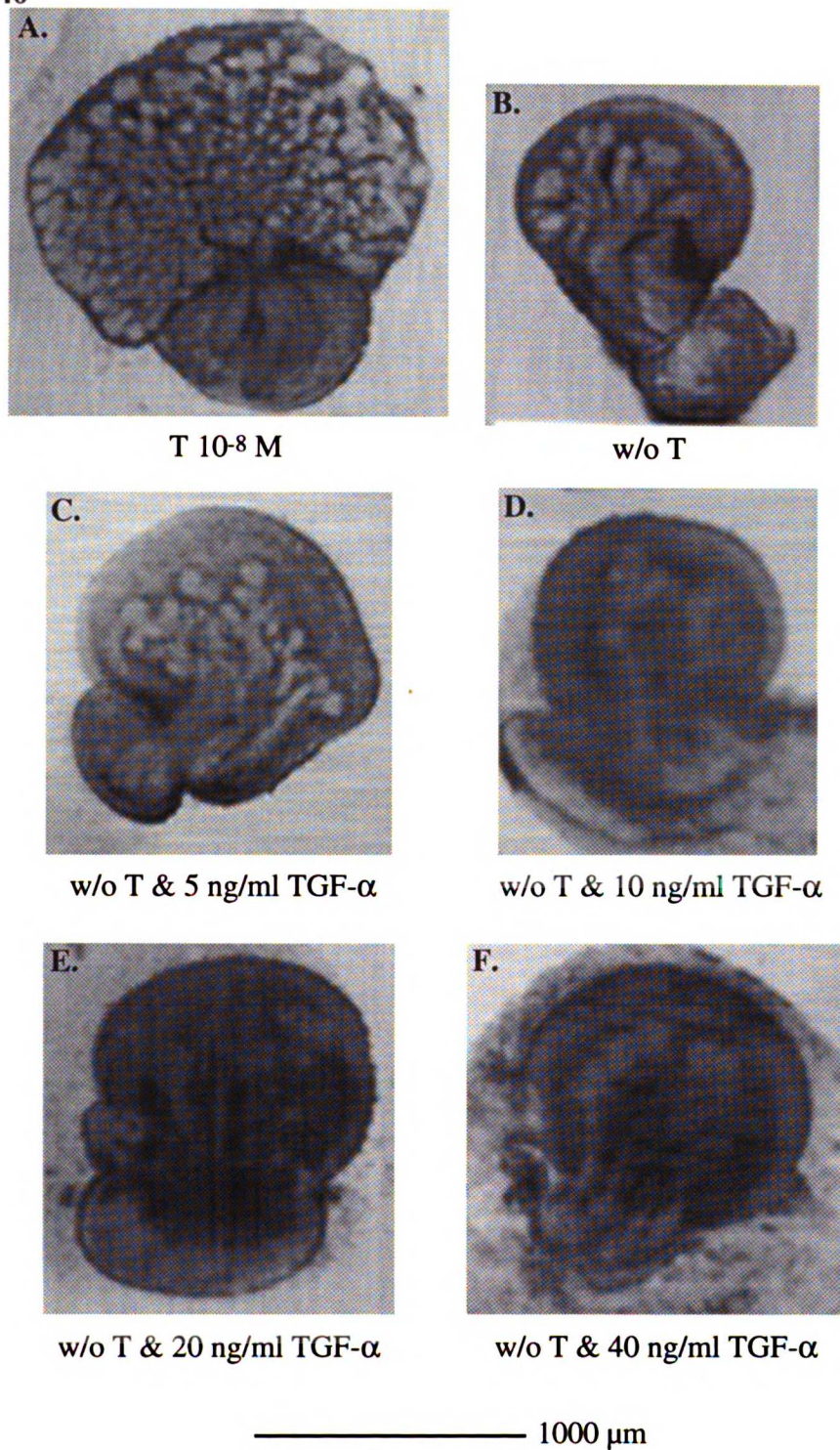
Representative images of neonatal rat VPs cultured 6 days in the absence of T (A.), with T 10^{-8} M (B.), or with T 10^{-8} M and EGF (C.-G).

Figure 39



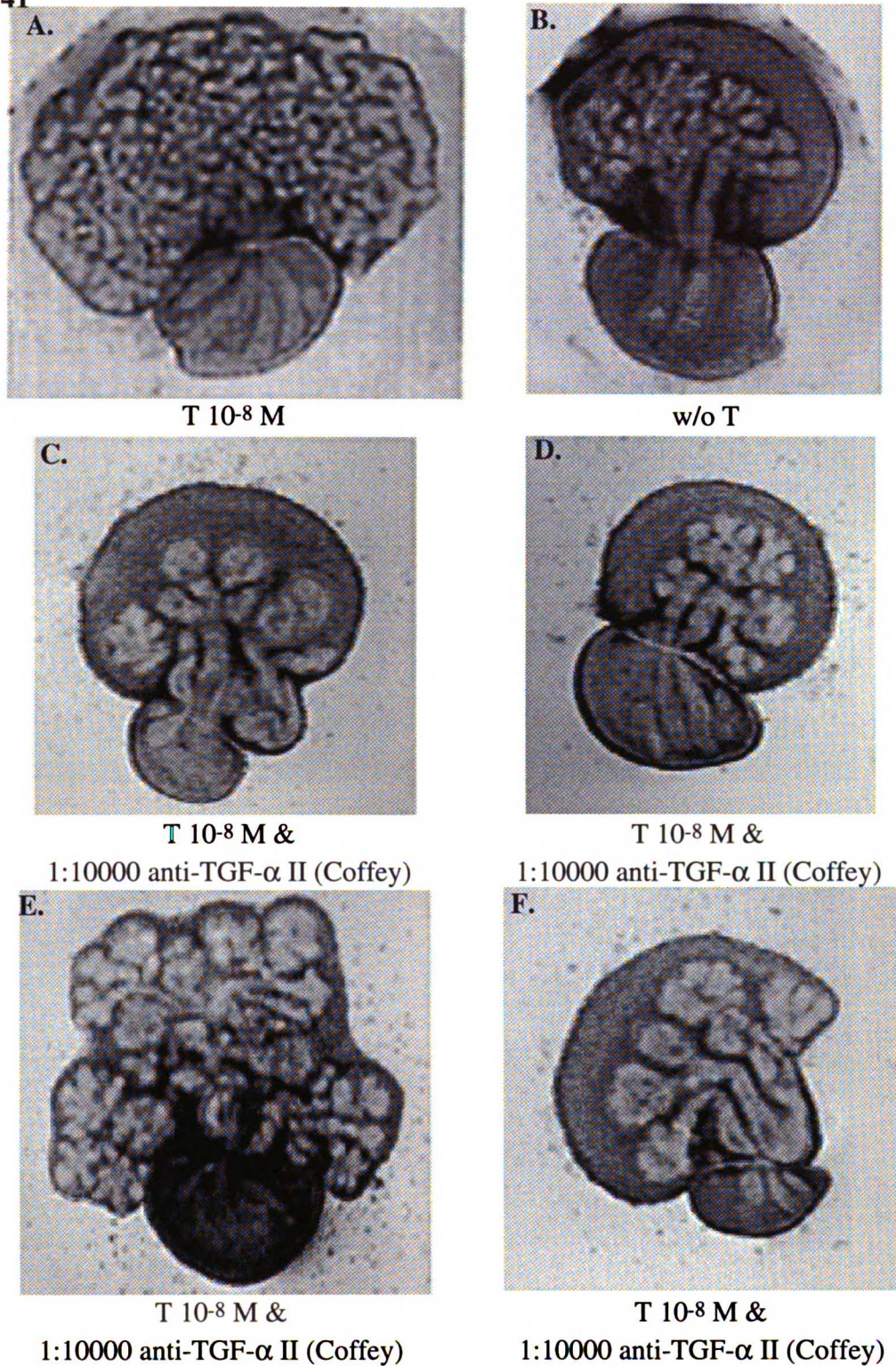
Representative images of neonatal rat VPs cultured 6 days with T 10^{-8} M (A.), in the absence of T (B.), with 100 ng/ml FGF-7/KGF and in the absence of T (C.), or with T 10^{-8} M and 100 µg/ml 1G4 (anti-KGF) (D.).

Figure 40



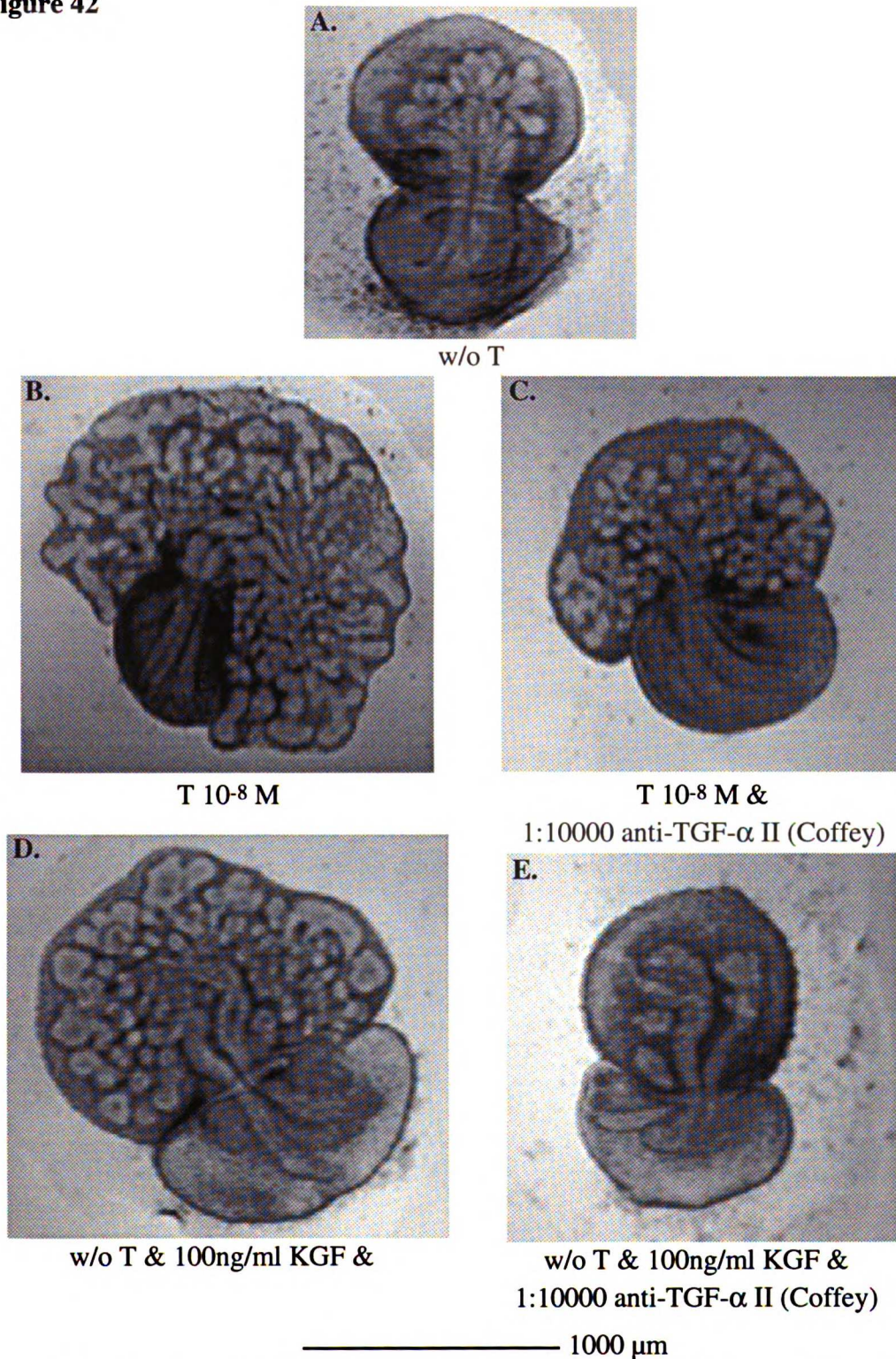
Representative images of neonatal rat APs cultured 6 days with T 10^{-8} M (A.), in the absence of T (B.), or with TGF- α and without T (C.- F.).

Figure 41



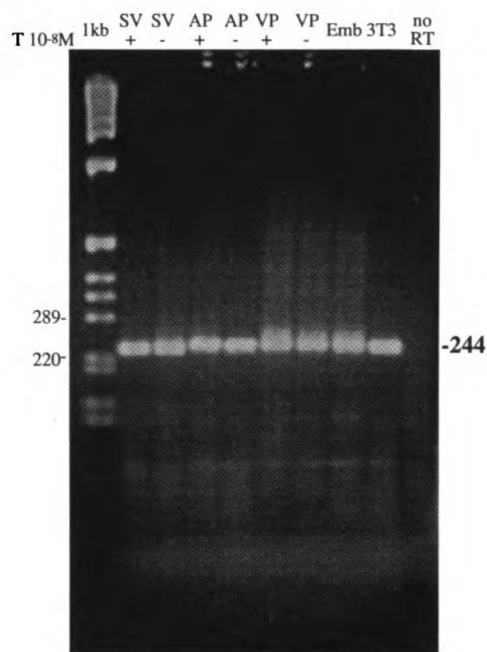
Representative images of neonatal rat VPs cultured 6 days with T 10^{-8} M (A.), in the absence of T (B.), with T 10^{-8} M and anti-TGF- α II from Dr. Coffey (Dilutions of 1:10000) (C.-F.). Note “grape cluster-like” appearance of ductal tips in glands cultured with anti-TGF- α II and T 10^{-8} M.

Figure 42

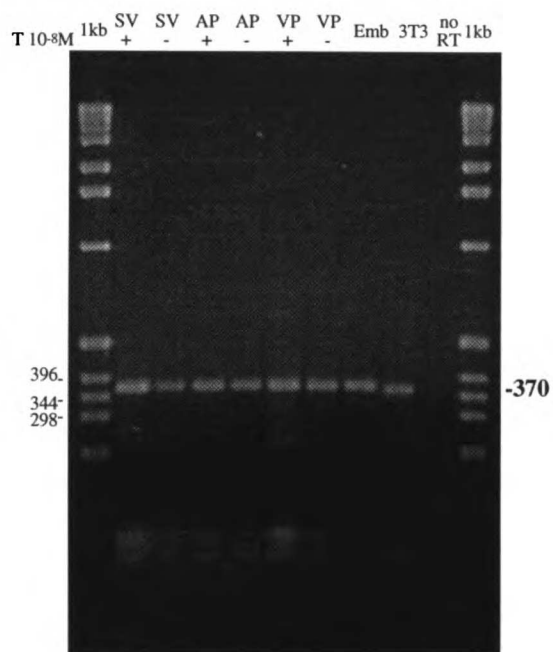


Representative images of neonatal rat VPs cultured 6 days in the absence of T (A.), with T 10^{-8} M (B.), with T 10^{-8} M and anti-TGF- α II from Dr. Coffey (Dilutions of 1:10000) (C.), with 100 ng/ml KGF and in the absence of T (D.), with 100 ng/ml KGF in the absence of T and anti-TGF- α II (Dilutions of 1:10000) (E.).

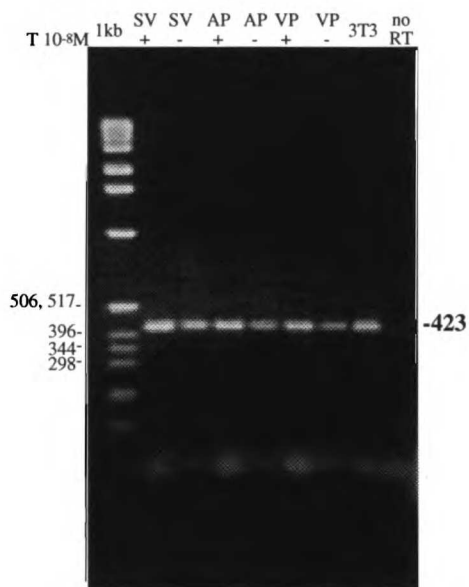
Figure 43 A. GAP



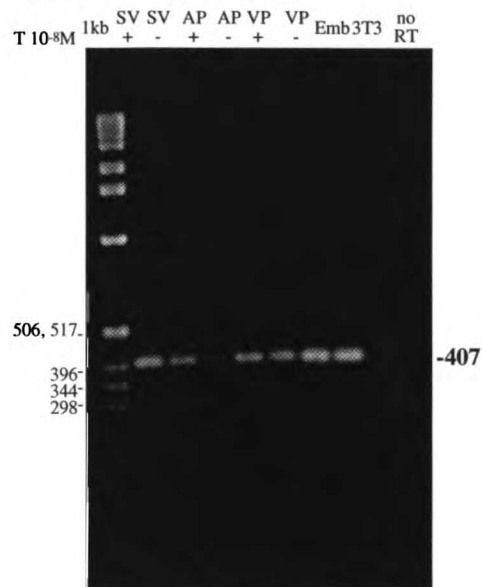
B. TGF- α



C. KGF



D. KGFR



Neonatal rat SVs, APs and VPs were cultured for 6 days with or without T 10-8 M and RT-PCR analysis to detect GAP (A.), TGF- α (B.), KGF (C.) and KGFR (D.) transcripts was performed. Whole 14 day rat embryo (emb) and 3T3 cells were used as tissue controls and the PCR reaction without reversed transcribed RNA (no RNA) was used as a negative control. The RT-PCR GAP product is 244bp, TGF- α product is 370bp, while KGF and KGFR products are 423bp and 407bp, respectively.

Figure 44

RNAse protection assay to detect epithelial specific cytokeratin 19 (CK19). The probe used protects a fragment which is 350 nucleotides long. An actin probe was included to detect differences in loading. The mRNA were loaded: Dunning epithelium and stroma (Dun. Epi. and Dun. Stroma), 0 day glands cultured 6 days in the absence of T (w/o), with T 10^{-8} M (T), 100 ng/ml FGF-7/KGF in the absence of T (KGF), rat AP, SV and VP respectively. SV cultured with T 10^{-8} M showed an increase in CK 19 expression, suggesting that during the culture period with T there was an increase in the epithelial to mesenchymal ratio.

Figure 44 Quantitation of Cytokeratin 19 by RNase Protection Assay

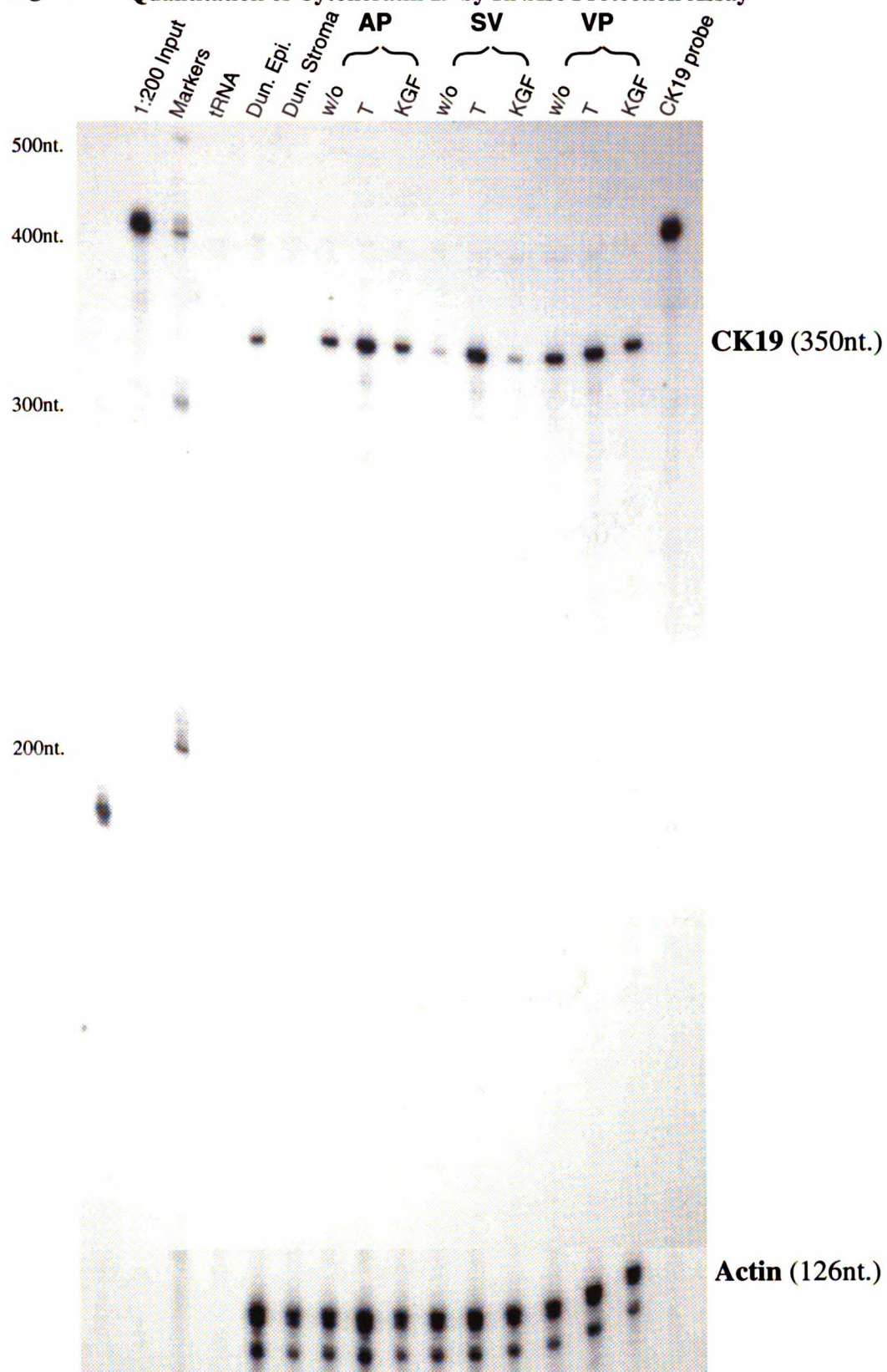
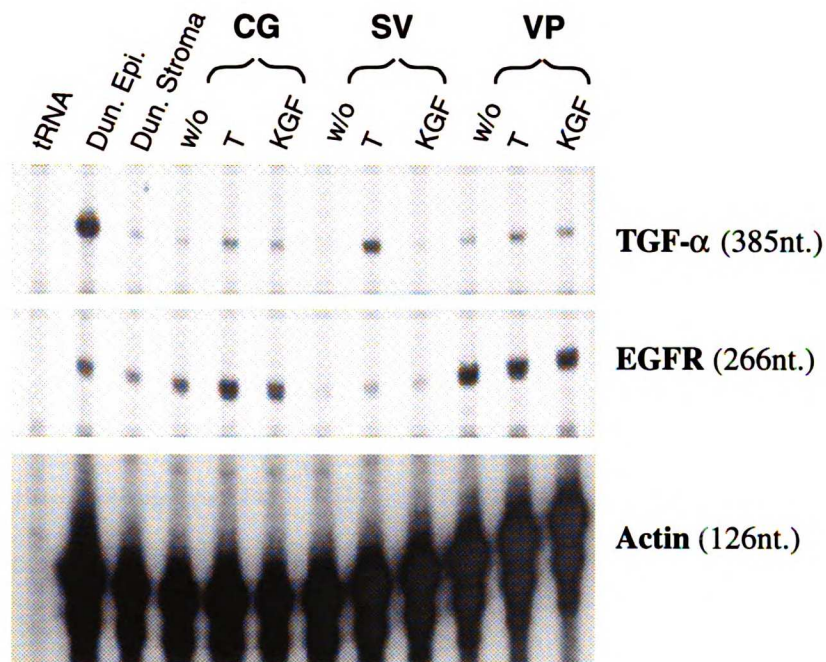


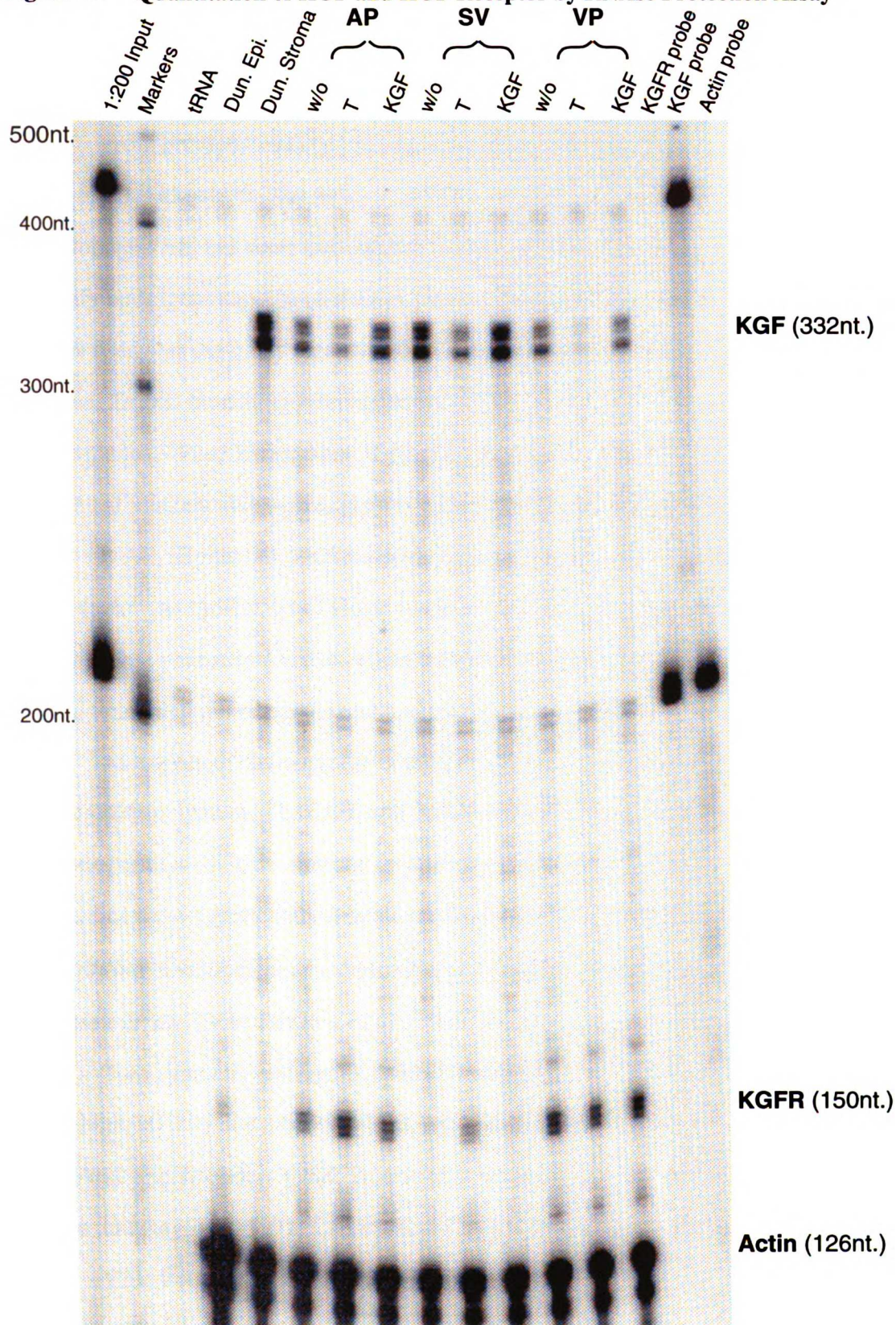
Figure 45
Quantitation of TGF- α and EGFR by RNase Protection Assay



RNase protection assay to detect TGF- α and EGFR. The TGF- α probe used protects a fragment which is 385 nucleotides long and the EGFR probe used protects a fragment which is 266 nucleotides long. An actin probe was included to detect loading differences. The mRNA were loaded: Dunning epithelium and stroma (Dun. Epi. and Dun. Stroma), 0 day glands cultured 6 days in the absence of T (w/o), with T 10^{-8} M (T), 100 ng/ml FGF-7/KGF in the absence of T (KGF), rat AP, SV, VP respectively.

Figure 46

RNAse protection assay to detect both FGF-7/KGF and its receptor FGFR2/IIIb (FGFR). The probe used to detect KGF protects a fragment which is 332 nucleotides long and the probe used to detect IIIb exon of FGFR2 protects a fragment which is 150 nucleotides. An actin probe was included to detect differences in loading. The mRNA were loaded: Dunning epithelium and stroma (Dun. Epi. and Dun. Stroma), 0 day glands cultured 6 days in the absence of T (w/o), with T 10^{-8} M (T), 100 ng/ml FGF-7/KGF in the absence of T (KGF), rat AP, SV and VP respectively.



DISCUSSION

Androgens

From previous studies it is known that the development of the prostate is androgen-dependent. However, the role of specific androgens on various aspects of prostatic development has not been well defined. The purpose of the studies presented here was to identify androgens capable of inducing ductal branching morphogenesis. The experimental model used was the 0 day rat anterior prostate (AP) grown for six days in serum-free organ culture. Ductal branching morphogenesis in this model mimics the *in vivo* development of these glands. Four parameters of epithelial morphogenesis were used to quantitate the effects of various androgens, growth factors and anti-growth factors on the developing 0 day rat AP. Epithelial node number, epithelial perimeter, epithelial area and epithelial form factor (an indication of shape complexity) were determined using Prism view software. These parameters are excellent quantitative indicators of the amount of growth and ductal branching morphogenesis that occurred in the glands during the six days of culture.

As expected, development of the AP was androgen-dependent using the serum-free organ culture system. T, DHT and 3α -diol were potent inducers of ductal branching morphogenesis in this system, while androsterone, androstanedione, 3β -diol and 5β 3α -diol were significantly less effective. All of these androgens are naturally present *in vivo* during development of the prostate as a result of androgen metabolism in the prostate (Baulieu et al., 1969; Baulieu et al., 1968; Bruchovsky and Wilson, 1968; Robel et al., 1971). Two synthetic androgens, MENT and R1881, which bind to the androgen receptor with high affinity but cannot be 5α -reduced to DHT, were also tested in this system (Agarwal and Monder, 1988; Bonne and Raynaud, 1975; Bonne and Raynaud, 1976; Bonne and Raynaud, 1977; Carlson and Katzenellenbogen, 1990; Kumar et al., 1992; Liao et al., 1973; Pousette et al., 1979). Both of these synthetic androgens were also potent inducers of prostatic development, eliciting a response which was comparable to that

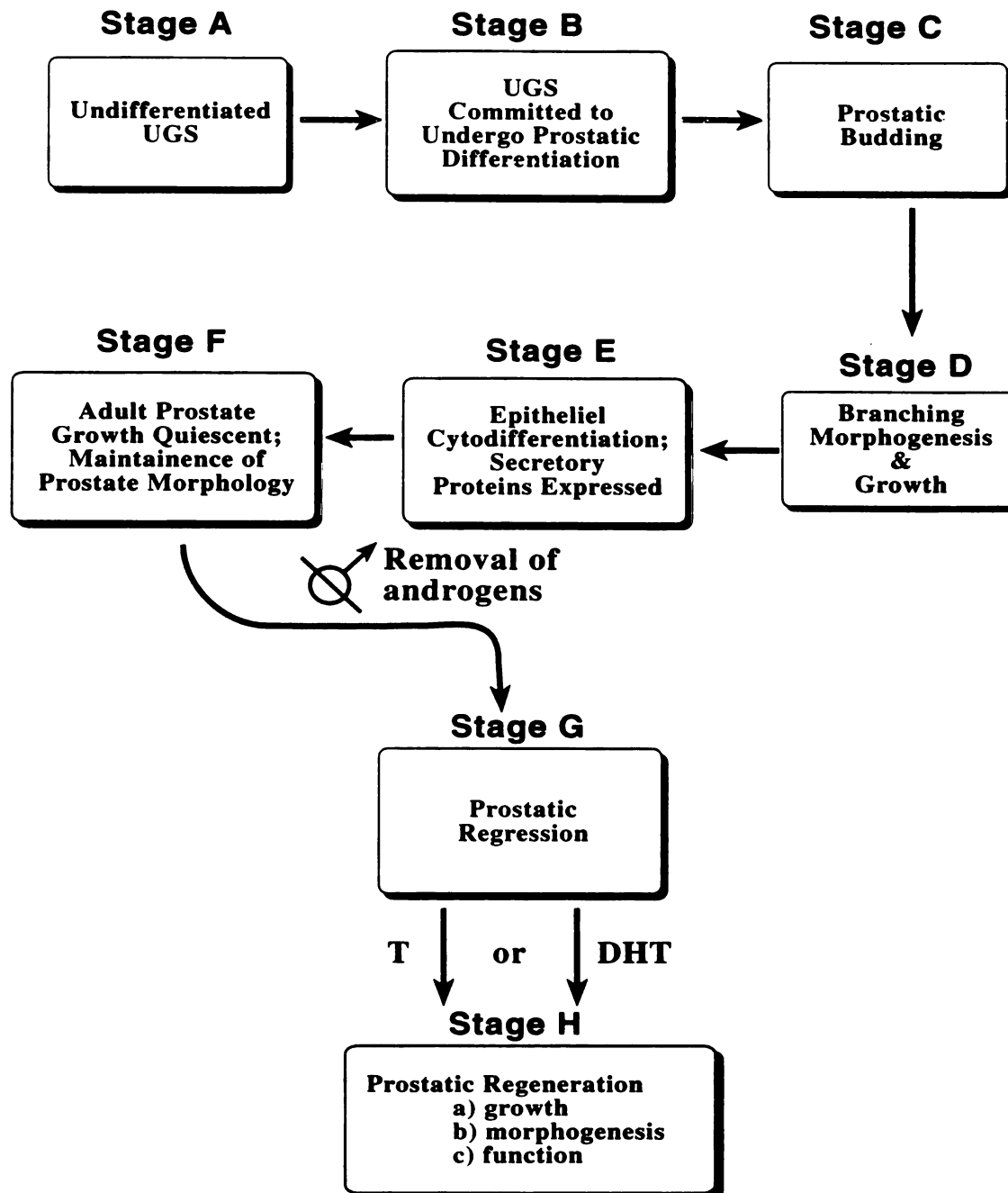
elicited by T or DHT. The novel aspect of this study is that developmental effects of androgens were assessed quantitatively by computer-interfaced morphometrics in a serum-free organ culture system. This fact allows for the first time a meaningful quantitative comparison of the effects of various androgens on prostatic development specifically during the stage in which the prostate undergoes ductal branching morphogenesis without the systematic complications associated with whole animal studies.

Prostatic development involves a coordinated sequence of events beginning with the undifferentiated embryonic UGS and culminating in the attainment of full adult function (See Fig. 47). The embryonic UGS starts as the undifferentiated, uncommitted prostatic anlagen (Stage A), which under the influence of androgens becomes committed to form prostate (Stage B). In Stage B the UGS has not expressed any morphological changes indicative of its commitment to undergo prostatic development. The first morphological change indicative of prostatic development is the formation of prostatic buds (Stage C). Under the continued influence of androgens the prostatic buds elongate, undergo ductal branching morphogenesis, and extensive growth (Stage D). During pubertal growth the epithelium undergoes functional cytodifferentiation and expresses prostate-specific secretory proteins as growth and branching morphogenesis continue (Stage E). The growth-quiescent adult prostate (Stage F) is androgen-dependent for maintenance of morphology and function. In adulthood the exceedingly low rate of cell proliferation is balanced by the low rate of cell death. Castration results in massive loss of epithelial cells due to apoptosis. The prostate regresses resulting in loss of functional morphology of the gland (Stage G). Administration of androgen to castrated males elicits prostatic growth, regeneration of ductal morphology, and induction of epithelial cytodifferentiation with the expression of prostatic specific-secretory proteins (Stage H).

DHT, a 5α -reduced metabolite of T, is thought to be the active androgen in the prostate. Evidence suggests that DHT is required for the early stages (A-C) of prostatic development. In adulthood DHT is believed to be required for maintenance of prostatic

Figure 47

Timeline of Prostatic Development



The prostate goes through many different stages in the course of its development from the uncommitted UGS anlage (Stage A) to the functional adult prostate (Stage F). Through the course of development the prostate is androgen-dependent and remains androgen-dependent in adulthood for the maintenance of morphology and function. Androgen withdrawal induces apoptosis and regression of the prostate (Stage G). Administration of androgens results in regeneration of the prostate (Stage H).

morphology and function (Stage F). DHT has also been implicated in regeneration of the prostate after androgen withdrawal (Stage G).

The conclusion that DHT, and not T, is the active androgen in prostatic development and that T by itself may be inadequate to elicit prostatic development is based on three lines of evidence: (1.) Formation of DHT occurs prior and during the time of prostatic development (Stages A-C) (Siiteri and Wilson, 1974; Wilson and Lasnitzki, 1971). (2.) Prenatal administration of 5 α -reductase inhibitors to pregnant rats inhibits prostatic bud formation in male fetuses (George and Peterson, 1988). (3.) Prostates are small or absent in patients having a defect in type II 5 α -reductase (5 α -reductase deficiency) (Cantu et al., 1980; Fisher et al., 1978; Imperato-McGinley et al., 1974; Imperato-McGinley et al., 1991; Imperato-McGinley et al., 1980; Imperato-McGinley et al., 1992b; Kuttann et al., 1979; Peterson et al., 1977; Price et al., 1984; Saenger et al., 1978; Walsh et al., 1974). While DHT clearly plays a role in the earliest stages (A-C) of prostatic development, the role of DHT in ductal branching morphogenesis (Stage D) has not been examined adequately. This is the focus of the studies presented here.

With regard to the production of DHT, 5 α -reductase activity is present in the developing prostate, including the undifferentiated UGS (Stage A), which is the embryonic prostatic anlagen. Siiteri and Wilson (1974) demonstrated that human embryonic UGS and genital tubercle have the capacity to form [3 H]-DHT from [3 H]-T. 5 α -reductase activity was detectable in human fetuses as early as 6-8 weeks of gestation, a stage before the testis synthesizes T, which begins about 8-10 weeks of gestation. While DHT production is demonstrable in the UGS from the earliest stages of sex differentiation (Stage A), DHT formation is not detectable in the Wolffian ducts until much later in development when the fetus is 13-14 weeks of gestation. At this stage the SV is clearly visible and undergoing morphogenesis. Thus, based on a total sample size of 21 fetuses the authors conclude that in the UGS "the capacity for DHT formation was maximal prior to the onset of male differentiation (Siiteri and Wilson, 1974)." Similar studies performed in the rabbit and rat

confirmed that in the UGS of these species the ability to convert T to DHT precedes both the onset of testosterone secretion by the fetal testis and sexual differentiation of the UGS (Bard et al., 1979; Wilson and Lasnitzki, 1971). These experiments assayed DHT formation during the time in which the UGS forms prostatic buds. Prostates from 3-4 day old rats, which are in the process of growth and ductal branching morphogenesis, also convert T to DHT (Wilson and Lasnitzki, 1971). Thus, DHT is produced in the developing prostate during postnatal ductal branching morphogenesis (Stage D) which is the focus of the studies presented here. In summary, DHT is produced in the developing prostate from the earliest stages and according to Siiteri and Wilson (1974) "DHT is probably the effective intracellular androgen that mediates male development in (UGS) tissue."

The second line of evidence indicating that DHT is required for prostatic development is that prenatal treatment of pregnant rats with inhibitors of 5α -reductase impairs prostatic development in their male pups (George and Peterson, 1988; Iguchi et al., 1991). Administration of a 5α -reductase inhibitor (N-(2-methyl-2-propyl)-3-oxo-4-aza- 5α -adrost-1-ene-17 β -carboxamide, 50 mg/kg day) from 14 to 22 days of gestation inhibited prostatic development in rats. For these studies treatment with 5α -reductase inhibitor was initiated at or before the earliest stages of prostatic development (Stages A-C) but treatment was terminated prior to the stage in which ductal branching morphogenesis of the prostate occurs (Stage D). Prostatic development was determined by histological examination of the reproductive tracts of newborn male pups at 100 μ m increments using as a reference point Wolffian duct entry into the UGS. Two methods were used to quantitate prostatic bud formation. Prostatic buds were counted and the amount of prostatic bud epithelium was determined as a percentage of total UGS-derived epithelium. At the level at which the Wolffian ducts enter the UGS (level of maximal budding) the control group (composed of 4 specimens) had on average 13 prostatic buds and approximately 50% of the UGS-derived epithelium comprised prostatic buds. The 5α -reductase inhibitor

treated group (composed of 6 specimens) had an average of 1 prostatic bud, and only 7% of the UGS-derived epithelium comprised prostatic buds. Furthermore, co-administration of DHT with the 5 α -reductase inhibitor restored prostatic development. Thus, the authors concluded that DHT formation is required for prostatic bud formation. The one specimen treated with both the 5 α -reductase inhibitor and DHT had 21 prostatic buds and approximately 70% of the UGS-derived epithelium comprised prostatic buds. Based on this one animal the authors conclude that DHT can "restore the normal pattern of prostatic budding." The variation within the treatment groups for the number of prostatic buds was not reported for these experiments. Thus, based on a total of six animals treated with 5 α -reductase inhibitor, four control animals and one 5 α -reductase inhibitor plus DHT treated animal the authors conclude that DHT formation is required for and is the active androgen in prostatic differentiation of the rat (George and Peterson, 1988). The stages of prostatic development actually studied were for the most part Stages A-C only and did not include Stage D in which ductal branching morphogenesis occurs.

Iguchi and collaborators (1991) performed similar experiments in the mouse. Pregnant mice were treated with the 5 α -reductase inhibitor 6-methylene-4-pregnene-3,20-dione (6-MP) (400 mg/day) starting on day 12 of gestation and continuing throughout the pregnancy (Stages A through C). Histological study of the urogenital tracts from newborn males pups prenatally treated with 6-MP revealed a marked suppression of the development of UGS derived structures (the prostate, bulbourethral gland (BUG), and AP), while the development of the SV was unaffected. The prostates of 6-MP treated newborn male pups had a decreased number of prostatic buds, and reductions in epithelial volume and mitotic index. However, prostatic development was not impaired in mice prenatally treated with a combination of 6-MP and DHT. Moreover, genital tract malformations observed at birth in prenatally 6-MP-treated mice were completely reversible. Mice prenatally treated with this drug had normal reproductive tracts when examined at 90 days of age (Stage F). Prenatally 6-MP exposed males also displayed normal sexual behavior and had normal reproductive

capacity, suggesting that prenatal exposure to 5 α -reductase inhibitors is reversible postnatally (Iguchi et al., 1991). However, the authors did not specifically investigate the effects of the 5 α -reductase inhibitor on ductal branching morphogenesis of the developing prostate.

Imperato-McGinley and collaborators (1985) performed a similar set of experiments in which they treated rats neonatally (Stages A-C) with 4-methyl-4-aza-5 α -pregnan-3-one-20 carboxylate, a 5 α -reductase inhibitor and assayed the effects on the prostates of newborn male pups. The authors reported that prostatic buds were present in newborn rats treated prenatally (12 to 21 days of gestation) with this drug at 3, 9, 12, 24, or 36 mg/kg day. Prostatic bud formation was determined by histological examination, but was not quantitated. Prostatic buds were present at all dosages (Imperato-McGinley et al., 1985). Since the authors did not quantitate prostatic bud formation, it cannot be determined if prostatic development was partially inhibited in this experiment. The compound used to inhibit 5 α -reductase activity, as well as, the concentration of the inhibitor were different than those used by George et al. (1988) and Iguchi et al. (1991). These differences could account for the discrepancies in results. Nonetheless, in the experiments by George et al. (1988) and Iguchi et al. (1991) prenatal inhibition of 5 α -reductase clearly inhibited prostatic bud formation (Stage C). It is important to recognize that in all three studies pregnant animals were treated with 5 α -reductase inhibitors beginning at Stage A prior to the formation of prostatic buds, and continued through Stage C in which prostatic buds appear. The effect of 5 α -reductase inhibitors on the postnatal development of the prostate was not examined, that is, during the period in which prostatic ductal branching occurs (Stage D). Thus, the role of DHT in prostatic growth and ductal branching morphogenesis was not examined.

Imperato-McGinley and collaborators (1992) also tested the effectiveness of finasteride, a 5 α -reductase inhibitor, to block prostatic development. Pregnant rats were treated with finasteride (25-320 mg/kg day) from 12 to 21 days of gestation (Stage A-C).

The pups were then allowed to grow to adulthood at which time the reproductive tracts were examined. Finasteride inhibited prostatic development as assayed by prostatic wet weight of the adult rat (Imperato-McGinley et al., 1992b). The status of the prostate was not examined in the newborn rat to determine to what extent, if any, prostatic buds had formed. Furthermore, the authors did not determine if the prostates of the finasteride-treated males produced prostatic specific secretory proteins. Histological examination of prostates from finasteride-treated males was not reported. In reality, the rationale of Imperato-McGinley's experiment involved examination of adult prostates (Stage F) of rats which had been treated prenatally with inhibitors of 5 α -reductase during the early stages of prostatic development (Stages A-C). These investigators then attempted to interpret effects as assessed in adulthood on a series of events occurring during the period of prenatal exposure to the drug. Since the period of exposure to the drug encompassed Stage A (undifferentiated UGS), Stage B (commitment to prostatic development), and Stage C (prostatic bud formation), it is impossible to determine the precise role of DHT in prostatic development from this study. The reduction in DHT levels through use of the 5 α -reductase inhibitor may have adversely affected events in Stage A (undifferentiated UGS), Stage B (commitment to prostatic development), or Stage C (prostatic bud formation). Effects at any one stage of prostatic development would impact the adult prostate. Thus, while DHT may be important in prostatic development, the stage at which DHT acts has not been addressed adequately. Most importantly, while these studies suggest that DHT production plays a role in early prostatic development (Stages A-C), the literature to date has not specifically addressed the relative roles of DHT versus T in neonatal growth and ductal branching morphogenesis of the prostate (Stage D). The data presented here, for the first time, assays the effectiveness of T versus DHT in inducing ductal branching morphogenesis in the developing prostate.

The third line of evidence that DHT is the active androgen in prostatic development comes from studies of patients with 5 α -reductase deficiency. Such individuals inefficiently

convert T to DHT and have feminized external genitalia. Their prostates are small or absent (Cantu et al., 1980; Fisher et al., 1978; Imperato-McGinley et al., 1974; Imperato-McGinley et al., 1991; Imperato-McGinley et al., 1980; Imperato-McGinley et al., 1992b; Kuttann et al., 1979; Peterson et al., 1977; Price et al., 1984; Saenger et al., 1978; Walsh et al., 1974). It should be emphasized that these individuals inefficiently convert T to DHT from the earliest stages of prostatic development and throughout the life of the individual. Detailed information on the status of the prostate of patients with 5 α -reductase deficiency is meager. Prostates of individuals with 5 α -reductase deficiency (ages 17-65 years) are difficult if not impossible to detect by digital palpation (Cantu et al., 1980; Imperato-McGinley et al., 1974; Imperato-McGinley et al., 1991; Imperato-McGinley et al., 1980; Kuttann et al., 1979; Peterson et al., 1977; Price et al., 1984). Laparotomy has also been used to assess the state of the internal genitalia. In one study patients were examined at 2, 11 and 14 years of age, but the status of the prostate was not reported (Walsh et al., 1974). In two other studies in which two prepubertal patients and two pubertal patients were examined the prostate was not found (Fisher et al., 1978; Saenger et al., 1978). Based upon analysis of a total of 59 individuals from 2 years up to 65 years of age, the presence of the prostate in individuals with 5 α -reductase deficiency is still questionable at best.

In one report by Imperato-McGinley and collaborators (1992), the status of the prostate of individuals with 5 α -reductase deficiency was examined by transrectal sonography in 14 males (ages 19-31 and one 70 year old subject). Six of these patients were also examined by magnetic resonance imaging (MRI), and 2 also had needle biopsies performed using direct visualization by transrectal sonography. "Prostatic volume" was calculated by both the ellipsoid and slice methods and compared with 14 socio-culturally and age matched males. The prostatic region of individuals with 5 α -reductase deficiency was significantly smaller (approximately one-tenth) than that of control patients. The two prostatic needle biopsies revealed fibrous connective tissue, smooth muscle, but not epithelial tissue. PSA (prostate-specific antigen) levels were undetectable for the four

subjects evaluated (Imperato-McGinley et al., 1992a). The sonographic method used to quantitate "prostatic" volume in this study is adequate to determine volumes of normal and hyperplastic prostate. However, it is questionable whether this method is sensitive enough to quantitate hypoplastic prostatic tissue. More importantly, this method cannot determine the histological phenotype of the tissue being measured. Therefore, while this study quantitated the volume of the "plate-like soft tissue structure posterior to the urethra, into which coursed the vas deferens or ejaculatory ducts", prostatic tissue was not confirmed. This is a particularly important point. If prostatic tissue is completely absent in patients with 5 α -reductase deficiency, one plausible interpretation is that the deficiency in DHT could have adversely affected Stages A, B, or C singly or collectively resulting in the failure of prostatic bud formation. Such a scenario would result in complete failure of prostatic development. By this scenario the interpretation that DHT is the active androgen in prostatic growth and ductal branching morphogenesis is entirely incorrect because of the failure of the prostatic rudiment to form in the first place. In contrast, if prostatic tissue is present in patients with 5 α -reductase deficiency, but diminutive in size, the interpretation is completely different. In this later scenario, the implication is that DHT is normally not required for Stages A-C and, thus, prostatic buds formed. In this case the defect would involve failure of growth of the prostatic anlagen, and thus, would implicate DHT in prostatic growth and ductal branching morphogenesis (Stage D). Taken as a whole, studies on both animals and humans suggest an important role of DHT in the prostate, even though its role in prostatic growth and ductal branching morphogenesis is far from clear.

Implicit in all of the animal and human studies of experimental or genetic 5 α -reductase deficiency is the premise that the elevated level of T associated with 5 α -reductase deficiency is somehow ineffective in eliciting prostatic development. If this is true, it is essential to determine the exact stages in which T by itself is incapable of eliciting prostatic development. For example, if prostates are truly absent in 5 α -reductase deficient patients, T may be ineffective in early stages of prostatic development (Stages A-C). However, if

prostates are present at birth, but smaller in 5 α -reductase deficient patients, then T may be ineffective in promoting prostatic growth and ductal branching morphogenesis (Stage D). Unfortunately the information available does not permit discrimination between these two radically different scenarios.

Inhibitors of 5 α -reductase have also been given postnatally to male rats. Brooks and collaborators (1982) treated prebutal male rats (200 grams) with the 5 α -reductase inhibitor, 17 β -N,N-diethylcarbamoyl-4-methyl-4-aza-5 α -androstan-3-one (4-MA), for 14 days with animals sampled at daily intervals. While prostatic wet weight of 4-MA treated-rats was decreased when compared to age-matched control rats at the end of treatment, in fact, prostatic wet weight of 4-MA treated-rats was unchanged when compared to prostates of rats at the start of the experiment. Thus, 4-MP did not cause an actual reduction in wet weight of the prostate, but rather almost completely blocked normal weight gain of the gland. This effect was completely reversible following discontinuation of treatment. Unfortunately, it is impossible to relate prostatic wet weight to biological function (Brooks et al., 1982).

In another report, neonatal rats were treated with the 5 α -reductase inhibitor, finasteride, from birth till 4 or 7 weeks of age (George et al., 1989). Prostatic development was inhibited as assayed by decreased wet weight of prostates of finasteride-treated males. These studies are difficult to interpret since the analysis of the circulating levels of androgens clearly indicated that finasteride had systemic endocrine effects. At 4 and 7 weeks of treatment with finasteride the circulating concentrations of DHT were decreased but were not significantly different from untreated controls. Furthermore, the circulating levels of T were 6 fold higher than the controls. Prostatic DHT levels were not reported. In this study, DHT formation was inhibited during the actual period of ductal branching morphogenesis (Stage D), and while the authors report an inhibition of prostatic development, this was based solely on prostatic wet weight, a rather meaningless parameter, as reduced prostatic wet weight could be caused by a number of reasons. The

effect of the 5 α -reductase inhibitor on actual prostatic growth and ductal branching morphogenesis (Stage D) was not determined. Thus, it is difficult to determine the respective effects of DHT versus T on developing prostates of the finasteride-treated rats.

From this review of the literature it is evident that the fine distinctions required to precisely interpret the role of DHT versus T on prostatic development have not been made and that the role of DHT in sex differentiation and prostatic development have been consistently over-interpreted. While strong evidence supports a role for DHT in prostatic development, DHT may only be important in the initial stages (A-C) in which prostatic bud formation occurs. Whether there is a unique role for DHT versus T in prostatic growth and ductal branching morphogenesis (Stage D) has not been actually examined until now. Although it is widely believed that DHT is the active androgen in prostatic development, the extensive studies presented herein demonstrate that T and DHT are equally effective in eliciting neonatal ductal branching morphogenesis and development of the prostate.

In summary, 5 α -reductase activity has been detected at the earliest stages of prostatic development in which the undifferentiated UGS (Stage A) commits to the pathway of prostatic development (Stage B) and forms prostatic ducts (Stage C), as well as, in the adult prostate (Stage F). Prenatal exposure to 5 α -reductase inhibitors during the earliest stages of prostatic commitment (Stages A and B) and bud formation (Stage C) reduced prostatic tissue at birth (George and Peterson, 1988; George and Wilson, 1978; Iguchi et al., 1991) and in adulthood (Imperato-McGinley et al., 1992b), while having no effect on fertility (Iguchi et al., 1991). Individuals with 5 α -reductase deficiency have low or undetectable levels of DHT from the earliest stage of prostatic development and throughout adulthood. Prostatic tissue was not been confirmed in these individuals. Neonatal treatment of rodents with 5 α -reductase inhibitors decreased prostatic wet weight. However, ductal branching morphogenesis has not been adequately assessed (George et al., 1989). Administration of 5 α -reductase inhibitors to pubertal rats impaired prostatic weight gain (Brooks et al., 1982). In adulthood 5 α -reductase inhibitors reduced prostatic

size and function. In castrated animals 5 α -reductase inhibitors impaired T-induced prostatic regeneration while having no effect on DHT-induced prostatic regeneration. In summary, DHT is clearly important in the earliest stages of prostatic development (Stage A and B), bud formation (Stage C), as well as in the later stages of maintenance of adult prostatic morphology and function (Stage F), including T-induced regeneration of the prostate following castration (Stage H). Based on the evidence from these two time frames, it has been assumed that DHT is the active androgen in all stages of prostatic development. However, studies to date have not examined the effectiveness of T versus DHT at the intermediate stages of development (Stages D and E) after formation of prostatic buds but prior to the adult stage.

The experiments presented here have used quantitative morphometrics to compare androgenic effects of various androgens on ductal growth and branching morphogenesis (Stage D) of the 0 day rat prostate in a serum-free organ culture system. The developmental time frame of the experiments presented here is after prostatic buds have emerged from the developing UGS (Stage C). Thus, for the first time the question can be asked: Is DHT essential for ductal growth and branching morphogenesis of the prostate and is there a quantitative difference between DHT and T in eliciting prostatic ductal elongation and branching?

Based on 25 separate dose-response experiments used in the current study utilizing 294 APs, DHT was clearly no more effective than T in inducing ductal branching morphogenesis in the developing prostate as quantitated by four parameters of epithelial morphogenesis. The effectiveness of T and DHT in inducing ductal branching morphogenesis was determined using a serum-free organ culture system in which systemic effects of the treatments were eliminated. In organ culture, the 0 day prostate recapitulates the normal androgen-dependent process of development as observed *in vivo*. Epithelial area, epithelial perimeter, node number and inverse form factor were equivalent when T or DHT were used at 10⁻¹⁰ M, a suboptimal concentration for both hormones. At higher

concentrations (10^{-9} M to 10^{-7} M) T appeared to be more effective than DHT in inducing prostatic branching morphogenesis as indicated by an increased number of nodes and inverse form factor. Our results appear to contradict the conclusions of the studies by George et al. (1988) and Iguchi et al. (1991) which demonstrated that treatment of pregnant rats with 5α -reductase inhibitors inhibited prostatic development, thus, implying that the elevated circulating levels of T were not sufficient to induce prostatic development (George and Peterson, 1988; Iguchi et al., 1991). A possible explanation of this apparent discrepancy is that DHT may be required for either commitment of the urogenital sinus (UGS) into the pathway of prostatic differentiation (Stage B) or for the actual formation of prostatic buds (Stage C) in the UGS, since in the *in vivo* studies the 5α -reductase inhibitor was given during early stages of prostatic development (A-C). Possibly, DHT may activate a different set of genes than T in the UGS, which is responsible for initiation of prostatic development.

DHT is believed to be the active androgen in prostatic development and has been suggested to act by amplifying the activity of T by as much as 10-fold, because DHT has a higher relative affinity for and forms a more stable complex with the AR (Grino et al., 1990b; Wilbert et al., 1983). However, in the studies presented here DHT was not more effective than T in inducing ductal branching. Similarities in the dose-response curves for DHT and T indicate that T was probably not exerting its androgenic effects by conversion to DHT. In order to explain the similar efficacy of T and DHT, nearly all of the T would have to be converted to DHT during the culture period. The metabolism studies presented here, which are in agreement with the studies by Wilson et al., indicate that only 52.6% of T is converted to DHT (Wilson and Lasnitzki, 1971). Given the presumed 10-fold higher efficacy of DHT over T the dose-response curves for T and DHT, particularly at the lower concentrations, should have been different, yet the dose-response curves of T and DHT are virtually superimposable (Fig. 4). Furthermore, if 50% of T is converted to DHT and DHT is the active androgen, then T 10^{-10} M should be equivalent to DHT 5×10^{-11} M. The

dose response curves for T and DHT clearly indicate this is not true. T 10^{-10} M induces a greater degree of ductal branching morphogenesis than DHT 5×10^{-11} M as assayed by node number, inverse form factor and epithelial perimeter (Fig 4). Thus, the ductal branching morphogenesis induced by T cannot be accounted for by conversion of T to DHT.

The supremacy of DHT in prostate development is further questioned by the ability of synthetic androgens, MENT and R1881, to support ductal branching morphogenesis of the 0 day rat prostate. MENT and R1881 cannot be 5α -reduced to DHT (Bonne and Raynaud, 1975; Kumar et al., 1992). However, both of these compounds were potent inducers of ductal branching morphogenesis in the developing prostate and elicited a response comparable to that of T and DHT. This is the first set of experiments to show that these synthetic androgens are able to induce androgen-dependent prostatic development. Thus, while 5α -reduction of T may produce androgens that are potent inducers of prostatic development, 5α -reduction to DHT is not required for ductal branching morphogenetic and growth of the rat neonatal prostate.

The biological system used in this study to assay the potency of the androgens (epithelial growth and ductal branching morphogenesis) is very complex and has a certain degree of variation in its endpoint. It is possible that the variation within the system could mask slight differences in the potencies of the androgens. Thus, slight differences between T and DHT would not have been detected. However, the expected 7-10 fold difference in activity between T and DHT would have been detectable (Deslypere et al., 1992; Grino et al., 1990a; Wilbert et al., 1983). The data presented here, based upon the largest data set examined to date, indicates that T and T-like molecules are just as effective as DHT at inducing ductal branching morphogenesis in the developing rat AP.

With regards to the relative potency of T versus DHT, Lasnitzki and Mizuno (1977) cultured rat urogenital sinuses (14.5-19.5 day gestation) with either T or DHT in serum-containing medium. The authors did not perform dose-response experiments comparing the effectiveness of T versus DHT. However, the effectiveness of T versus DHT to induce

prostatic bud formation in urogenital sinuses from 14.5 to 19.5 day fetal rats was compared at the same pharmacological concentration ($1.5 \mu\text{g/ml} = 5 \times 10^{-6} \text{ M}$). DHT was more effective than T at the earliest time points. At 14.5 days of gestation DHT induced 2.9 prostatic buds per UGS, while T was unable to induce prostatic buds. Furthermore, at 15.5 and 16.5 days of gestation DHT was more effective than T at inducing prostatic buds. However, at 17.5 days of gestation T and DHT were equivalent in their ability to induce prostatic buds in the UGS. The authors interpreted the difference between T and DHT in the 15.5 and 16.5 day UGS as indicating that DHT is the active androgen in masculine differentiation of the UGS. The authors further suggest that at 14.5 days T was not able to elicit prostatic bud formation because of the low content or absence of 5α -reductase at this stage and suggest this ability is acquired in the period between 14 and 15 days of gestation (Lasnitzki and Mizuno, 1977). The exact timing of 5α -reductase activity in the developing rat UGS has not been determined. The studies by Lasnitzki and Mizuno suggest that DHT may be the active androgen in initial prostatic bud formation. By 17.5 days of gestation, at which time the UGS is known to have 5α -reductase activity (Bard et al., 1979; Wilson and Lasnitzki, 1971), T and DHT are equally effective at inducing prostatic bud formation. Further studies are needed to determine the dose-response effectiveness of T and DHT both in the commitment of the UGS into the prostatic pathway of differentiation and in prostatic bud formation of the UGS, especially since the dose used by Lasnitzki and Mizuno was in the toxic range.

DHT is accepted as being a more potent androgen than T. DHT interacts with the AR with higher affinity than T and is reported to be more effective at mediating AR-dependent events. Deslypere and collaborators (1992) examined the potencies of T versus DHT to stimulate AR dependent transcription of a reporter gene (chloramphenicol acetyltransferase, CAT) containing a mouse mammary tumor virus (MMTV) promoter (MMTV-CAT). While the MMTV promoter may not be representative of all androgen-responsive elements, it is capable of conferring androgen-dependent regulation of gene

expression. The authors report that DHT is 10 times more potent (half maximal of 0.018 nM) than T (half maximal of 0.2 nM) in inducing CAT activity, while the maximal activity is the same for the two hormones (Deslypere et al., 1992). This is in agreement with the reported K_d values for the interaction of T and DHT with the AR (Grino et al., 1990b). Grino and collaborators (1990) using fibroblasts from an individual with 5 α -reductase deficiency examined the interactions of T and DHT with the AR. Saturation analysis indicates similar amounts of binding with T and DHT. However, DHT has about a 2-fold greater affinity for the AR than T. Furthermore, T also exhibits a 5-fold faster dissociation rate from AR than DHT. These authors suggest that the weaker androgenic properties of T compared the DHT reside in the weaker interaction of T with the AR. These authors also examined up-regulation of AR levels after T and DHT treatment. Up-regulation of AR by 20 nM of T was slightly less than that observed with 2 nM of DHT, indicating that DHT has a 10-fold greater potency than T (Grino et al., 1990b). These studies are in agreement with the previously reported higher K_d values or binding affinity of DHT versus T for the AR (Carlson and Katzenellenbogen, 1990; Liao et al., 1973; Maes et al., 1979; Wilbert et al., 1983; Wilson and French, 1976; Wilson and Gloyna, 1970).

At birth circulating levels of androgens are at their post-natal nadir. Androgen levels rise at puberty and remain high through adulthood (Corpechot et al., 1981). It has been hypothesized that 5 α -reductase activity is required for prostatic development because the low circulating levels of T at birth are inadequate to drive prostatic development (George and Peterson, 1988; George et al., 1989; Grino et al., 1990a; Wilbert et al., 1983). Local formation of DHT may serve to amplify the androgen signal by enhancing the interaction between hormone and AR. DHT may be required to induce prostatic development in the embryonic UGS because of the low circulating levels of T present during the time of prostatic bud formation and subsequent ductal branching morphogenesis (Grino et al., 1990b; Wilbert et al., 1983). However, the circulating level of T in the plasma of newborn rats is ~0.3 ng/ml, which is approximately T 10^{-9} M, and increases to

~3 ng/ml in adulthood ($T\ 10^{-8}\ M$) (Corpechot et al., 1981). In the studies presented here the dose-response curve for T-induced ductal branching of the 0 day rat AP indicates that the circulating level of T in the newborn rat is more than sufficient to induce maximal ductal branching morphogenesis in the developing prostate. Thus, formation of DHT should not be required to enhance the activity of T during this time period of prostatic development.

In the studies presented here 3α -diol was also a potent inducer of prostatic ductal branching morphogenesis *in vitro*. This is in agreement with others who have reported that 3α -diol is a potent androgen. Following castration 3α -diol restores male sexual behavior and increases VP and SV weight (Isaacs and Coffey, 1981; Morali et al., 1994). 3α -diol is a potent inducer of benign prostatic hyperplasia in dogs (DeKlerk et al., 1979; Gloyna et al., 1970; Isaacs and Coffey, 1981; Walsh and Wilson, 1976). 3α -diol is believed to work by backconversion to DHT, because DHT is the primary androgen detected in the nucleus of prostatic cells when 3α -diol is administered to adult dogs (Anderson and Liao, 1968; Gloyna and Wilson, 1969). If 3α -diol was eliciting its effect solely through backconversion to DHT, an equivalent efficacy of DHT and 3α -diol would require a nearly 100% backconversion of 3α -diol to DHT (Fig. 4). Metabolism studies presented here indicate that approximately 85% of 3α -diol was converted to DHT, and the dose-response curves for 3α -diol and DHT were almost identical. Thus, the data presented herein are consistent with the hypothesis that the androgenic activity of 3α -diol is due to backconversion to DHT.

Androsterone, androstanedione and 3β -diol are also able to be converted to DHT (Isaacs and Coffey, 1981). In the studies presented here these androgens were able to induce ductal branching morphogenesis, but to a lesser degree than either T or DHT. This may be due to the lower affinity of these compounds for the AR (Hammond, 1978; Isaacs and Coffey, 1981; Liao et al., 1973). Androsterone, androstanedione and 3β -diol were not as potent at inducing ductal branching as 3α -diol, which also has a low affinity for the AR. This was likely due to the fact that androsterone, androstanedione and 3β -diol are not

as rapidly converted to DHT as 3α -diol (Hammond, 1978; Isaacs et al., 1979a; Isaacs et al., 1979b). $5\beta,3\alpha$ -diol was the least effective in eliciting ductal branching in the developing prostate and this was likely due to $5\beta,3\alpha$ -diol's low affinity for the AR, as well as, its inability to be converted to the more potent androgen, DHT (Isaacs and Coffey, 1981).

Summary of the Effects of Androgens

The studies presented herein quantitated ductal branching morphogenesis in the AP prostate of the newborn rat. To date this is the most extensive and detailed study of the effectiveness of various natural and synthetic androgens on postnatal development of the prostate. Nine androgens were tested using 808 newborn rat APs in 68 dose-response experiments. Based on these studies T is just as effective as DHT in supporting ductal branching morphogenesis in the developing rat AP. Furthermore, the activity of T could not be accounted for by metabolism of T to DHT. Synthetic androgens, MENT and R1881 which cannot be 5α -reduced to DHT, also induced extensive ductal branching and elicited responses comparable to T and DHT. This suggests that while DHT is sufficient for prostatic development, it is not necessary for postnatal branching morphogenesis and growth of the prostate. 3α -diol was particularly potent in inducing ductal branching morphogenesis, eliciting a response comparable to T and DHT. However, 3α -diol was extensively backconverted to DHT during the culture period which could account for its androgenic activity. Androsterone, androstenedione, 3α -diol and $5\beta,3\alpha$ -diol induced ductal branching morphogenesis, but to a lesser extent than either T or DHT. These studies challenge the assumption that DHT is critically essential for prostatic ductal branching morphogenesis.

Growth Factors

Branching morphogenesis is a complex series of events (Hardman and Spooner, 1992). The epithelium synthesizes a proteoglycan-rich basal lamina which accumulates in the clefts between lobules due to stabilization by interstitial collagens (type I and III) (Bernfield and Wessells, 1970). The mesenchyme produces hyaluronidase which regulates GAG turnover in the basal lamina (Bernfield et al., 1984). Enzymatic action at the distal lobules could potentially release proteoglycan-bound growth factors, which then act locally to stimulate cell growth (Hardman and Spooner, 1992). Growth factors associated with the ECM of the clefts would presumably be protected from enzymatic release. Thus, a low rate of proliferation would occur at the clefts and stalks, sites of slower ECM turnover, while the ductal tips, sites of faster ECM turnover, would exhibit a higher rate of proliferation. This is exactly the pattern of cell proliferation seen in the developing prostate (Prins and Birch, 1995; Sugimura et al., 1986). Additionally, collagenase regulates the site of stabilization of branch points during branching morphogenesis. TGF- β , EGF, TGF- α and FGF-1/aFGF have been implicated as growth factors in branching morphogenesis. TGF- β is known to regulate ECM by stimulating ECM deposition, stimulating ECM-degrading enzymes, inhibiting degradation of ECM, and regulating cell adhesion molecules (Massagué, 1990; Matrisian et al., 1992; Roberts and Sporn, 1990). EGF and TGF- α have also been implicated in regulating matrix-degrading metalloproteinases in the developing lung (Ganser et al., 1991). EGF, TGF- α and FGF-1/aFGF are also thought to be involved in cell proliferation and ductal outgrowth in developing glands (Goldin and Opperman, 1980; Hardman and Spooner, 1992; Nogawa and Takaaki, 1995; Seth et al., 1993; Snedeker et al., 1991; Warburton et al., 1992). The experiments undertaken in this project were designed to explore the role of growth factors in androgen-induced prostatic development.

The adult prostate is dependent on androgens for maintenance of morphology and function. Castration results in apoptosis (programmed cell death) of the androgen-

dependent prostatic epithelium resulting in involution of the gland (Kyprianou and Isaacs, 1989a; Lee, 1981). In the VP by 4 days post-castration there is a dramatic loss of total protein, DNA and RNA content (Lee, 1981) and by 10 days post-castration approximately 93% of prostatic glandular epithelial cells are lost (English et al., 1989).

In contrast to the adult prostate, the response of the newborn prostate to androgen deprivation is markedly different. Based upon analysis of neonatal rat prostate grown in vitro without androgen, it is evident that the epithelium of 0 day prostate is not dependent on androgens for survival. Indeed, the epithelial area was essentially the same for APs at the start of culture and those cultured in the absence of T, suggesting that the epithelium of the 0 day AP in the absence of T does not undergo massive apoptosis as does the adult prostate. Isaacs (1987) has postulated that the adult prostate contains a hierarchy of cells: stem cells, amplifying cells, transit cells and postmitotic cells. Stem cells are defined as relatively undifferentiated and pluripotent, capable of self maintenance of cell number, and essentially have unlimited proliferative potential. Amplifying cells have a more restricted potential, arise from stem cells and can proliferate for only a limited number of cell divisions. Transit cells are derived from amplifying cells and have a significantly reduced proliferative capacity. Post-mitotic cells cannot divide and are terminally differentiated. Both stem cells and possibly amplifying cells are androgen-independent for their survival, while transit cells and postmitotic cells absolutely require a critical level of androgen for their survival (Isaacs, 1987). Thus, castration in adulthood causes the loss of transit and postmitotic prostatic epithelial cells which comprise the vast majority of the cells present. The results of the experiments presented here suggest that the unbranched prostatic epithelial ducts of the newborn rat may be comprised primarily of stem cells and amplifying cells that are androgen-independent for survival.

TGF- β has been implicated in the apoptotic response of the prostate to androgen withdrawal. Within the first day post-castration TGF- β mRNA expression increases dramatically (~10 fold) and by 4 days post-castration TGF- β mRNA expression levels have

increased ~40 fold (Kyprianou and Isaacs, 1989b). Furthermore, exogenous TGF- β induces apoptosis when administered directly into the ventral prostate of adult rats *in vivo*. Moreover, addition of 20 ng/ml of TGF- β to cultures of adult prostatic tissue elicited apoptosis to nearly the same extent as removal of T from the culture media (Martikainen et al., 1990). Thus, in the adult prostate androgen withdrawal increases TGF- β expression which activates a program of cell death (apoptosis) in prostatic epithelium.

The studies presented here indicate that TGF- β does not have the same effect on the developing 0 day prostate. TGF- β is a potent inhibitor of T-induced prostatic ductal branching morphogenesis and epithelial growth (as assayed by epithelial area), but does not appear to induce apoptosis in the 0 day prostate. At low concentrations (2.5 and 5 ng/ml) TGF- β in the presence of T distorted ductal branching morphogenesis, but epithelial area, an indicator of epithelial growth, was comparable to that of glands grown with T. This indicates that the 0 day AP did not undergo apoptosis in response to TGF- β . At intermediate doses (10, 20 and 40 ng/ml) TGF- β severely inhibited T-induced ductal branching morphogenesis, while the epithelial area was comparable to that of glands grown with T. This indicates that while TGF- β at intermediate concentrations (10, 20 and 40 ng/ml) inhibited ductal branching morphogenesis, these concentrations of TGF- β also did not induce apoptosis in the 0 day prostate. At high concentrations (50 and 80 ng/ml) TGF- β completely inhibited ductal branching morphogenesis in the presence of T, but the epithelial area was comparable to that of glands at the start of culture. Thus, even at high concentrations, TGF- β probably did not induce massive apoptosis in the 0 day the prostate, even though the expected T-induced increase in epithelial area was completely inhibited. Clearly, the newborn and adult prostate respond differently to androgen deprivation and exogenous TGF- β , especially in regard to the induction of apoptosis which in adulthood may be dependent on the presence of complex cell lineages (stem cells, amplifying cells and transit cells) that emerge during and after puberty.

TGF- β is known to inhibit development of other organs. TGF- β inhibits ductal branching morphogenesis in the developing mammary gland (Daniel et al., 1989) and lung (Serra et al., 1994). It has been suggested that remodeling of the ECM is required during ductal branching morphogenesis (Bernfield et al., 1984; Hardman and Spooner, 1992). Exogenous TGF- β may act to inhibit T-induced ductal branching morphogenesis by inappropriately regulating ECM components, resulting in accumulation of the ECM and inhibition of ductal branching morphogenesis. Preliminary studies by Simon Hayward in the laboratory examining the histology of SVs or APs grown with T 10^{-8} M plus or minus TGF- β suggest that there was an increased or precocious differentiation of the mesenchymal cells into smooth muscle following TGF- β treatment. This could profoundly affect the pattern of ductal branching at low concentrations of TGF- β and inhibit ductal branching at higher concentrations. Further investigation is needed to elucidate how TGF- β inhibits T-induced ductal branching in the developing prostate.

The highly orchestrated process of ductal branching morphogenesis requires both stimulation and inhibition of branching to achieve proper ductal patterning. Endogenous TGF- β may be important in local inhibition of branching in both the clefts and stalks of the developing gland by regulating ECM deposition and degradation. Northern analysis by Timme and collaborators were able to detect TGF- β 1, - β 2 and - β 3 expression in the developing prostate, yet the authors were only able to detect TGF- β 1 expression in the intact adult prostate (Timme et al., 1994). Further studies are needed to determine the pattern of TGF- β expression in the developing prostate, its relationship to focal areas of proliferative activity and branching morphogenesis, as well as, its hormonal regulation.

EGF and TGF- α have been implicated in branching morphogenesis of the developing salivary gland, lung and mammary gland and are also known mitogens for prostatic epithelial cells (Hofer et al., 1991; McKeehan et al., 1984; Peehl and Stamey, 1986; Sherwood et al., 1992). Salivary gland epithelium grown in Matrigel undergoes ductal branching morphogenesis in the absence of mesenchyme when cultured with either

EGF or TGF- α (Nogawa and Takahashi, 1991). However, using the same culture system EGF cannot replace the mesenchyme and induce ductal branching in lung epithelium grown in Matrigel (Nogawa and Takaaki, 1995). These studies suggest that while the underlying process of ductal branching morphogenesis may be the same in various organ systems, the signaling molecules involved in branching may be different. In the present studies, EGF and TGF- α were not able to induce ductal branching morphogenesis in the intact AP in the absence of T. Furthermore, EGF and TGF- α inhibited or distorted ductal branching morphogenesis in the presence of T. The present results suggest that while the developing prostate can respond to exogenous EGF and TGF- α , this is not sufficient to induce and coordinate ductal branching morphogenesis in the absence of T.

In the experiments presented here exogenous EGF and TGF- α distorted and inhibited the pattern of ductal branching in the developing rat prostate grown in the presence of T as assayed by epithelial node number, inverse form factor and epithelial perimeter. The similarity in the results obtained with TGF- α and EGF are not surprising since both act through the EGFR. Recent studies demonstrate the presence of EGFR in the developing rat prostate (A. Thomson and B. A. Foster, unpublished). It is quite likely that both the epithelium and mesenchyme of neonatal rat prostates express EGFR. Both EGF and TGF- α have also been demonstrated to influence embryonic lung development. In organ explants of embryonic lungs, Ganser et al. reported that exogenous EGF and TGF- α increased explant size but decreased the number of ductal branch points. Furthermore, the authors demonstrated that EGF and TGF- α treatment induced matrix-degrading metalloproteinases activity and that inhibition of branching by EGF and TGF- α was blocked by specific inhibitors of metalloproteinases (TIMP) (Ganser et al., 1991). Thus, these authors suggest that EGF and TGF- α act in lung development by inducing the expression of ECM-degrading enzymes.

Development of the prostate involves non-uniform proliferation of the epithelium during ductal branching morphogenesis. The epithelium has a higher rate of proliferation at

the ductal tips than in the proximal ducts (Prins and Birch, 1995; Sugimura et al., 1986). The distortion of T-induced ductal branching produced by exogenous EGF and TGF- α could be due to global stimulation of cell proliferation, resulting in the thicker ducts observed in these studies. Alternatively, EGF and TGF- α may be globally regulating the activity of ECM degrading enzymes, thus distorting finely tuned focal regulation and having dramatic effects on the pattern of ductal branching. Further studies are needed to determine the mechanism by which EGF and TGF- α distort and inhibit ductal branching morphogenesis in the developing prostate. While the present studies suggest that the developing prostate can respond to exogenous EGF, these studies do not indicate if EGF is actually involved in development of the prostate *in vivo*. RT-PCR analysis indicates that the UGS of male and female mice do not express EGF (B. A. Foster, unpublished). Thus, it is unlikely that EGF is expressed in the prostate of the newborn rodent. Further studies are needed to determine if EGF is expressed during prostatic development and to elucidate its role in prostatic ductal branching morphogenesis.

The present studies indicate that TGF- α is expressed and is an important molecule in the development of the rat prostate. Branching morphogenesis was inhibited when the activity of endogenous TGF- α was inhibited with neutralizing antisera. This inhibition was reversible and not due to toxicity. TGF- α is a mitogen for prostatic epithelial cells and has been reported in rat prostatic epithelial cells (28 days to adulthood) (Hofer et al., 1991; Sherwood et al., 1992; Taylor and Ramsdell, 1993). TGF- α may be involved in epithelial cell proliferation and ductal outgrowth during prostatic development. Local activity of TGF- α could account for the non-uniform pattern of proliferation reported in the developing prostate, in which proliferation is greatest at the ductal tips (Prins and Birch, 1995; Sugimura et al., 1986). The present experiments indicate that exogenous TGF- α cannot induce ductal branching in the absence of androgen. Moreover, like EGF, exogenous TGF- α distorted the pattern of ductal branching in the presence of T. As suggested above, the distortion of the ductal branching pattern could be due to global

stimulation of proliferation throughout the developing gland, resulting in the thickened ducts throughout. Alternatively, TGF- α could be affecting the activity of metalloproteinases as discussed above.

Taylor and collaborators (1993) detected the mature form of TGF- α by immunocytochemistry in VP from as early as 28 days post-partum and up to 9 weeks of age. However, TGF- α was not detected in prostate glands from 10 to 21 day old rats. Thus, Taylor et al. could not detect TGF- α expression until well into the pubertal phase of prostatic development. Therefore, these authors suggest that TGF- α is not the primary growth stimulatory factor in the developing prostate. Rather, they suggest that TGF- α may function to promote differentiation, not proliferation, of prostatic epithelial cells (Taylor and Ramsdell, 1993). Problems with this interpretation are 2-fold: 1.) Proliferative activity in the prostate is high both before and after 28 days. 2.) Prostatic epithelial differentiation and initial secretion of prostatic specific proteins begins well before 28 days (Erin Lopez, unpublished data).

In the present studies TGF- α was detected by RT-PCR in newborn rat APs and VPs after 6 days of culture both in the presence and absence of T. RNase protection assays confirmed that TGF- α and its receptor, EGFR, were expressed in APs and VPs grown in both the presence and absence of T and that TGF- α mRNA expression was not regulated by T in the developing prostate. The apparent discrepancy in results between the present studies and those of Taylor et al. is probably due to the differences in detection methods. The antibody used by Taylor et al. recognizes only the mature form of the TGF- α protein, while the highly sensitive methods used in the present studies detect TGF- α messenger RNA. The failure to detect TGF- α protein by Taylor et al. is not due to a regulatory step in the translation of the messenger RNA into protein, because the present studies also showed that blocking the activity of endogenous TGF- α with neutralizing antisera inhibited ductal branching morphogenesis. The results presented here indicate that

TGF- α is expressed and is functional during the early postnatal period of prostatic development and plays an important role in the development of the rat prostate.

The present studies also indicate that TGF- α is important in FGF-7/KGF-induced development of the rat VP. TGF- α has been implicated in mediating FGF-7/KGF-induced mitogenic response of keratinocytes (Dlugosz et al., 1994). In these studies TGF- α mRNA expression and secretion increased in keratinocytes treated with FGF-7/KGF. Preincubation of conditioned medium obtained from keratinocytes treated with FGF-7/KGF with neutralizing antibodies to both FGF-7/KGF and TGF- α blocked cell growth, while preincubation with FGF-7/KGF antibody alone did not affect cell growth. The authors concluded that the predominant mitogen in conditioned medium from keratinocytes grown in the presence of FGF-7/KGF is TGF- α , not FGF-7/KGF, and that TGF- α is a downstream effector of FGF-7/KGF action. The present studies support such a role for TGF- α as a mediator of FGF-7/KGF effects in the developing rat VP.

FGF family members have been implicated in the development of a diverse number of biological systems (Mason, 1994). These include mesoderm induction in *Xenopus* (Slack, 1994), early embryogenesis and body axis patterning in mammals (Orr-Urtreger et al., 1993), spatial patterning in the developing central nervous system (Crossley and Martin, 1995; Slack, 1994) and limb (Fallon et al., 1994; Niswander et al., 1993; Vogel and Tickle, 1993; Vogel et al., 1990), development of the respiratory systems of both mammals and arthropods (Klambt et al., 1992; Mason et al., 1994; Reichman-Fried et al., 1994), as well as, mesenchymal-epithelial interactions in developing glands (Mason et al., 1994). Presently nine members of the FGF family, encoded by nine different genes, and four FGFR genes have been identified. A wide range of diversity can be generated among the FGF ligands and receptors through the use of alternative initiation codons for translation, alternative splicing and post-translational modification such as glycosylation, methylation, phosphorylation, proteolytic cleavage and dimerization (Mason, 1994). The cellular response elicited by members of the FGF family could depend in part on the

differentiated state of the cell. The differentiated state of the cell would determine which "flavor" of FGFR the cell expresses and, in turn, the internal signaling pathway available for receptor activation. Therefore, in order to understand the functional role of FGFs in a biological system, it is not sufficient to examine just the expression of the FGF ligand and receptor. The following must also be determined: 1.) the exact splice variant of FGF ligand, 2.) the precise splice variant(s) of the FGF receptor, including dominant negative forms of the receptors, and 3.) the internal signaling pathways with which the FGFR may be interacting with intracellular machinery.

Several members of the FGF family have been implicated in the development of the male reproductive tract. FGF-2/bFGF (basic FGF) is involved in the development of the epididymis. A neutralizing antibody to FGF-2/bFGF inhibited development of the epididymis, but did not effect prostatic development (Alarid et al., 1991). FGF-7/KGF has been implicated in androgen-dependent development of the seminal vesicle (SV). A neutralizing antibody to FGF-7/KGF inhibited T-induced ductal branching morphogenesis of the developing SV. Moreover, FGF-7/KGF partially replaces T and induces SV growth (Alarid et al., 1994).

The present studies indicate that FGF-7/KGF is an important mediator of T-induced ductal branching morphogenesis in the developing rat VP. FGF-7/KGF was expressed in the developing VP as assayed by both RT-PCR and RNase protection assays and blocking of FGF-7/KGF activity with a neutralizing antibody or peptide inhibited T-induced ductal branching morphogenesis of the VP. Moreover, FGF-7/KGF induced development of the VP in the absence T as assayed by ductal branching morphogenesis and growth. Surprisingly, FGF-7/KGF did not seem to be involved in the branching morphogenesis of the closely related AP. FGF-7/KGF in the absence of T did not induce ductal branching in the developing rat AP, even though at high concentrations FGF-7/KGF induced growth of the AP as indicated by increased epithelial area and distortion of the normal pattern of T-induced ductal branching of the AP. Molecular analysis indicated that the developing rat

AP expressed both FGF-7/KGF and its receptor, FGFR2-IIIb. This raises an intriguing question: What is the difference in the signaling mechanism for FGF-7/KGF through its receptor FGFR2-IIIb (KGFR) in the developing rat VP versus the AP? One possibility is that, while both glands express the splice variant of FGFR2 containing the IIIb exon, the functionality of the receptor in the different lobes of the rat prostate may not be equivalent. Johnson and collaborators have reported a possible secreted form of FGFR1 which contains the extracellular IgG-domains involved in ligand binding, but lacks the transmembrane and intracellular domains involved in signal transduction. While such a form has not been reported for FGFR2, the conserved structural arrangement found between FGFR1 and FGFR2 genes suggests that such a form exists for FGFR2 (Johnson et al., 1991). The function of the secreted form of FGFR remains to be elucidated. It has been proposed that the secreted FGFR protein may act as an extracellular reservoir of FGF. Alternatively, the secreted form of the receptor may act as a dominant negative receptor by preventing FGF from interacting with membrane-spanning signal transducing FGFR.

Yan and collaborators have recently reported an alternatively spliced variant of FGFR2 which has an unique COOH-terminus that lacks an autophosphorylation site (tyrosine 789) required for interaction with phospholipase C γ 1 due to deletion of exon 16 (Yan et al., 1993b). This splice variant is expressed in normal adult prostatic tissue. The authors suggest that the two COOH-terminal splice variants may signal through different signal transduction pathways. The authors also suggest that the two COOH-terminal isoforms of FGFR2 may explain the dual role of FGF-7/KGF in cell growth and differentiation. However, the authors did not determine whether the alternatively spliced form of the FGFR2 lacking tyrosine 789 included exon IIIb which defines FGFR2 as the receptor for FGF-7/KGF.

The AP and VP offer an unique system to study the differences in signaling pathways for FGFs. Both AP and VP express FGF-7/KGF and the FGFR2 splice variant containing exon IIIb (KGFR). However, the biological response to FGF-7/KGF is not

equivalent in these glands. Further experiments are needed to determine the complete structure of the FGFRs expressed in the developing AP and VP. It is an intriguing possibility that the AP may produce a truncated form of the FGFR2 gene that acts as a dominant negative receptor, while the VP may express full length FGFR which is capable of transducing a signal into the cell. Targeted expression of a dominant negative FGFR2 to the developing lung blocks ductal branching morphogenesis and epithelial differentiation (Peters et al., 1994). Thus, a truncated form of the FGFR2 lacking the internal signaling domain can act as a dominant negative receptor and block FGF function. However, it remains to be proven if this is a mechanism of action for FGFR2 *in vivo*. Alternatively, the COOH-terminus of FGFR2 may be different in the AP and VP, such that FGFR2 signals through different transduction pathways in these two glands. Further experiments are needed to elucidate the difference in FGFR2 signaling mechanism between VP versus AP.

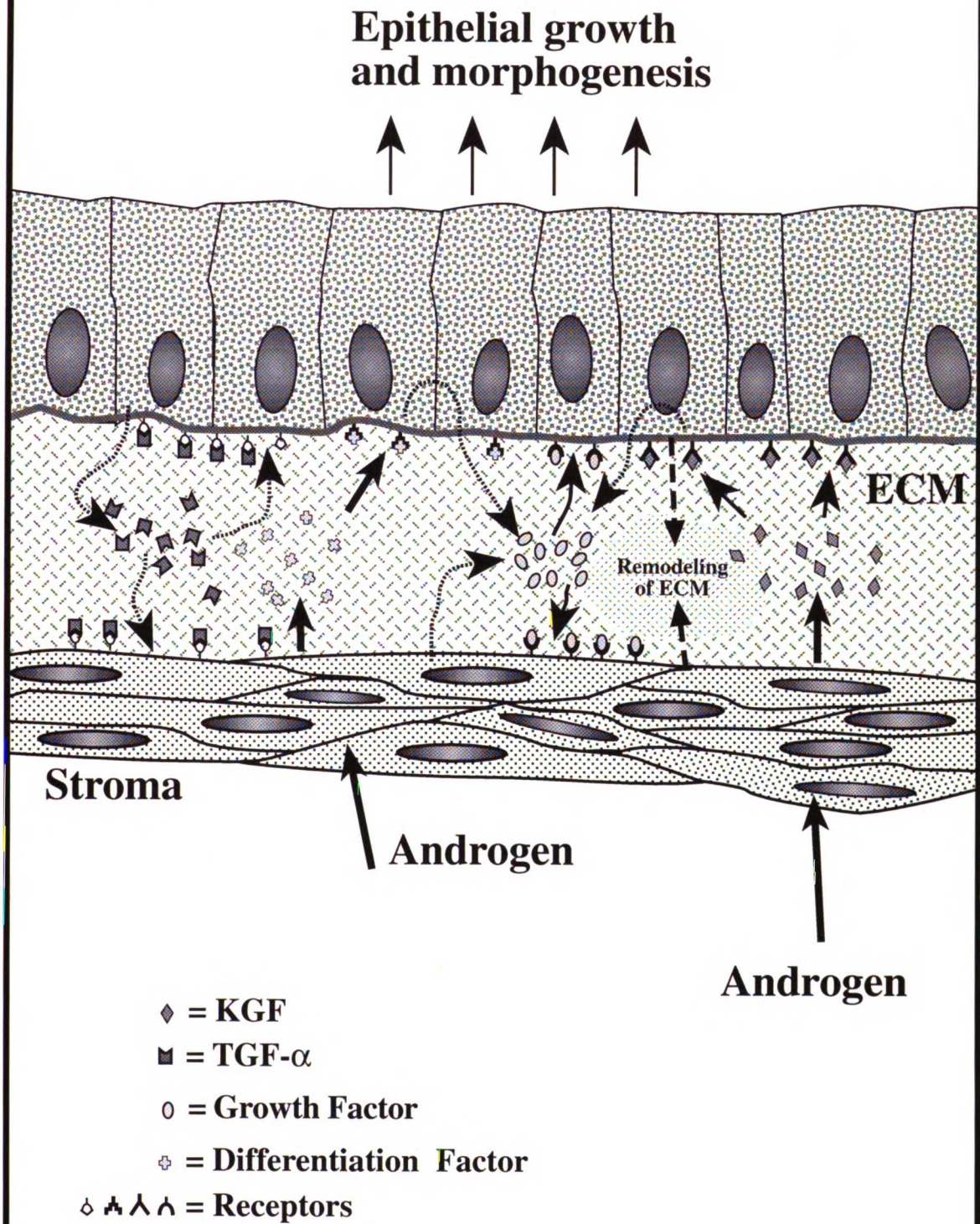
The present studies indicate that FGF-7/KGF is important in the development of the rat VP. However, in the developing rat AP FGF-7/KGF does not appear to play a role. The morphogenetic effects of two other members of the FGF family, FGF-1/aFGF and FGF-2/bFGF, were assayed in the developing rat AP. FGF-2/bFGF did not affect ductal branching morphogenesis as assayed by epithelial area, epithelial perimeter, inverse form factor and node number, but FGF-2/bFGF appeared to increase the mesenchymal compartment of the AP (Fig. 33). FGF-2/bFGF is produced by and is a mitogen for prostatic stromal cells (Story, 1991; Story et al., 1989). Thus, exogenous FGF-2/bFGF may stimulate growth of mesenchymal cells, but not affect ductal branching morphogenesis of the epithelium. Indeed, FGF-1/aFGF did not affect the ductal branching morphogenesis of the developing AP. This is in contrast to dramatic effects of FGF-1/aFGF on ductal branching morphogenesis of the developing lung. When embryonic lung epithelium was grown in Matrigel FGF-1/aFGF elicited epithelial growth and ductal branching morphogenesis even though the pattern of branching morphogenesis was abnormal (Nogawa and Takaaki, 1995). An antibody to FGF-1/aFGF completely blocked bronchial

epithelial growth and branching induced by FGF-1/aFGF. Surprisingly, the effective dose of FGF-1/aFGF on lung epithelium was 500 ng/ml. In the experiments presented here FGF-1/aFGF and FGF-2/bFGF were used at concentrations of 3 or 30 ng/ml. Thus, the concentrations of FGF-1/aFGF and FGF-2/bFGF used here may not have been high enough to elicit an effect. Further studies are needed to determine if other members of the FGF family are involved in the development of the AP or the VP.

Model of Prostate Development (Figure 48)

Androgens act as a hormonal signal to induce masculine development of the reproductive tract. At birth, circulating levels of T in the rat are sufficient to induce ductal branching morphogenesis in the developing prostate. Thus, it is not necessary to invoke a mechanism by which T is metabolized to DHT in order to amplify the androgenic signal of T. Furthermore, T and the synthetic androgens MENT and R1881, which cannot be 5α -reduced to form DHT, are as effective as DHT in supporting prostatic development. Androgens bind the androgen receptor and act as transcriptional activators to elicit a program of differentiation. Part of the program of development for the prostate includes the cascade of events which results in ductal branching morphogenesis. The mesenchyme mediates the effects of androgens on the epithelium and directs the specific patterns of ductal branching morphogenesis. Growth factors are likely to be involved in the epithelial-mesenchymal interactions which result in prostatic development. FGFs may be involved in the patterning of the different lobes of the rat prostate. The data presented here suggests that FGF-7/KGF is involved in the development of the 0 day rat VP, but not the AP. However, other members of the FGF family may be involved in development of the AP. TGF- α is an important mediator of prostatic development and is likely to be involved in proliferation during ductal branching morphogenesis and possibly regulation of ECM degradation. TGF- β may act as a brake during branching morphogenesis by regulating ECM remodeling resulting in stabilization of clefts. Further investigation is needed to determine how androgens initiate and regulate these complex interactions.

Figure 48



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1872. The first of the year was a very dry one, and the crops were much injured. The weather was very hot, and the crops were much injured. The weather was very hot, and the crops were much injured.

The second of the year was a very wet one, and the crops were much injured. The weather was very cold, and the crops were much injured. The weather was very cold, and the crops were much injured.

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