Progression of Prostate Cancer from a Subset of p63-Positive Basal Epithelial Cells in FG/Tag Transgenic Mice

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Abstract

Transgenic mice that allow targeting of SV40 T antigen (Tag) to the prostate provide a unique model to identify cancer-initiating cells and follow their progression from a normal cell phenotype into prostate cancer cells. We have developed the FG/Tag transgenic mouse model of prostate cancer using the human fetal globin (FG) promoter linked to Tag. Immunohistochemistry results show that before the development of prostate intraepithelial neoplasia (PIN), a subset of p63+ basal epithelial cells expresses Tag. As in the case of human prostate cancer, there is a loss of p63+ basal cells with neoplastic progression, and a long period of time is required for PIN lesions to develop into palpable prostate tumors. Other immunohistochemistry results show cellular heterogeneity in FG/Tag PIN lesions and primary tumors with neuroendocrine differentiation. Cell lines derived from primary prostate tumors showed characteristics of a neuroendocrine-epithelial intermediate cell type. The FG promoter has high transcriptional activity in intermediate (DU 145, PC-3) and p63+ basal epithelial (LHSR-AR) prostate cancer cells. Therefore, the unexpected development of prostate cancer in the FG/Tag mice may be due to the presence of DNA elements in the FG promoter that can target Tag to specific basal or intermediate cells. We conclude that FG/Tag mouse is a unique model of prostate cancer because the initiating cells are a subset of p63+ basal (possibly stem cells), which may be the true cells of origin for carcinogenesis in aggressive human prostate cancer. (Mol Cancer Res 2007;5(11):1171–9)

Introduction

A better understanding of the types of epithelial cells present in the normal prostate gland and of the cellular alterations important in the initiation and progression of prostate cancer should result in the development of more effective chemopreventive and therapeutic agents. The prostate is a classic exocrine gland consisting of ductal-acinar structures embedded in stromal tissue (1). The acini are lined by well-differentiated secretory or luminal epithelial cells, which are androgen dependent and secrete proteins like prostate-specific antigen into the lumen of the duct. These cells are surrounded by an underlying layer of proliferating nonsecretory basal epithelial cells that are primarily androgen independent and rest on the basement membrane separating the epithelial cells from the surrounding stroma. In addition, rare neuroendocrine cells are present in the normal prostate, but their function is not well understood (2).

There is evidence suggesting that luminal, basal, and neuroendocrine epithelial cells of the prostate originate from the differentiation of stem cells present in the basal compartment (3-8). Transit amplifying and intermediate cells coexpressing characteristics of luminal, basal, and/or neuroendocrine cells have been identified in developing and normal adult prostate and in prostate cancer. It is postulated that there are androgen-independent prostate stem cells that give rise to androgen-responsive intermediate cells, which develop into terminally differentiated androgen-dependent luminal epithelial cells. The ability of the prostate to undergo multiple cycles of regression and regeneration supports the presence of stem cells in the basal epithelial cell compartment (9, 10).

The clinical progression of prostate cancer involves the development of androgen-dependent cancer, typical of luminal epithelial cells, to an undifferentiated androgen-independent cancer with some features of basal epithelial and stem cells (3-8). Because the major prostate cancer cell type expresses luminal cytokeratins 8 and 18 but not basal cytokeratins 5 and 14, it is commonly thought that carcinogenesis originates from well-differentiated luminal epithelial cells that express prostate-specific antigen (11-13). Because prostate cancer increases with age and stem cells are present throughout life, prostate stem cells located in the basal epithelial compartment are also considered as origins of carcinogenesis (3-8). However, because prostate cancer rarely contains cells expressing basal cytokeratin, intermediate or transit-amplifying cells are also considered as origins of carcinogenesis (4). Overall, there is little agreement as to which cell is the origin for carcinogenesis in prostate cancer.

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The cancer stem cell hypothesis states that most if not all cancers originate from tissue-specific stem or progenitor cells, and tumors are driven by components that display stem cell properties (14). Recent studies suggest that prostate cancer stem cells representing a small percentage of the total tumor mass are much more tumorigenic than their progeny cells (15, 16). The principal therapy for men with advanced prostate cancer is androgen ablation, but most of these patients eventually progress to androgen-independent disease that is resistant to chemotherapy (17). It is possible that chemotherapy kills prostate cancer stem cell progeny, resulting in tumor regression; however, the relapse of prostate cancer probably is initiated from remaining stem cells that were not eradicated by chemotherapy. The resulting tumors are more metastatic and resistant to chemotherapy and result in the death of the patient. Support for the hypothesis that more aggressive and metastatic cancers express stem cell–like genes comes from the identification of the 11-gene signature as a powerful predictor of unfavorable patient prognosis in 11 distinct types of cancer, including prostate cancer (18).

The expression of SV40 T antigen (Tag) oncogene targeted to the prostate has resulted in the development of several transgenic mouse models of prostate cancer (19-24). The androgen-dependent prostate-specific promoter probasin targets the well-differentiated luminal epithelial cells, and prostate cancers develop initially as androgen-dependent and progress into androgen-independent prostate cancer (AI-PC) with neuroendocrine differentiation (22, 24). The TRAMP and LADY models (probasin Tag) have been used by a variety of investigators to study molecular events in carcinogenesis of the prostate and for preclinical testing of new therapies (25, 26). However, the targeted androgen-dependent luminal epithelial cells may not be the true initiation site of transformation in prostate cancer. In TRAMP mice, there is no luminal epithelial cell expression of Tag in a small subset of basal epithelial cells (27). In addition, targeted deletion of PTEN in mouse prostate using a modified probasin promoter results in the accumulation of basal and intermediate epithelial cells before tumor formation (28). Overall, these results suggest that alterations in basal and intermediate cell compartments are associated with the initiation of prostate cancer.

We have developed the FG/Tag transgenic mouse model of AI-PC using the human fetal globin (FG) promoter linked to Tag (previously referred to as Gv/T-15; refs. 29-32). The progression of prostate cancer in the FG/Tag mice is similar to that in humans, i.e., it originates from high-grade prostate intraepithelial neoplasia (PIN) and progresses to advanced metastatic carcinomas (31, 32). The purpose of the present study was to better identify the initiation cells of carcinogenesis in FG/Tag mice and to investigate the cellular changes involved in the development of PIN lesions and advanced prostate tumors. Our results show that before the production of PIN, Tag is targeted to a subset of p63+ basal epithelial cells, which are required for the development of basal and luminal cells during normal differentiation of the prostate and may function as adult prostate stem cells (33). Once PIN develops, p63 is lost, and there is a long period before the generation of prostate tumors. Our findings suggest that the FG/Tag mouse is a unique model of AI-PC because it allows targeting of prostate basal/stem cells.

Results

Initiation Cells of Carcinogenesis in FG/Tag Transgenic Mice

Our previous results showed that before the development of palpable prostate tumors in adult FG/Tag transgenic males, mRNA for Tag is detected in prostates, but not in other tissues. Therefore, this offers an explanation why prostate tumors develop in FG/Tag mice. Furthermore, prostate tumors still develop in castrated males, suggesting that tumors initiate from androgen-independent cells located in the basal epithelial compartment (31). To localize putative initiation cells in FG/Tag mice, prostataes without visible tumors were analyzed for Tag expression by immunohistochemistry. Because our previous results showed Tag+ prostate cells located in the basal epithelial layer of the prostate, we also immunostained for the basal epithelial marker p63 (32, 34). Interestingly, p63+ basal cells are required for the development of basal and luminal cells during normal differentiation of the prostate, suggesting that they may function as adult prostate stem cells (33). Results show that in a prostate of a FG/Tag mouse, a cluster of Tag+ cells also express p63 (Fig. 1A) before the formation of PIN lesions, indicating that the targeted cells are basal epithelial cells. However, not all p63+ basal epithelial cells express Tag. In addition, another prostate of a FG/Tag mouse showed Tag+ cells above the basal layer that do not express p63 (Fig. 1B). These Tag+/p63+ cells have enlarged nuclei and may be an early neoplastic prostate cell. Using double immunofluorescence immunohistochemistry, our results more clearly show that before PIN formation, there are epithelial cells that coexpress Tag and p63 (yellow cells in merge; Fig. 1C). These results suggest that the cells of origin for the initiation of prostate cancer are a subset of p63+ basal epithelial cells.

Immunohistochemistry of another prostate of a FG/Tag mouse before the development of PIN lesions showed an expansion of synaptophysin-positive neuroendocrine cells (also Tag+; not shown) that expressed low levels of androgen receptor (AR) compared with synaptophysin+ cells (Fig. 1D). In prostates of FG/Tag mice that did not express Tag, we detected synaptophysin+ nerve bundles, but no staining of epithelial cells. By double immunofluorescence, we did not detect prostate epithelial cells coexpressing p63 and synaptophysin (data not shown). Neuroendocrine epithelial cells in normal prostate are rare compared with basal and luminal epithelial cells (2). In addition, normal prostate neuroendocrine cells do not express p63 or AR, making it unlikely that the initiation cells of carcinogenesis in FG/Tag mice are neuroendocrine cells. A more likely possibility is that the expression of Tag, which normally inactivates the p53 and Rb tumor suppressor proteins, may force the differentiation of prostate basal/stem cells toward the neuroendocrine phenotype (35).

Loss of p63 in FG/Tag PIN Lesions

Compared with other Tag-expressing transgenic mouse models of prostate cancer, in which luminal epithelial (TRAMP, LADY, C3) and neuroendocrine prostate cells (cryptdin) are targeted, the FG/Tag transgenic mice are a unique model because a subset of basal (or possibly stem) epithelial cells is targeted (19-23). We determined how early Tag+ cells can be detected by performing immunohistochemistry on FG/Tag...
express little or no p63 (Fig. 2C). FG/Tag prostate ducts that contain Tag+ cells in an adjacent prostate duct from the same mouse did not express cytokeratin. These Tag+/cytokeratin− cells proliferate from the underlying basal compartment and push into the cytokeratin− luminal compartment toward the lumen (Fig. 4B). These results suggest that there is heterogeneity in the types of PIN lesions that develop in FG/Tag mice. It is also likely that expression of Tag rapidly converts p63+ cells into neuroendocrine cells, resulting in the loss of cytokeratin.

Heterogeneity in FG/Tag PIN Lesions

To gain further insight into the cellular and molecular changes occurring in the progression of PIN lesions in FG/Tag mice, we did immunohistochemistry analysis of the epithelial marker pan-cytokeratin. Results showed that Tag+ cells in PIN lesions expressed cytokeratin, although not as strongly as Tag+ luminal epithelial cells (Fig. 4A). However, PIN lesions containing Tag+ cells in an adjacent prostate duct from the same mouse did not express cytokeratin. These Tag+/cytokeratin− cells proliferate from the underlying basal compartment and push into the cytokeratin− luminal compartment toward the lumen (Fig. 4B). These results suggest that there is heterogeneity in the types of PIN lesions that develop in FG/Tag mice. It is also likely that expression of Tag rapidly converts p63+ cells into neuroendocrine cells, resulting in the loss of cytokeratin.

Prostate Cancer Initiation Cells

FIGURE 1. Initiation cells of carcinogenesis in FG/Tag transgenic mice are a subset of p63+ basal epithelial cells. A, Immunostaining for Tag and p63 of consecutive sections from a FG/Tag prostate (21 wk) without a visible prostate tumor. A cluster of non-neoplastic Tag+ cells (arrows) coexpress p63, as shown in the consecutive section of the same cells (hematoxylin, ×400). B, Immunostaining for Tag and p63 of consecutive sections from a FG/Tag prostate (10 wk) showing some Tag+ p63− cells (arrows) coming up from the basal layer toward the luminal layer (hematoxylin, ×400). This may be the earliest sign of a neoplastic cell phenotype with an enlarged nucleus. C, Double immunofluorescence of Tag (green) and p63 (red) on the same section from a FG/Tag prostate (10 wk). Merge, cells that coexpress (yellow) Tag and p63 (>200). D, Immunostaining for AR and synaptophysin (Syp) of consecutive sections from a FG/Tag prostate (33 wk). Clusters of non-neoplastic synaptophysin+ cells (arrows), which are also Tag+ (data not shown), express lower levels of AR compared with synaptophysin+ cells, typical of luminal epithelial cells (hematoxylin, ×200).

Prostates at 3, 6, 10, and 14 weeks, at or before palpable prostate tumors are detected (13 weeks; refs. 31, 32, 36). At 3 weeks, no Tag− cells were detected (n = 4; Fig. 2A). Mouse prostates at 3 weeks are not fully developed and contain a continuous layer of p63+ cells, which is in contrast to the discontinuous layer of p63+ cells present in fully developed adult prostates (≥6 weeks; Fig. 1). At 6 weeks, PIN lesions containing Tag− epithelial cells were detected in 50% of prostates (n = 8; Figs. 2B and 3). The frequency of Tag− prostate cells in FG/Tag mice increased to 65% to 75% by 10 (n = 8) and 14 weeks (n = 8; Fig. 3). Thus, as in the case of human prostate cancer, there is a latent period (≥7 weeks) between the formation of PIN lesions at 6 weeks and the development of palpable prostate tumors at ≥13 weeks in FG/Tag mice. Our results also showed that Tag− cells in PIN lesions do not express p63, and that there is a depletion of p63+ basal epithelial cells (Fig. 2B). Another FG/Tag prostate containing a small PIN lesion showed the presence of Tag− cells that express little or no p63 (Fig. 2C). FG/Tag prostate ducts that contained Tag+ cells often contained fewer p63+ cells compared with prostates without Tag+ cells. Similarly, p63 was not expressed in advanced FG/Tag prostate tumors, indicating that they are not basal cell carcinomas (data not shown). In human prostate cancer, p63 is not expressed in PIN and in advanced prostate cancers (34). Therefore, as in human prostate cancer, the loss of p63+ basal cells is a common characteristic of prostate carcinogenesis in FG/Tag transgenic mice.

FIGURE 2. Loss of p63 in Tag+ FG/Tag PIN lesions. A, Immunostaining for Tag and p63 of consecutive sections from a FG/Tag prostate (3 wk). No Tag+ cells were detected (n = 4); p63+ cells form a continuous basal cell layer (hematoxylin, ×200). B, Immunostaining for Tag and p63 of consecutive sections from a FG/Tag prostate (6 wk) showing a PIN lesion (arrow) containing Tag+ cells that do not coexpress p63 and p63− cells that do not express Tag (hematoxylin, ×400). C, Double immunofluorescence of Tag (green) and p63 (red) on the same section from a FG/Tag prostate (22 wk) with early PIN. Merge, cells that are Tag+/p63− (yellow), Tag+/p63+ (green), and Tag−/p63+ (red; ×400).
Heterogeneity in FG/Tag Primary Prostate Tumors

Once FG/Tag prostate tumors become initially palpable, there is rapid growth and nearly 100% of metastases to lymph nodes after 2 weeks (32, 36). To elucidate the molecular changes occurring in FG/Tag primary prostate tumors, we did immunohistochemistry analysis of epithelial (cytokeratin and E-cadherin), neuroendocrine (synaptophysin), and AR. As in TRAMP and LADY 12T-10 transgenic mice, advanced AI-PC in FG/Tag mice expressed little or no cytokeratin or E-cadherin (22, 24). An embedded prostate inside the tumor showed strong immunostaining for cytokeratin and E-cadherin (Fig. 5A and B). Also, similar to TRAMP and LADY 12T-10 mice, AI-PC in FG/Tag mice strongly expressed synaptophysin, although there was variability in the expression levels between different tumors (Fig. 5C and D). There was a low but consistent level of AR in primary prostate tumors; embedded prostates showed much higher expression of AR (Fig. 5E). Interestingly, some prostate tumors showed a pattern of strongly AR+ tumor cells located near blood vessels and AR– tumor cells located further away from blood vessels, suggesting a differentiation-promoting effect of the blood (Fig. 5F). These results suggest that similar to human prostate cancer, there is cellular heterogeneity in primary prostate tumors of FG/Tag mice. Unlike most human prostate cancers, there is stronger neuroendocrine differentiation in FG/Tag, TRAMP, and LADY 12T-10 mice, possibly due to the effect of Tag on p53 and Rb (35).

Cell Lines Derived from FG/Tag Primary Prostate Tumors

We established multiple cell lines from FG/Tag primary prostate tumors to better identify its molecular and cellular characteristics. Cells were cultured from the prostate tumors using media that is preferential to epithelial cells but without additional dihydrotestosterone (DHT) already present in 10% fetal bovine serum (see Materials and Methods). Cells with epithelial-like morphology were cloned and analyzed for expression of epithelial and neuroendocrine markers by Western blot analysis (Fig. 6A and B). Interestingly, none of the 10 cloned FG/Tag cell lines expressed Tag, as in the case of the...
Negative controls for CD44, CD133, p63, and GAPDH are mouse prostate; it is the housekeeping RNA control. Positive control RNA in FG/Tag cell lines or primary prostate tumors. GAPDH Neither p63 nor Oct4 stem cell markers were detected. Stem cell markers CD44 and CD133 were detected in FG/Tag cell lines 15, 17, 21, and primary prostate tumor (1° PT) express Bmi-1, CD44, and AR. PC-3 is the positive control (+C) for the Bmi-1/CD44 blots and LNCaP for the AR blot. RT-PCR analysis showing that prostate stem cell markers CD44 and CD133 were detected in FG/Tag cell line 21 and primary prostate tumors. Neither p63 nor Oct4 stem cell markers were detected in FG/Tag cell lines or primary prostate tumors. GAPDH is the housekeeping RNA control. Positive control RNA for CD44, CD133, p63, and GAPDH is mouse prostate; for Oct4, we used mouse testis. Negative controls (−RT) contain Tag polymerase, whereas +RT contains SuperScript III. Numbers to the left, expected sizes in nucleotides of the amplified fragments. Similar results were obtained in FG/Tag 15 and 17 cell lines.

TRAMP cell lines derived from TRAMP primary prostate tumors (37). Similar to the human LNCaP, DU 145, and PC-3 prostate cancer cells, FG/Tag cell lines expressed luminal epithelial cytokeratin 8. Unlike human prostate cancer cells, there was a lower expression of cytokeratin 18 in FG/Tag cells (Fig. 6B). FG/Tag cell lines expressed much higher neuroendocrine synaptophysin and similar levels of neuron-specific enolase (NSE) compared with human prostate cancer cells. In addition, similar to primary prostate tumors, the FG/Tag cell lines express low levels of AR (Fig. 6C); E-cadherin and p63 was not detected in FG/Tag cell lines (data not shown). These results suggest that FG/Tag cell lines represent a neuroendocrine/luminal epithelial intermediate prostate cancer cell with some similarities to the established human prostate cancer cell lines (38).

We determined by Western blot and reverse transcription-PCR (RT-PCR) whether FG/Tag cell lines express stem cell markers. Bmi-1, a member of the Polycomb group of repressors that has an important role in cancer and normal stem cell self-renewal (39), is expressed in FG/Tag cell lines and primary prostate tumors, as well as PC-3 human prostate cancer cells (Fig. 6C). Results from RT-PCR showed that the FG/Tag cell lines and primary prostate tumor tissue express CD44 and CD133, previously shown to be expressed in prostate stem cells (3-8, 15, 16). Expression of CD44 was confirmed by Western blot (Fig. 6C). Similar to human prostate cancer, there was no expression of p63 and the Oct4 stem cell marker in FG/Tag cell lines or primary prostate tumors (refs. 34, 40; Fig. 6D). These results indicate that stem and intermediate cell markers are expressed in FG/Tag cell lines and primary prostate tumor tissues.

**FG Promoter Is Highly Active in Prostate Cancer Basal/Intermediate Cells**

One possible explanation underlying prostate tumor formation in FG/Tag transgenic mice involves the presence of DNA elements in the FG promoter that serve as targets for prostate transcription factors to activate Tag transcription to a subset of p63+ basal epithelial cells. We previously showed that the FG promoter has high activity in the DU-145 and PC-3 AI-PC cell lines but low activity in the androgen-dependent LNCaP prostate cancer cell line (30). Our results showed that there was higher expression of the intermediate prostate cell marker cytokeratin 19 (41) and Bmi-1 in AI-PC cell lines DU 145 and PC-3 than in LNCaP cells (Fig. 7A). These results suggest that the FG promoter is more active in intermediate-like prostate cancer cells compared with well-differentiated luminal prostate cancer cells.

To further investigate the activity of the FG promoter in prostate cells, we used stable clones of human PrEC cells that have been transduced with Tag (large and small), hTERT, AR, and H-ras (LHSR-AR; ref. 42). These cells express p63 and are considered to be a model of prostate basal epithelial cells that can differentiate into luminal epithelial-like cells in the presence of DHT (42, 43). After 2 days treatment with DHT (10 nmol/L), there was a 2-fold reduction of p63 protein (Fig. 7B). The FG promoter had a very high activity without DHT (390-fold above promoterless plasmid) that was decreased 2.4-fold when treated with DHT for 2 days (Fig. 7C). In contrast, the FG promoter is not regulated by DHT in LNCaP or PC-3 cells (data not shown). As a comparison, the FG promoter has a 50- to 100-fold activity in DU 145, PC-3, and FG/Tag cells (30). These results suggest that the FG promoter is most active in p63+ prostate cells and support the hypothesis that Tag is targeted to a subset of p63+ cells in the FG/Tag mice.

**Discussion**

The generation of transgenic mice in which the expression of Tag can be specifically targeted to the prostate has increased the opportunities to better define the cellular origins of prostate cancer (25, 26). Our results indicate that the FG/Tag
Overall, our results indicate that p63 + basal cells (possibly stem cells) due to positive regulatory DNA elements present in the FG promoter. In contrast, in the TRAMP, LADY, and cryptdin models of prostate cancer, Tag is targeted to normal prostate luminal and rare neuroendocrine epithelial cells (25, 26). Initiation and progression of prostate cancer in FG/Tag mice is similar to human prostate cancer because (a) there is a loss of p63+ cells in PIN and advanced prostate tumors, and (b) a long period of time (relative to mouse model) is required for normal prostate epithelial cells in transgenic mice to a neuroendocrine cancer (35). Inactivation of the p53 and Rb tumor suppressors frequently occurs in progression of human prostate cancer (46, 47). Similar to our results in FG/Tag mice, the resulting prostate cancers are highly aggressive, poorly differentiated, and metastatic carcinomas that express neuroendocrine synaptophysin and luminal cytokeratin 8. Cell lines derived from FG/Tag primary prostate tumors also show neuroendocrine and luminal differentiation (Fig. 6). Because Tag inactivates both p53 and Rb proteins, a similar mechanism may occur in FG/Tag, TRAMP, and LADY 12T-10 mice, i.e., the expression of Tag blocks differentiation of prostate cells at the neuroendocrine/luminal intermediate stage.

Another recently reported transgenic mouse model suggesting that prostate cancer originates from p63+ basal cells is the conditional PTEN knock-out model (28). This model is generated by crossing PTEN flox with Pb-Cre4, which can target Cre recombinase to the prostate using the modified probasin ARR2 promoter (48). Interestingly, PTEN deletion occurs in both p63+ basal and p63+ luminal cells. Early carcinogenic cellular events in these mice are similar to our results in the transgenic mice are a unique model of AI-PC because they allow targeting of a subset of normal p63+ prostate basal epithelial cells. In contrast, in the TRAMP, LADY, and cryptdin models of prostate cancer, Tag is targeted to normal prostate luminal and rare neuroendocrine epithelial cells (25, 26). Initiation and progression of prostate cancer in FG/Tag mice is similar to human prostate cancer because (a) there is a loss of p63+ cells in PIN and advanced prostate tumors, and (b) a long period of time (relative to mouse model) is required for PIN lesions to develop into palpable prostate tumors. Advanced primary prostate tumors in FG/Tag mice show a strong neuroendocrine differentiation, which may be due to the effect of Tag on inactivating p53 and Rb proteins in p63+ basal cells (35). We suggest that the FG promoter targets a subset of p63+ basal epithelial cells (possibly stem cells) due to positive regulatory DNA elements present in the FG promoter. Overall, our results indicate that p63+ basal epithelial cells are a cell of origin for carcinogenesis in prostate cancer that can lead to the development of aggressive and lethal disease.

Stem cells located in the basal layer of the prostate duct are thought to be present throughout life and are considered to be involved in the initiation of prostate cancer (3-8). Given that p63+ prostate epithelial cells are located in the basal layer, in vivo studies have been conducted to determine if p63+ basal epithelial cells have properties of prostate stem cells. Grafting of p63+/−urogenital sinus tissue to the renal capsule results in prostate-like tissue containing luminal and neuroendocrine but not basal cells, suggesting that prostate stem cells do not express p63 (33, 44). However, growth of p63+/−urogenital sinus in non-prostatic kidney tissue may have resulted in differentiation into intestinal epithelium, which does not require p63 for normal development (33). In contrast, complementation of p63+/−mouse blastocysts with p63+/+ embryonic stem cells shows that p63−basal cells are required for the development of both basal and luminal cells during normal prostate differentiation. In addition, a recent publication identifies p63 as a key lineage-specific determinant of the proliferative capacity in stem cells of stratified epithelia, including the prostate (45). These results suggest that p63+ prostate basal cells may function as adult prostate stem cells (33). The presence of a subset of p63+ cells that are Tag+ in FG/Tag prostates before development of PIN lesions suggests that p63+ basal cells are the cells of origin of carcinogenesis (Fig. 1). We are currently investigating whether these Tag+/p63+ cells are prostate stem, transit-amplifying, or intermediate cells.
FG/Tag mice, i.e., a fraction of p63+ cells bud out from the basal layer, often showing a decrease in p63 perhaps because of an increase in the neoplastic phenotype (Figs. 1B and 2C). In contrast to our results in the FG/Tag mice, however, there is an accumulation of p63+ cells in PTEN conditional knock-out mice (28). Overall, these results strongly suggest that basal or transit-amplifying/intermediate cells, but not luminal cells, are the initiation cells of carcinogenesis in the FG/Tag and conditional knock-out mice, and differences may be due to the expression of Tag, which directs differentiation toward the neuroendocrine/luminal intermediate cell phenotype.

In TRAMP mice, there is non-luminal epithelial expression of Tag in cells that are positive for breast cancer resistance protein, a member of the ATP-binding cassette transporter family associated with adult stem cells (27). This leaky expression of Tag to non-luminal epithelial cells is possibly due to the expression of Foxa2 transcription factor in a small subset of basal epithelial cells coexpressing cytotkeratin 14 and synaptophysin (49). Foxa2 is a member of the forkhead box family known to regulate the probasin promoter (50). Similarly, the use of the modified probasin AR2 promoter to target Cre recombinase to these small subsets of basal epithelial cells in the p53/Rb and conditional knock-out mice may also explain the observed effects on the initiation and progression of prostate cancer. We suggest that the FG promoter contains DNA elements that target a subset of p63+ cells in the FG/Tag mice. In contrast to the androgen-dependent basal promoter, the FG promoter has higher transcriptional activity in intermediate cell-like prostate cancer cells DU 145 and PC-3 compared with well-differentiated LNCaP (Fig. 7; refs. 30, 51). In addition, the FG promoter is most active in p63+ prostate cancer basal LHSR-AR cells compared with cells treated with DHT to reduce p63 and increase luminal epithelial cell differentiation (Fig. 7). Chromosomal position effect is an unlikely factor in the prostate tumors because such an effect is observable in more than one transgenic line (30). Our preliminary data suggest that the FG promoter contains DNA elements that target a subset of p63+ cells in the FG/Tag mice.

In conclusion, the FG/Tag transgenic mice are a unique model of AI-PC because the initiation cells are a subset of p63+ basal (possibly stem cells), which may be the true cells of origin of carcinogenesis for human prostate cancer. Transgenic models using the probasin promoter, which predominantly targets differentiated prostate luminal epithelial cells, may not be ideal to study prostate intermediate or stem cells. The FG/Tag transgenic mice should increase the opportunities to understand the role of prostate intermediate/stem cells in progression to AI-PC and to identify chemotherapeutic agents that kill prostate stem cells and potentially cure prostate cancer.

**Materials and Methods**

**Reagents**

Protease inhibitor cocktail tablets were purchased from Roche Applied Sciences. Coomassie blue was purchased from EMD Chemicals, Inc. All other chemicals were purchased from Sigma-Aldrich.

**FG/Tag Transgenic Mice**

We used the FG/Tag (previously referred to as Gyr/T-15) transgenic mouse model of AI-PC to investigate the early molecular events involved in the neoplastic transformation of normal prostate epithelial cells (30-32). Transgenic mice (CBA × C57) were identified by DNA slot blot analysis as previously described (30, 31). These mice, bred to homozygosity, begin to develop palpable prostate tumors at ≥13 weeks of age, an earlier time than in hemizygous mice (16 weeks; ref. 36). All animal studies were carried out with the approval of the Institutional Animal Care and Use Committee of the Miami Veterans Affairs Medical Center (Association for Assessment and Accreditation of Laboratory Animal Care accredited) and conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

**Prostates and Tumors**

Homozygous male transgenic mice were palpated in the urogenital region to detect prostate tumor mass. Mice without palpable prostate tumor mass were killed (n = 45, at 16 to 32 weeks) and ventral and dorsal-lateral prostate lobes removed under a dissecting microscope, fixed overnight in 10% buffered formalin, dehydrated, embedded in paraffin, sectioned at 5 μm, and baked at 55°C overnight. Prostates from transgenic males at 3 (n = 4), 6 (n = 8), 10 (n = 8), and 14 (n = 8) weeks were similarly processed. Small portions of primary prostate tumors (n = 10) were also fixed overnight in formalin and processed for immunohistochemistry.

**Immunohistochemistry**

For immunostaining, endogenous peroxides were blocked using 3% H₂O₂ in methanol for 5 min. Antigen retrieval was done by incubating sections in hot 10 nmol/L citrate buffer (pH, 6.0) for 20 min. Immunostaining for Tag on consecutive sections was done using a 1:30 dilution of mouse monoclonal antibodies specific for SV40 large Tag (PAb101; BD Biosciences PharMingen) and 1:100 dilution of p63 (4A4) and pan-cytokeratins (C11; Santa Cruz Biotechnology) and the Vector Mouse on Mouse (MOM) Peroxidase Kit (Vector Laboratories) following the manufacturer’s instructions. Immunostaining for AR (N-20; Santa Cruz Biotechnology) and synaptophysin (Invitrogen) was done using a 1:10 (AR) and 1:100 (synaptophysin) dilution of rabbit polyclonal antibody and a 1:100 dilution of biotinylated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (Vector Laboratories).

Immunostaining for E-cadherin (1:800 dilution, clone 36; BD Biosciences) was done similarly to Tag and p63. All primary and secondary antibodies were incubated for 30 min at room temperature. Specific color was developed with the Vector ABC kits and 3,3’-diaminobenzidine substrate kit (Vector Laboratories), and the sections were lightly counterstained with hematoxylin, dehydrated, and glass coverslipped. For the negative controls, we used the same concentration of mouse or rabbit IgG (Santa Cruz Biotechnology) instead of specific primary antibodies, resulting in the lack of immunostaining (data not shown).

**Double Immunofluorescence**

For double immunofluorescence, after first immunostaining for Tag, we used Fluorescein Avidin DCS (1:300 dilution;
Vector Laboratories) for 5 min, followed by avidin/biotin blocking for 15 min, immunostaining with p63, and detection with Texas Red DCS for 5 min. Images were captured using a Nikon fluorescence microscope with FITC and Texas Red filters and merged using IP Lab software.

**Isolation of Cell Lines from FG/Tag Primary Prostate Tumors**

Transgenic mice with primary prostate tumors were killed (n = 4), and small outer tumor portions were removed under sterile conditions. Tumor pieces were washed with PBS, minced into smaller pieces (1 mm³), centrifuged, resuspended in growth media, and treated with 100 units/mL of collagenase II (Invitrogen), with gentle rocking overnight at 37°C. The growth media consists of RPMI 1640, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin, 10 µg/L epidermal growth factor, 10 mg/L bovine pituitary extract, 4 mg/L insulin (Invitrogen), 10 µg/L cholera toxin (Calbiochem), 1 µg/L hydrocortisone (Sigma), and 10% fetal bovine serum (Hyclone). Clumps were centrifuged to remove single cell fibroblasts and maintained in growth media. A differential trypsinization (1 min) was done to further remove fibroblasts. After 3 to 4 weeks, cells with epithelial-like morphology were cloned, expanded, and analyzed by Western blot. Three cell lines (FG-Tag-15, 17, and 21) were further grown for >10 passages without changes in morphology.

**Human Prostate Cancer Cell Lines**

Human prostate carcinoma cell lines LNCaP, DU 145, and PC-3 were obtained from the American Type Culture Collection (38). These cells were maintained in RPMI 1640 (Invitrogen) with 5% fetal bovine serum (Hyclone), 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin (Invitrogen). LHSR-AR cells were obtained from William Hahn (Harvard Medical School, Boston, MA) and maintained in PREGM (CC-3166; Cambrex) and 100 units/mL penicillin, 10 µg/mL streptomycin, and 0.25 µg/mL amphotericin. A differential trypsinization (1 min) was done to attach overnight. The next day, cells were seeded in 12-well plates and allowed to attach overnight. The next day, cells were seeded in 12-well plates and allowed to attach overnight. The next day, LHSR-AR cells were seeded in 12-well plates and allowed to attach overnight. The next day, –1261FG/Luc; ref. 30). For transfection into LHSR-AR, cells were seeded in 12-well plates and allowed to attach overnight. The next day, –1261FG/Luc or promoterless pGL4-Basic plasmid was cotransfected with CMV/β-galactosidase plasmid (Clontech) using FuGene 6 HD (Roche Applied Sciences) following the manufacturer’s instructions. After 24 h, media was removed, and fresh media containing 10 nmol/L DHT or 0.1% ethanol was added. Luciferase activity was measured after 48 h in culture in an AutoLumat LB 953 luminometer with a luciferase assay kit (Promega); measurements were normalized to β-galactosidase activity. Luciferase activity values were expressed as light units/β-galactosidase and the relative values were divided by promoterless activity. All plasmids used in transfections were purified with the Maxi tip-500 kit (Qiagen Inc.).

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**References**


**RT-PCR**

RNA was isolated from mPT-15, 17, and 21 cell lines using QIAshredder and RNeasy miniprep kit (Qiagen Inc.). RNA was isolated from FG/Tag mouse prostate tumor and non-transgenic mouse prostate and testis using the LiCl-urea method (53). All RNAs were treated with RNase-free DNase (Ambion) to remove possible DNA contamination. The following mouse-specific DNA oligonucleotides (Operon Technologies) were used for RT-PCR: CD44, sense 5'-AATGTAAAC-CTGCCGCTACG-3' and antisense 5'-GGAGGTGGTGAGCT-GAC-3'; CD133, sense 5'-ACACACACCAAGAACAGGC-3' and antisense 5'-GGAGCTGACTGAATTAGGG-3'; p63, sense 5'-GGAATAAATGCCCCAGACT-3' and antisense 5'-GATTGGAGAGAGGCGATCAA-3'; Oct4, sense 5'-GGC-GTTTTCTTITGGAAGGTGTC-3' and antisense 5'-CTCGAACCACATCCTTCTCT-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5'-TGGCATTGGAGG-GCTCATGC-3' and antisense 5'-ATGCCATGAGCTCAGTTCGAGC-3'. Conditions for RT-PCR were 2 min at 94°C for 1 cycle; 1 min at 94°C, 1 min at 55°C for GAPDH; 57°C for p63; 60°C for CD44/CD133; 62°C for Oct4, and 2 min at 72°C for 35 cycles; 7 min at 72°C for 1 cycle using SuperScript III (Invitrogen). Predicted sizes of the amplified products were visualized on a 2% agarose gel. Negative controls were addition of Taq DNA polymerase (Promega) instead of SuperScript III.

**Transfection of FG/Luc Reporter Plasmid**

The same FG 5'-flanking sequence used in the FG/Tag mice was cloned into pGL4-Basic luciferase reporter plasmid (Promega). A 1.3-kb SacI-BglII DNA fragment containing the FG promoter (–1261 to +51) was excised from –1261G/ Luc (pGL2-Basic) and cloned into a SacI-BglII–digested pGL4-Basic plasmid (–1261FG/Luc; ref. 30). For transfection into LHSR-AR, cells were seeded in 12-well plates and allowed to attach overnight. The next day, –1261FG/Luc or promoterless pGL4-Basic plasmid was cotransfected with CMV/β-galactosidase plasmid (Clontech) using FuGene 6 HD (Roche Applied Sciences) following the manufacturer’s instructions. After 24 h, media was removed, and fresh media containing 10 nmol/L DHT or 0.1% ethanol was added. Luciferase activity was measured after 48 h in culture in an AutoLumat LB 953 luminometer with a luciferase assay kit (Promega); measurements were normalized to β-galactosidase activity. Luciferase activity values were expressed as light units/β-galactosidase and the relative values were divided by promoterless activity. All plasmids used in transfections were purified with the Maxi tip-500 kit (Qiagen Inc.).


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