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Exon Switching and Activation of Stromal and Embryonic Fibroblast Growth Factor (FGF)-FGF Receptor Genes in Prostate Epithelial Cells Accompany Stromal Independence and Malignancy

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Stroma and the heparin-binding fibroblast growth factor (FGF) family influence normal epithelial cell growth and differentiation in embryonic and adult tissues. The role of stromal cells and the expression of isoforms of the FGF ligand and receptor family were examined during malignant progression of epithelial cells from a differentiated, slowly growing, nonmalignant model rat prostate tumor. In syngeneic hosts, a mixture of stromal and epithelial cells resulted in nonmalignant tumors which were differentiated and slowly growing. In the absence of the stromal cells, epithelial cells progressed to malignant tumors which were independent of the stroma and undifferentiated. The independence of the malignant epithelial cells from stromal cells was accompanied by a switch from exclusive expression of exon IIIb to exclusive expression of exon IIIc in the FGF receptor 2 (FGF-R2) gene. The FGF-R2(IIIb) isoform displays high affinity for stromal cell-derived FGF-7, whereas the FGF-R2(IIIc) isoform does not recognize FGF-7 but has high affinity for the FGF-2 member of the FGF ligand family. The switch from expression of exclusively exon IIIb to exclusively exon IIIc in the resident FGF-R2 gene was followed by activation of the FGF-2 ligand gene, the normally stromal cell FGF-R1 gene, and embryonic FGF-3 and FGF-5 ligand genes in malignant epithelial cells. Multiple autocrine and potentially intracrine ligand-receptor loops resulting from these alterations within the FGF–FGF-R family may underlie the autonomy of malignant tumor cells.

The development of prostate tumors is age related and believed to progress by a series of genetic changes and selection of cells with increasing malignant character. Most marked is the shift from relatively slowly growing, nonmetastatic, androgen-sensitive tumors to a rapidly growing, androgen-independent, highly malignant stage (15). The androgen-sensitive tumors are subject to antiandrogen therapies which can result in regression of the tumor, but frequently appearance of a highly malignant tumor that is resistant to treatment follows such therapies. A lack of knowledge of mechanisms underlying the appearance of the malignant tumors has hampered design of strategies for prediction and prevention of their appearance as well as intervention after they appear. The epithelial cell compartment of slowly growing, androgen-sensitive tumors usually exhibits some degree of morphological differentiation that distinguishes it from the stromal compartment. In contrast, malignant tumors are undifferentiated and exhibit no apparent relationship between epithelial and stromal cells (15). Mesenchyme plays an active role in growth and differentiation of the epithelium during prostate development (4, 6), and the stroma has been implicated in maintenance of adult epithelium (5, 9). However, the role of the stroma in development and progression of tumors has received less attention. Advances in isolation and maintenance of specific prostate cell types in vitro and the identification of purified polypeptide regulators that act directly on them has renewed optimism for understanding the cellular and molecular basis of the progression of prostate tumors (25, 27–30, 39). Isolated epithelial and stromal cells from both normal prostate tissue and slowly growing, androgen-sensitive tumors are insensitive to androgen, yet they are responsive to multiple polypeptide regulators (30). The paradox can be explained by an androgen-sensitive and directionally specific communication system from stromal to epithelial cells that supports growth and differentiation of epithelial cells (48). A candidate for one of the andromedic polypeptides is heparin-binding fibroblast growth factor type 7 (FGF-7), whose expression is limited to stromal cells and is androgen sensitive (8, 21, 48). Stromal cells display no receptor for FGF-7, while epithelial cells exhibit a specific receptor for the stroma-derived ligand (21, 31, 32, 48). The specificity of the epithelial cell receptor for FGF-7 arises by exclusive splicing of one of three alternate exons coding for the COOH terminus of the extracellular domain of the FGF receptor 2 (FGF-R2) gene (18, 21, 31).

To better understand the role of stromal cells during progression of epithelial cells to malignancy, epithelial and stromal cells from an androgen-responsive, differentiated, slowly growing, transplantable rat prostate tumor (Dunning R3327PAP, hereafter called the DT tumor) were isolated (25, 30) and transplanted into male hosts. The resulting tumors were compared with the parent DT tumor and an androgen-independent, malignant derivative (Dunning R3327AT3, hereafter called the AT3 tumor) that arises from DT tumors after castration and serial passage through castrated and female hosts (15). In the absence of stromal cells, the DT tumor-derived epithelial cells progressed to fast-growing,

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undifferentiated, malignant tumors (called E tumors for epithelial cell derived) similar to tumors that arise from the parent DT tumor after castration and serial passage in vivo. However, implantation of a mixture of DT tumor-derived epithelial and stromal cells resulted in tumors (called SE tumors for stromal plus epithelial) composed of well-differentiated epithelial and stromal compartments with growth properties similar to those of the parent DT tumor. No tumors arose from stromal cells. A switch from exclusive expression of the FGF-R2(IIIb) isoform to exclusive expression of the FGF-R2(IIIc) isoform of the epithelial cell receptor gene occurred concurrently with the progression of epithelial cells to the stromal cell-independent, malignant state. The FGF-R2(IIIc) isoform does not recognize FGF-7 but has a high affinity for FGF-2 and potentially for other FGF ligands (8, 17, 18, 31, 32, 48). Activation of the FGF-2 gene, the embryonic FGF-3 and FGF-5 genes, and the stromal cell FGF-R1 gene also occurred in the malignant epithelial cells.

MATERIALS AND METHODS

Prostate tissues and cells. Normal rat ventral or dorsal prostate DT and AT3 tumor tissues were obtained from 8- to 15-week-old Copenhagen male rats (Harlan Sprague-Dawley, Inc.) as previously described (25). Cultures of epithelial and stromal cells from the DT tumor and the single-cell type from the AT3 tumor were prepared and maintained as previously described (25, 29, 30). Periodically, subcultures were prepared from single-cell clones of the DT and AT3 tumor-derived cells to ensure a homogenous cell population exhibiting the FGF and FGF-R expression pattern characteristic of primary or low-passage cultures. DT tumor-derived stromal and AT3 tumor cells were maintained in RITC medium (21) supplemented with 5% fetal bovine serum. Primary and serial cultures of E tumor cells and epithelial cells from the reconstituted SE tumors were established by the same methods described for the DT tumor epithelial cells, except that recombinant rat FGF-7 was substituted for the pituitary or hypothalamic extracts (29, 30). Primary and serial cultures from the E tumors were prepared by methods described for the AT3 tumors.

Tumor cell implantation and histochemical analysis. Cultured cell types indicated in the text were harvested by trypsinization, collected by centrifugation at 100 × *g*, resuspended in 1 ml of phosphate-buffered saline (PBS; pH 7.2), and injected subcutaneously into the flank of 8- to 15-week-old Copenhagen male rats with 18-gauge needles. Animals were observed continuously for appearance and progress of tumors for the periods indicated in the text. For histochemical analysis, tumors were excised, fixed in 4% formaldehyde in PBS, and embedded in paraffin. Tissues were sectioned and stained with hematoxylin and eosin.

PCR analysis of FGF and FGF-R. Poly(A)$^+$ RNA was isolated as previously described (25). Single-strand cDNA was prepared from poly(A)$^+$ RNA (10 μg) with the polymerase chain reaction (PCR) reverse transcription kit from InVitrogen, recovered by precipitation with ethanol, and dissolved in 100 μl of water. A 1-μl aliquot of the solution was used as a template in the standard 100-μl reaction mixture from the Perkin-Elmer Cetus PCR kit. Reaction mixtures were optimized for each FGF in regard to specificity and sensitivity. The addition of 0.1 mM tetramethylammonium chloride enhanced the analysis of FGF-3 and FGF-7, and 10% dimethyl sulfoxide enhanced analysis of FGF-5. After 40 cycles for 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C, 25 μl of each reaction was analyzed on 1.5% agarose gels with ethidium bromide. The following 5′ and 3′ primers, respectively, were used for analysis of FGF genes (nucleotide sequences within the FGF genes are in capital letters): FGF-1 (542 bp), cgagaattCAAAGAACCAGCATC TGACCTGTC and gtggaattcCTTAGTCAGAAGATACCGG GAG; FGF-2 (465 bp), GCTTAAAGAGAGTCAGCTCTTA GCAGA and AGCATCACTTCGCTTCCCGCACTGC; FG F-3 (444 bp), CATGAACAAGAGAGGACGGCTGTATGC TT and CAGGCCACCAGTCCACCTGTTA; FGF-4 (339 bp), GACTACGTGCTGGGCATCAAGCGGCTG and TCA CAGCCTGGGGAGGAAGTGGGTGACCTT; FGF-5 (435 bp), GCTGTGTCTCAGGGGATTGTAGGAATA and TAT CCAAAGCGAAACTTGAGTCTGTA; and FGF-7 (693 bp), AATCTACAATTCACAGATAGGA and TTAAGTTATTG CCATAGGAAGAAAGTG.

Northern (RNA) blot hybridization. Poly(A)$^+$ RNA (5 μg) was separated on 1.5% agarose gels containing 1.2% formaldehyde, transferred to nitrocellulose paper, prehybridized, and hybridized as previously described (25). Ethidium bromide was included in each sample to ensure that RNA loads were equal prior to blotting. cDNA probes were labeled to a specificity of 4×10^9 cpm/μg with a random primer DNA labeling kit from Boehringer-Mannheim.

Nuclease protection analysis. Nuclease protection analysis of rat FGF-7 (49), FGF-R1, FGF-R2, and β-actin was performed as previously described (21, 48). To distinguish the FGF-R2 exon IIIb and IIIc variants, a 178-bp *Alu*I-*Alu*I restriction fragment coding for 159 bp of common FGF-R2 sequence upstream of the 5′ splice site and 19 bp of exon IIIb was cloned into the *Eco*RV cloning site of pBluescript SK vector and the antisense RNA probe was generated with the T7 promoter. To determine the expression of FGF-R1, a 360-base antisense probe was synthesized from an *Eco*RI-*Rsa*I fragment (50). Total RNA in the protection assays was standardized by comparison with β-actin. Protection probes (10^5 cpm) were hybridized with 20 μg of total cellular RNA or yeast tRNA overnight at 50°C in 50% formamide, and the samples were digested with RNases T_1 and A for 30 min at 30°C and analyzed on 5% acrylamide DNA sequencing gels. Sizes of protected fragments were estimated from a parallel run of a DNA sequencing reaction.

RESULTS

Stromal cells promote differentiation and delay malignant progression of nonmalignant tumor epithelial cells. Epithelial and stromal cells from the transplantable model rat prostate DT tumor were isolated and characterized as previously described (25, 28–30). Epithelial cells implanted subcutaneously in the flank of syngenic Copenhagen male rats in absence of stromal cells gave rise to E tumors with wet weights ranging from 20 to over 100 g after 7 to 10 months (Table 1; Fig. 1A). In contrast, when mixtures of the epithelial and stromal cells at the 70:30 ratio in the parent DT tumors (15) were implanted, significantly smaller SE tumors appeared at the injection site at a rate similar to that of 1- to 3-mm^3 pieces of the transplantable parent DT tumor (Table 1; Fig. 1B). No reconstituted SE tumors exceeded 10 g even after an additional 2 to 3 months. No tumors arose from stromal cells alone (Fig. 1C) over a 2-year period. Histochemical analysis revealed that the slowly growing SE tumors exhibited distinct epithelial and stromal cell compartments similar to those of the parent DT tumors. In contrast to the parent DT tumor, which exhibits predominantly a gland-like morphology (Fig. 2a and b) (15) and only rare foci

TABLE 1. Effects of stromal cells on malignant progression of epithelial cells[a]

Rat host	Tumor wt (g)			
	E	SE	E°	SE°
1	117.50	7.80	109.21	3.10
2	26.90	5.71	64.54	0.28
3	40.95	1.89	78.73	0.91
4	70.10	1.57	81.46	0.53
5	69.92	1.30	55.75	2.13
6	93.15	0.67	47.88	1.49
Mean ± SD	69.75 ± 30.26	3.16 ± 2.64	72.92 ± 20.06	1.40 ± 0.97

[a] Primary tumors were established by subcutaneous implantation of 1×10^7 epithelial cells alone (E tumors) or the same number of epithelial cells with 3×10^6 stromal cells (SE tumors) into six host animals numbered 1 to 6. Tumors were excised and weighed after 10 months. Secondary SE and E tumors (SE° and E°) were established from 1- to 5-mm^3 pieces of primary tumor tissue. Secondary tumors were excised and weighed 70 days after implantation. The mean and standard deviation among the six individual hosts in each group are indicated.

of squamous-type structures (Fig. 2c and d), the SE tumor consisted of intensely keratinized squamous-type structures with only a remnant of glandular organization (Fig. 2e and f). In addition, the SE tumors exhibited rare foci of morphologically disorganized, undifferentiated epithelial cells (Fig. 2g and h) which were not observed in the parent DT tumors. In contrast to the SE tumors, the E tumors resulting from the DT tumor-derived epithelial cells exhibited an undifferentiated sarcomatous morphology interspersed with areas of necrosis (Fig. 2i and j). Although secondary SE tumors of 0.2 to 4 g developed upon transplantation (2 to 5 mm^3) within 2 to 3 months (Table 1), the transplanted SE tumors maintained the differentiated morphological characteristics similar to those of the primary SE tumors shown in Fig. 1. As did the parent DT tumors, both primary and secondary SE tumors continued to grow slowly, reaching wet weights after

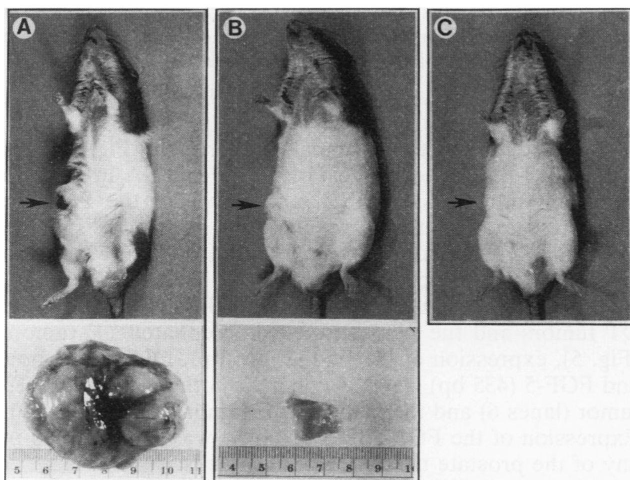

FIG. 1. Reconstituted tumors from differentiated, nonmalignant DT tumor epithelial and stromal cells. Cultured DT tumor-derived epithelial cells (1×10^7) (A), a mixture of 1×10^7 epithelial with 3×10^6 stromal cells (B), or 3×10^6 stromal cells alone (C) were implanted at the indicated site in six male Copenhagen rats per group (Table 1). Rats were sacrificed at 10 months, and the tumors were excised, weighed, and used for the analyses described in the text.

1 year equal to that of the host, but without obvious effect beyond ulceration at the site of injection. In contrast, secondary tumors of 50 to over 100 g developed upon transplantation of the primary E tumors within 1 to 2 months (Table 1) and killed the hosts after 2 months. Histochemical examination of the secondary E tumors revealed the relatively homogeneous undifferentiated, anaplastic characteristics of undifferentiated, highly malignant tumors (Fig. 2k and l) which arise from DT tumors in castrated male hosts and serial passage through castrated or female hosts (15). In contrast to the parent DT and the SE tumors which remain localized at the subcutaneous injection site, secondary E tumors gave rise to satellite tumors in numerous organs of the hosts, including spleen, liver, and lung (not shown). Appearance of metastatic lesions is also a characteristic of tumors (AT3) that arise from the parent DT tumors after extensive serial passage of the tumors in castrated or female hosts in vivo (16). Cells (SE-E) from the reconstituted SE tumors were morphologically similar (Fig. 3A) and exhibited a pattern of expression of keratin (Fig. 3B) similar to that of the parent DT tumor-derived epithelial cells (DT-E), while the E tumor cells (E-C) were similar to cells from the malignant AT3 tumors (AT3-C) that arise from passage of DT tumor tissue in castrated hosts in vivo.

To determine whether stromal cells could reverse the growth rate and malignancy of E tumor-derived cells, we implanted primary E tumor cells with the stromal cells in a 70:30 ratio. Results in Table 2 indicate that the stromal cells had no effect on size of tumors that developed from the E tumor cells. Separate experiments also revealed that the DT tumor stromal cells had no effect on growth rate and malignancy of the AT3 tumor cells even when implanted at a ratio of stromal to AT3 cells of up to 100 to 1.

Exon switching in the epithelial cell-specific FGF-R2 gene accompanies stromal independence and malignant progression. The stromal independence of the composite cells and the lack of a clearly defined stromal compartment is a hallmark that distinguished the undifferentiated, malignant E and AT3 tumors from the differentiated normal prostate and nonmalignant DT tumors. Since normal prostate and DT tumor tissue as well as DT tumor-derived epithelial cells express exclusively the stromal cell-derived FGF-7-specific FGF-R2(IIIb) splice variant (48) (Fig. 4, lanes 3 to 6), we examined the relative expression of the exon III variants of the FGF-R2 gene in the reconstituted SE and E tumors and derived cells. Nuclease protection analysis with a probe spanning 159 bp common to both FGF-R2(IIIb) and FGF-R2(IIIc) variants and 19 bp specific to exon IIIb indicated that, while both exons IIIb and IIIc were expressed in the reconstituted SE tumors (lane 8), exon IIIc was expressed exclusively in the malignant E tumors (lanes 9 and 10). As did the E tumors, the AT3 tumor (lane 7) also expressed exclusively the exon IIIc variant of FGF-R2. The appearance of FGF-R2(IIIc) correlates with the appearance of foci of undifferentiated epithelial cells in the reconstituted SE tumors (Fig. 2g and h). However, a complete switch from expression of the stromal cell-derived FGF-7-specific exon IIIb to exclusively FGF-2-specific exon IIIc in the FGF-R2 gene occurs in the stroma-independent, completely undifferentiated malignant E and AT3 tumors.

Activation of FGF-2 and embryonic FGF-3 and FGF-5 ligand genes in epithelial cells accompanies progression to malignancy. Although the exon switch in the FGF-R2 gene may render epithelial cells independent of stroma-derived FGF-7 and the stroma-derived growth and differentiation response mediated by it, the single alteration is likely insuf-

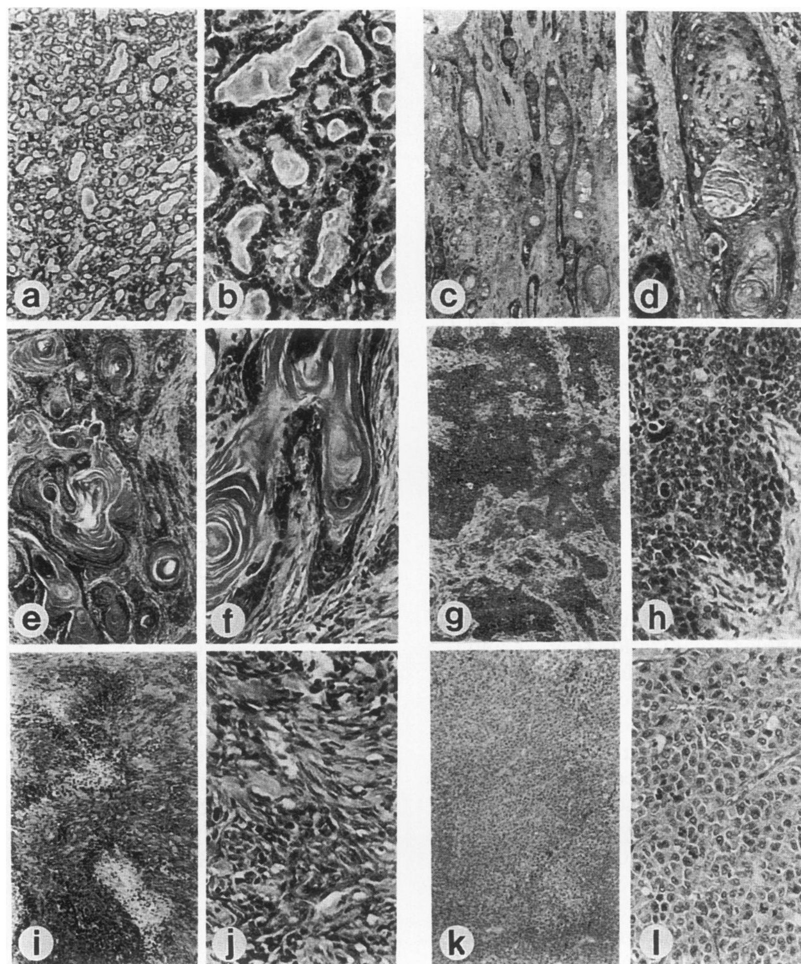

FIG. 2. Histological analysis of tumors. Tumor tissues were fixed, sectioned, and stained with hematoxylin and eosin. Panels a plus b and c plus d are representative of the predominant tubular and rare squamous structures, respectively, that compose the well-differentiated, nonmalignant parent DT tumor. Panels e plus f and g plus h show the predominant intensely keratinized squamous structures and the rare foci of undifferentiated epithelial cells from the reconstituted SE tumors, respectively. Panels i and j show the homogenous morphology of the primary E tumors derived from epithelial cells alone. Panels k and i show the homogenous, undifferentiated morphology of the malignant AT3 tumor. Panels a, c, e, g, i, and k are a ×100 magnification, and panels b, d, f, h, j, and l are a ×400 magnification.

ficient to confer autonomous growth on the epithelial cells without concurrent activation of one or more FGF ligands within the epithelial cells. To determine expression of FGF genes in the prostate tissues and derived cells, we employed a PCR analysis (44) with primers that spanned the coding sequences for FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, and FGF-7 (Fig. 5). FGF-1 (542 bp) was expressed in all tissues and cells examined. Expression of the FGF-7 (693 bp) gene was limited to the stromal cells from normal prostate and the DT tumors (48) (Fig. 5, lanes 4). As an expected consequence of the presence of well-defined stromal compartments, normal prostate tissue (lane 2) and both the differentiated parent DT (lane 3) and the reconstituted SE (lane 7) tumors exhibited strong FGF-7 signals. Nuclease protection analysis confirmed that the expression of FGF-7 correlates with the presence of a well-defined stromal compartment and differentiated state of the tissues (Fig. 6). Although the PCR analysis detected weak FGF-7 signals in the undifferentiated, malignant AT3 and E tumor tissues (Fig. 5, lanes 6 and 8), nuclease protection analysis indicated that the expression of the FGF-7 gene is very low in the undifferentiated tumors which exhibit no distinct stromal compartment (Fig. 6, lanes 7, 9, and 10). The low levels of FGF-7 mRNA in the two malignant tumor tissues that require use of the PCR for detection may reflect a low level of host stromal cells within the tumors rather than activation of the FGF-7 gene in epithelial cells that compose the tumor.

Although silent in normal prostate, differentiated parent DT tumors and the reconstituted differentiated SE tumors (Fig. 5), expression of the FGF-2 (465 bp), FGF-3 (444 bp), and FGF-5 (435 bp) genes is apparent in the malignant AT3 tumor (lanes 6) and the malignant E tumors (lanes 8 and 9). Expression of the FGF-4 (399 bp) gene was not detected in any of the prostate tissues or derived cells.

The stromal FGF-R1 receptor gene is also activated in undifferentiated malignant tumors. Expression of the FGF-R1 gene is normally limited to the nonparenchymal cells from adult tissues (21, 48) and partitions with mesenchymal cells in embryonic development (37, 41). Both normal prostate and the differentiated parent DT tumors and reconstituted SE tumors, which exhibit well-defined stromal compartments, display a strong FGF-R1 signal which is limited

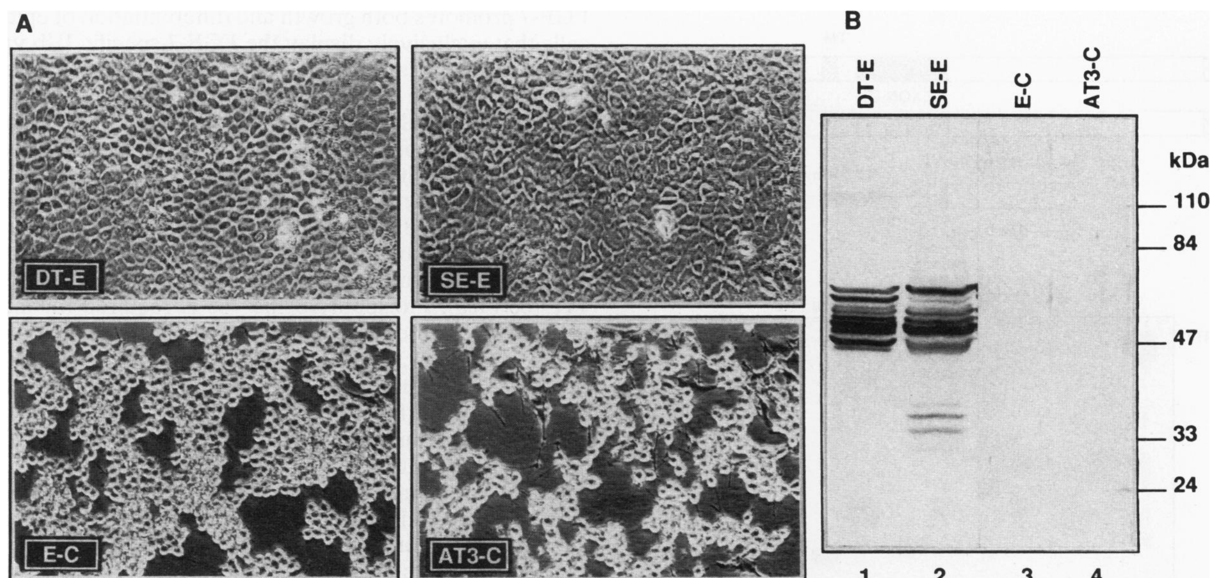

FIG. 3. Morphology and cytokeratin expression of different tumor epithelial cells. (A) Morphology, DT-E, DT tumor epithelial cells (passage 80); SE-E, SE tumor epithelial cells (passage 5); E-C, E tumor cells (passage 5); AT3-C, AT3 tumor cells (passage 5). (B) Cytokeratin expression. The cultured tumor cells in panel A were dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and 25 μg of protein was analyzed by protein immunoblotting with a polyclonal antibody against human heel keratins (25).

to the stromal cells from each of the tissues (48) (Fig. 7A). Failure to detect an FGF-R1 signal by the more sensitive PCR analysis with several rat FGF-R1 paired primers confirmed that the FGF-R1 gene is silent in the epithelial cells derived from both the parent DT and reconstituted SE tumors (data not shown). However, both the undifferentiated, malignant AT3 (Fig. 7A, lane 5) and E (Fig. 7A, lanes 7 and 8) tumors which have no distinct stromal cell compartment express the FGF-R1 gene. Intensity of the FGF-R1 signal increased in secondary E tumors (E°) (Fig. 7A, lane 8). The activation of the FGF-R1 gene in the malignant tumor epithelial cells was further confirmed by RNase protection analysis of cloned cell lines derived from both the AT3 (47, 48) and E tumors (Fig. 7C). The malignant tumor cells continue to express the epithelial cell-specific FGF-R2 gene (Fig. 7B) which is exclusively the exon IIIc isoform described earlier (48) (Fig. 4). These data indicate that progression of the DT-derived epithelial cells to the stroma-independent, malignant state also coincides with activation of the normally stromal cell FGF-R1 gene.

TABLE 2. Stromal independence of E tumor epithelial cells[a]

Rat host	Tumor wt (g)	
	E tumor cells	E tumor cells + stromal cells
1	15.72	20.19
2	19.47	22.69
3	14.54	18.60
4	14.48	13.64
5	18.23	15.53
6	19.17	16.07
Mean ± SD	17.00 ± 2.02	19.28 ± 5.26

[a] Cultured E tumor-derived cells (1×10^7) were implanted alone or with DT tumor-derived stromal cells (3×10^6) into six individual hosts. Tumors were excised and weighed after 15 days.

DISCUSSION

After castration of male hosts or passage through female hosts, slowly growing, androgen-responsive, nonmalignant rat prostate tumors (type I) which are composed of differentiated epithelial and stromal cell compartments progress into rapidly growing, androgen-independent, highly malignant tumors (type II) which are composed of a relatively homogenous undifferentiated cell type (15). Here we show that isolated and cloned epithelial cells from type I tumors progress to type II tumors in syngeneic hosts. In contrast, type I tumor stromal cells do not give rise to tumors, nor do they survive in the same hosts. However, when implanted together with epithelial cells, the stromal cell population expanded together with the epithelial cells to result in type I tumors with well-differentiated epithelial and stromal cell compartments. These results suggest that (i) stromal cell growth and survival in vivo depends on the epithelial cells, (ii) stromal cells are the source of an inducer of epithelial cell differentiation, and (iii) a key step in the progression of type I tumor epithelial cells to the type II phenotype may be the loss of sensitivity to a stroma-derived inducer of terminal differentiation. A switch from exclusive expression of alternate exon IIIb to exon IIIc in the epithelial cell-specific FGF-R2 gene; activation of FGF-2, FGF-3, and FGF-5 genes; and activation of the stromal cell-specific FGF-R1 gene accompany progression of the epithelial cells to malignancy. None of the changes associated with the type II tumor cells have been observed in type I tumor-derived epithelial cells in vitro even in cells that have been extensively cultured. This suggests that host factors support progression of the epithelial cells to the type II state. The facts that type II tumors can arise from cultures of type I tumor epithelial cells in which the FGF-R2(IIIc), FGF-2,

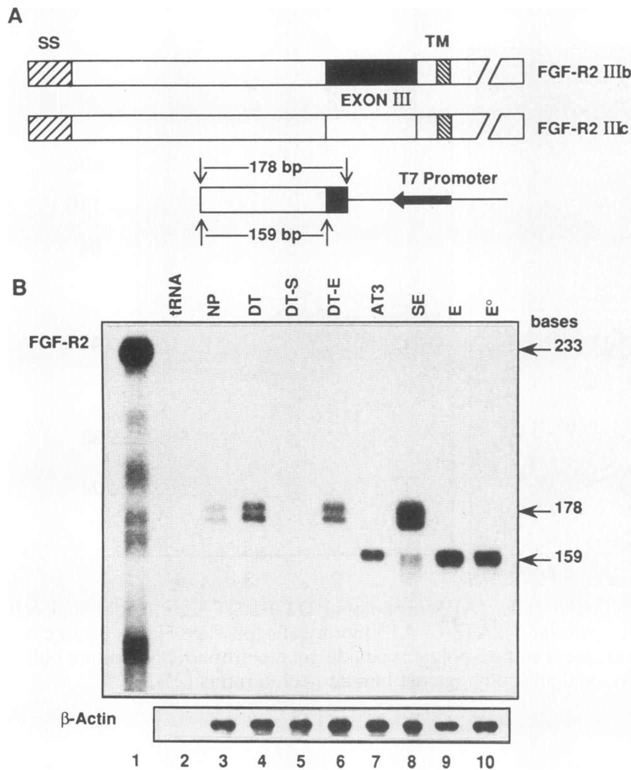

FIG. 4. Expression of exons IIIb and IIIc in the FGF-R2 gene. (A) Probe design. A RNase protection probe composed of 159 bases of the common FGF-R2 sequence preceding alternate exons IIIb and IIIc and 19 bases specific for exon IIIb (shaded) was designed. The probe protected a 178-base sequence in the FGF-R2(IIIb) mRNA and a 159-base sequence in the FGF-R2(IIIc) mRNA. SS, hydrophobic secretory signal sequence; TM, transmembrane domain. (B) Nuclease protection. Lane 1, the protection probe (3,000 cpm); lane 2, 20 μg of tRNA. Total cellular RNA (20 μg) was analyzed from the following tissues and cells: lane 3, normal prostate tissue (NP); lane 4, parent DT tumor tissue; lane 5, DT tumor-derived stromal cells (DT-S); lane 6, DT tumor-derived epithelial cells (DT-E); lane 7, AT3 tumor tissue; lane 8, reconstituted SE tumor tissue; lane 9, E tumor tissue; lane 10, secondary E tumor (E°). RNA loads were standardized by analysis with a β-actin protection probe.

FGF-3, FGF-5, and FGF-R1 genes cannot be detected and that a significant time is required for emergence of the type II tumors suggest that progression to malignancy may involve genetic changes in the epithelial cells rather than only selection and amplification of preexistent clones of cells exhibiting the type II phenotype.

The switch from exclusive expression of exon IIIb to exon IIIc in the FGF-R2 gene which renders epithelial cells insensitive to stroma-derived FGF-7 may underlie the insensitivity of type II tumor cells to stroma. In mouse skin keratinocytes, FGF-7 induces expression of specific keratins associated with terminal differentiation in addition to its mitogenic activity (26). Although expression of the FGF-7-responsive FGF-R2(IIIb) variant is predominant and the expression of FGF ligand genes is similar to that in normal prostate and type I tumor tissues, appearance of the FGF-7-independent FGF-R2(IIIc) splice variant correlates with appearance of rare foci of undifferentiated cells in the type I SE tumors that were reconstituted from isolated type I tumor epithelial and stromal cells. The mechanism of how FGF-7 promotes both growth and differentiation of epithelial cells that exclusively display the FGF-7-specific IIIb variant of the extracellular domain of FGF-R2 remains to be established. Alternate splicing in the FGF-R2 gene results in variant intracellular domains with potential to differentially interact with signal transducers (3, 10, 14). We have identified a variant in the FGF-R2 gene that is expressed in normal prostate and type I tumors that results from alternate splicing of exon 16 (18, 48a). The splice variant which skips exon 16 results in a unique COOH-terminal sequence which is devoid of two candidate phosphotyrosine sites, Tyr-730 and Tyr-766 (48a). Phosphotyrosine 766 is required for interaction and activation of the *src* homology 2 (SH2) domain signal transducer, phospholipase Cγ1, by the FGF-R kinase (33, 34, 40, 45). In the FGF-R1 gene, combinatorial splicing of the α exon in the extracellular domain, which determines ligand affinity and interaction with the FGF-R cofactor heparan sulfate (19, 20, 45), with an alternate acceptor site splice in the intracellular domain which affects interaction with signal transducers is regulated (45, 47). Whether combinatorial alternate splicing of alternate exons coding for variant extracellular domains of the FGF-R2(IIIb) isoform and exon 16 in the intracellular domain of FGF-R2 is controlled and whether the intracellular domain variants of FGF-R2 interact with different signal transducers to differentially affect growth and differentiation are under investigation. It should be noted that we have been unable to induce either glandular or squamous differentiation in vitro in mixed cultures of epithelial and stromal cells under a variety of test conditions. This suggests that, if stromal cell-derived FGF-7 acting on epithelial cell FGF-R2(IIIb) prevents progression of type I tumor epithelial cells by driving them into the differentiated state, permissive factors from the animal hosts are involved.

The activation of the FGF-2, FGF-3, and FGF-5 genes in type I tumor epithelial cells that give rise to type II tumors is consistent with previous reports that FGF ligand activity is elevated and the FGF-2 gene in particular is activated during progression of type I tumors to the type II malignant tumors in castrated or female host animals (25). FGF-2 is normally expressed prior to limb bud formation in embryos (1a, 13) and appears to be associated with cells of the vascular and nervous systems from adults (2, 24, 46). Conceivably, an autocrine loop between abnormally expressed FGF-2 and FGF-R2(IIIc) is sufficient to confer growth autonomy and malignancy on the stroma-independent type I tumor epithelial cells. However, the normally stromal FGF-R1 gene is also activated and coexpressed with the FGF-R2 exon IIIc variant in the type II tumor cells. Among isoforms of the FGF-R1 gene that are activated in the malignant type II tumor cells are the three- and two-immunoglobulin-like loop extracellular domain splice variants, FGF-R1α and -β, which have low and high affinity, respectively, for FGF-2 (14, 47). A significant portion of mRNAs resulting from alternate splicing of the 189-bp α exon coding for the unique NH_2-terminal immunoglobulin loop of the low-affinity isoform of FGF-R1, FGF-R1α, is spliced at an alternate acceptor site in the α exon (50). The predicted product of this isoform is an alternately translated two-immunoglobulin loop high-affinity receptor isoform, FGF-R1γ, which is not translocated to the membrane as a consequence of lack of a NH_2-terminal membrane translocation signal. Overexpression of FGF-R1γ together with FGF-2 and FGF-1, whose mechanism and degree of access to external transmembrane receptors are in contention (22, 42, 43), may result in a constitutive intracrine loop within the malignant type II tumor cells. Relative to

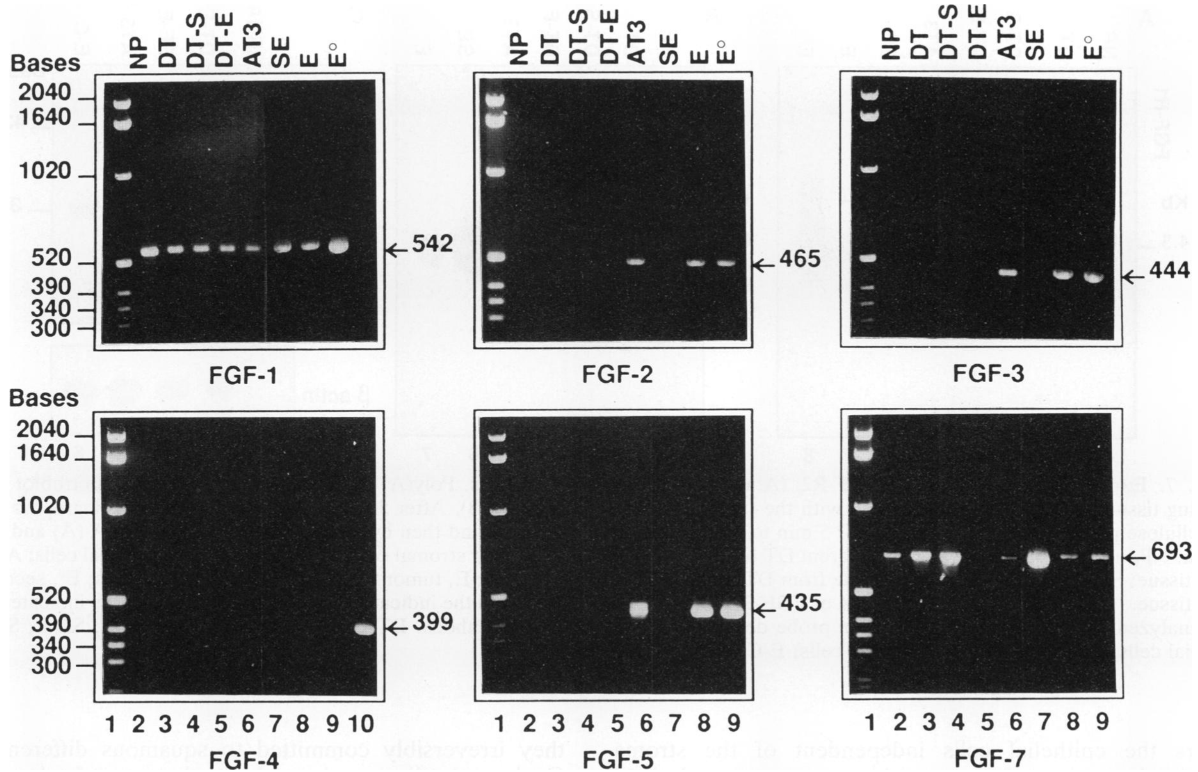

FIG. 5. Expression of FGF ligand genes. cDNA templates from the indicated tissues and cells were used with FGF-specific primers in the PCR described in Materials and Methods. Lanes 1, size standards in base pairs; lanes 2, normal prostate tissue (NP); lanes 3, parent DT tumor tissue (DT); lanes 4, stromal cells from DT tumors (DT-S); lanes 5, epithelial cells from DT tumors (DT-E); lanes 6, malignant AT3 tumor tissue (AT3); lanes 7, reconstituted tumors from DT tumor-derived epithelial and stromal cells (SE); lanes 8, tumors derived from DT epithelial cells (E); lanes 9, secondary E tumor tissue (E°). Lane 10 in the FGF-1 panel is from a reaction mixture containing no template. Lane 10 in the FGF-4 panel is a mixture containing a cDNA template from a sea urchin embryo. The indicated analysis of the six FGF ligands was performed on 1-μl portions of the same preparation of cDNA template from the indicated sources. The positive signal generated by paired primers for FGF-1 in all samples serves as an internal standard.

cDNAs coding for transmembrane isoforms of FGF-R1, overexpression of the FGF-R1γ cDNA has a potent transforming activity in conventional transformation assays (50). Finally, activation of the embryonic FGF-3 and FGF-5 genes also occurs in the highly malignant type II tumors. Both gene products are actively secreted and FGF-5 is recognized by FGF-R1 transmembrane isoforms that are expressed in the type II tumors (36, 51). Lack of knowledge about the receptor isotype specificity of the FGF-3 ligand hampers a prediction of the impact of activation of FGF-3 in the type II tumor cells. In addition to expression during embryogenesis, FGF-3, also called *int-2*, is activated in mouse mammary tumor virus-induced mammary tumors (7). Noteworthy is the fact that the mouse mammary tumor virus-promoted FGF-3 transgene is activated and causes epithelial cell hyperplasia in prostate tissue in addition to its activation in mammary tissue (35). The FGF-3 gene is a weak inducer of anchorage independence in mouse 3T3 mesenchymal cells which presumably express isoforms of stromal cell FGF-R1 (7). The fact that prostatic hyperplasia in transgenic animals bearing the FGF-R3 gene is limited to prostate epithelial cells which express the FGF-R2(IIIb) isoform suggests that FGF-3 may cross-react with the specific isoform of the FGF-R2 gene. However, cross-reactivity of FGF-3 with and expression of the FGF-R3 (23) or FGF-R4 (38) genes in prostate epithelial cells cannot be eliminated. Intracrine activities of FGF-3 as a consequence of alternate translational initiation have also been proposed (7).

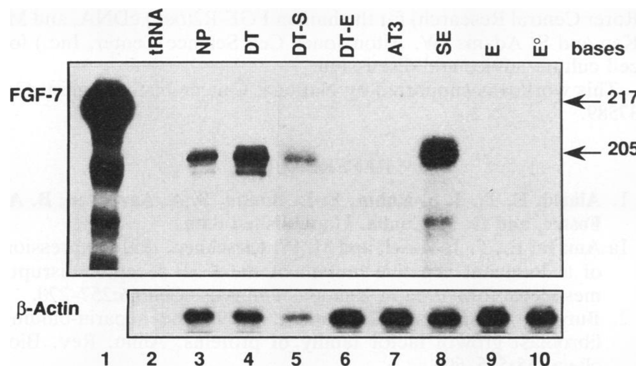

FIG. 6. FGF-7 expression in prostate tissues and cells. Lane 1, nuclease protection probe (3,000 cpm); lane 2, 20 μg of tRNA. Total RNA (20 μg) from the following tissues and cells was analyzed: lane 3, normal prostate tissue (NP); lane 4, DT tumor tissue; lanes 5, DT tumor-derived stromal cells; lane 6, DT tumor epithelial cells; lane 7, AT3 tumor tissue; lane 8, SE tumor tissue; lane 9, E tumor tissue; lane 10, secondary E (E°) tumor tissue.

In sum, we propose that exon switching in the resident epithelial FGF-R2 gene may be an early, potentially irreversible event in the progression of malignant tumors that

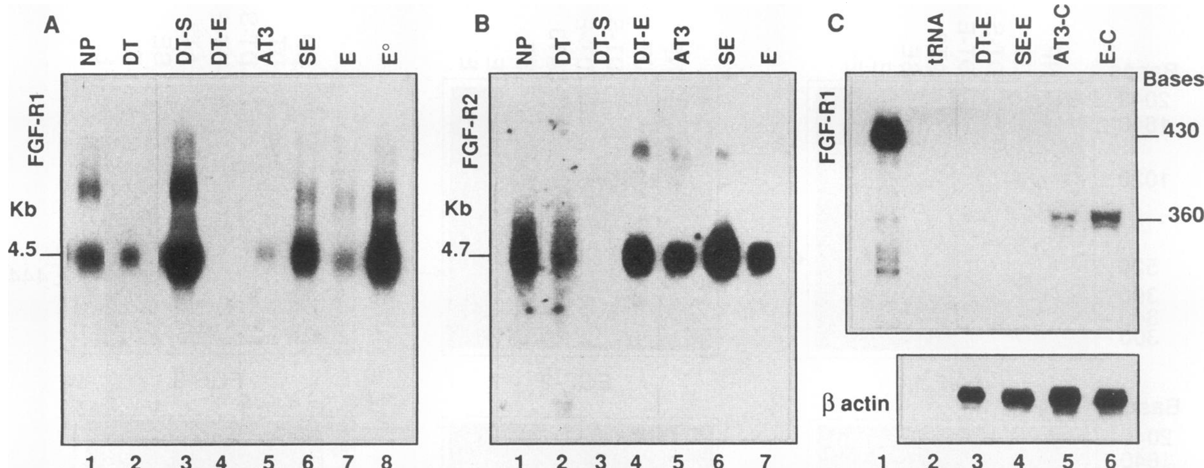

FIG. 7. Expression of FGF-R1 and FGF-R2. (A and B) Northern blot analysis. Poly(A)$^+$ RNA (5 μg) on the same electroblot from the following tissues and cells was hybridized with the ^{32}P-labeled FGF-R2 cDNA (B). After 5 days of exposure to autoradiographic film, the nitrocellulose paper was boiled in water for 5 min to remove the FGF-R2 probe and then hybridized with FGF-R1 cDNA (A) and exposed for 12 h. NP, normal prostate tissue; DT, parent DT tumor tissue; DT-S, DT tumor stromal cells; DT-E, DT tumor epithelial cells; AT3, AT3 tumor tissue; SE, reconstituted tumor tissue from DT stromal and epithelial cells; E, tumor tissue from DT epithelial cells; E°, secondary E tumor tissue. (C) RNase protection analysis of FGF-R1: Total RNAs (20 μg) from the indicated cell types derived from the indicated tumors were analyzed with the FGF-R1 protection probe described in Materials and Methods. DT-E, DT tumor epithelial cells; SE-E, SE tumor epithelial cells; AT3-C, cloned AT3 tumor cells; E-C, cloned E tumor cells.

renders the epithelial cells independent of the stroma through abrogation of responsiveness to stroma-derived FGF-7, which not only supports growth of the epithelial cells but also drives them into a growth-arrested, differentiated state. However, this single change is insufficient for autonomous growth and the fully malignant phenotype. Subsequent activation of the FGF-2, FGF-3, and FGF-5 genes and the stromal cell FGF-R gene in the FGF-7- and stroma-independent epithelial cells creates multiple and potentially redundant autocrine and intracrine loops between FGF ligand and FGF-R isoforms in type II tumors. Although we contend that exon switching in the resident epithelial cell FGF-R2 gene may be an early change within the FGF–FGF-R family that occurs during progression of epithelial cells to malignancy, it is of interest whether activation of the stromal FGF-R1 and the ectopic FGF ligand genes occurs in a random or temporal and stepwise mode during tumor progression. These questions should be clarified by temporal and clonal analysis of expression of the FGF–FGF-R isoforms in cells which compose the progressively malignant model tumors described here.

Although the growth rates, the nonmalignant character, the differentiated state, the maintenance of distinct relationships between epithelium and stroma, and the pattern of FGF and FGF-R expression in the transplantable parent DT tumor and the reconstituted SE tumors derived from DT tumor epithelial and stromal cells are similar, the nature of the differentiated compartments of the two tumors is quite different. The differentiated compartment of the parent tumors is predominantly gland-like with only rare foci of squamous structures, whereas the SE tumors consist of predominantly intensely keratinized squamous structures and rare foci of undifferentiated epithelial cells. Key questions concerning the lineage relationships of epithelial cells which give rise to gland-like and squamous structures arise. Are the cultured DT tumor-derived epithelial cells used in this study multipotent and capable of glandular or squamous differentiation in the proper inductive environment, or are they irreversibly committed to squamous differentiation? Cunha and colleagues have shown that intact fetal urogenital or neonatal seminal vesicle mesenchyme maintains the glandular phenotype of cell suspensions from the DT tumors in subcapsular renal grafts (11, 12). The effect of the intact mesenchymal tissue on cytodifferentiation was concurrent with an eightfold reduction in tumor growth rate and an apparent loss of tumorigenicity. FGF-7 has been implicated in the androgen-stimulated induction of neonatal seminal vesicle epithelial cell growth and branching morphogenesis of the secretory glandular phenotype (1).

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