Role for Stromal Heterogeneity in Prostate Tumorigenesis

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Abstract
Prostate cancer develops through a stochastic mechanism whereby precancerous lesions on occasion progress to multifocal adenocarcinoma. Analysis of human benign and cancer prostate tissues revealed heterogeneous loss of TGF-β signaling in the cancer-associated stromal fibroblastic cell compartment. To test the hypothesis that prostate cancer progression is dependent on the heterogeneous TGF-β responsive microenvironment, a tissue recombination experiment was designed in which the ratio of TGF-β responsive and nonresponsive stromal cells was varied. Although 100% TGF-β responsive stromal cells supported benign prostate growth and 100% TGF-β nonresponsive stromal cells resulted in precancerous lesions, only the mixture of TGF-β responsive and nonresponsive stromal cells resulted in adenocarcinoma. A computational model was used to resolve a mechanism of tumorigenic progression in which proliferation and invasion occur in two independent steps mediated by distinct stromally derived paracrine signals produced by TGF-β nonresponsive and responsive stromal cells. Complex spatial relationships of stromal and epithelial cells were incorporated into the model on the basis of experimental data. Informed by incorporation of experimentally derived spatial parameters for complex stromal–epithelial relationships, the computational model indicated ranges for the relative production of paracrine factors by each cell type and provided bounds for the diffusive range of the molecules. Because SDF-1 satisfied model predictions for an invasion-promoting paracrine factor, a more focused computational model was subsequently used to investigate whether SDF-1 was the invasion signal. Simulations replicating SDF-1 expression data revealed the requirement for cooperative SDF-1 expression, a prediction supported biologically by heterotypic stromal interleukin-1β signaling between fibroblastic cell populations. The cancer stromal field effect supports a functional role for the unaltered fibroblasts as a cooperative mediator of cancer progression. Cancer Res; 71(10); 3459–70. © 2011 AACR.

Major Findings
A computational model was used to resolve a mechanism of prostate epithelial tumor initiation dictated by paracrine signaling. Model simulations and biological experiments supported fibroblast responsiveness to TGF-β as an initial tumor suppressor and subsequent mediator of tumor progression. TGF-β nonresponsive stromal cells permitted the initial proliferative step. Cooperativity of both TGF-β nonresponsive and responsive stromal cells was required for the secondary invasion step. Thus, stromal heterogeneity in TGF-β responsiveness enables a paracrine-mediated tumorigenesis.

Introduction
Organ development and epithelial tumor progression are the result of reciprocal and sequential stromal–epithelial interactions. Prostate cancer, the most common noncutaneous cancer in men, is initiated by the transformation of luminal epithelial cells that line the prostactic ducts resulting in altered proliferation leading to prostatic intraepithelial neoplasia (PIN; ref. 2). Loss of cell polarity and invasion through the basement membrane into the surrounding prostatic stroma by the transformed epithelia mediate the establishment of prostatic adenocarcinoma. The sequential progression of epithelial differentiation to adenocarcinoma is dictated by its inherent genetic stability and the cues provided by the surrounding microenvironment (3–5). Tgfrb2cko mice, with a conditional knockout (KO) of the TGF-β type II receptor (TβRII) gene (Tgfr2) in stromal fibroblasts, develop PIN lesions and subsequently prostate adenocarcinoma (6, 7). Human prostate cancer is analogously associated with the heterogeneous loss of TβRII expression in the stromal compartment (7, 8).

We used a flexible, biologically intuitive, hybrid computational model to investigate mechanisms for cell–cell communication in stromal and epithelial compartments for tumor initiation and progression. Hybrid cell-based mathematical modeling has developed into a means of explaining biological mechanisms, combining explicit cell representations with...
Quick Guide to Equations and Assumptions

Our computational model couples a discrete, stochastic description of cells at the macroscopic scale with a continuous, deterministic description of paracrine factor diffusion at the microscopic scale.

Discrete, stochastic description of cells

A spatially explicit model for cell positions incorporates the spatial scales of epithelial and stromal cells located at discrete lattice locations, based on their positions in a histologic cross section of mouse prostate (epithelia circumscribing prostate ducts surrounded by stromal fibroblastic cells). Fibroblasts were randomly identified as dominant point sources for either $M_1$ or $M_2$ paracrine factors, with probabilities depending on the simulated fraction of wild-type (WT) and Tgfbr2-KO fibroblasts, respectively.

Paracrine factor production

Forty-four stromal cells were point sources for paracrine factors $M_1$ and $M_2$. We denote the set of WT [normal (N)] and Tgfbr2-KO [altered (A)] stromal cell positions as $S_N$ and $S_A$ in locations $S_N = \{(x_i, y_i)|i = 1, 2, \ldots, n\}$ and $S_A = \{(x_i, y_i)|i = 1, 2, \ldots, a\}$, with $n + a = 44$. Let $m_1$ and $m_2$ be the concentrations (μmol/L) of paracrine factors $M_1$ and $M_2$, respectively. $M_1$ and $M_2$ are dominantly produced by Tgfbr2-KO and WT fibroblastic cells, respectively, and their production rates ($\dot{q}m_1/\partial t$ and $\dot{q}m_2/\partial t$) are proportional to the number of Tgfbr2-KO cells and WT cells.

Paracrine factor diffusion

To model diffusion in the context of the complex geometry of the mouse prostate, paracrine factors diffuse in the region between and surrounding (but not within) irregularly shaped ducts. In normal prostate, the basement membrane provides a diffusion barrier between epithelial and stromal cells. The effects of the basement membrane as a barrier to diffusion and the erosion of the basement membrane were not modeled here. Instead, the model considers the steady-state levels of paracrine factors and their effect on epithelial cells with the assumption that any erosion of the basement membrane had already occurred. Because $M_1$ and $M_2$ diffuse freely in the interductal space, with certain metabolic decay rates, the kinetics of $m_1$ and $m_2$ can be described as uncoupled reaction diffusion:

$$\frac{\partial m_1}{\partial t} = k_1 \sum_{i=1}^{n} \delta(x_i, y_i) - k_d m_1 + D_1 \nabla^2 m_1$$

$$\frac{\partial m_2}{\partial t} = k_2 \sum_{i=1}^{a} \delta(x_i, y_i) - k_d m_2 + D_2 \nabla^2 m_2$$  \hspace{1cm} (A)

where $k_1$ and $k_2$ are production rates (μm/s), $k_d$ are decay rates (μm/s), and $D_1$ and $D_2$ are the diffusion coefficients (μm$^2$/s) of paracrine factor $M_1$ and $M_2$, respectively. The Dirac delta function $\delta(x, y)$ is defined as follows:

$$\delta(x, y) = \begin{cases} \infty & \text{if } (x, y) = (x_i, y_i) \\ 0 & \text{otherwise} \end{cases}$$

The initial conditions for Equation (A) were given by $m_1(x, y, 0) = 0$ and $m_2(x, y, 0) = 0$. No-flux boundary conditions on the ducts and the outer rectangle are given by:

$$\frac{\partial m_1}{\partial n} = 0$$

$$\frac{\partial m_2}{\partial n} = 0$$

where $n$ indicates the outward facing normal of the boundary.

Paracrine factor response

Epithelial cells respond to paracrine factors by transforming from normal to proliferative at threshold levels of $M_1$ and from proliferative to invasive at threshold levels of $M_2$. Levels of paracrine factors are measured at steady-state concentrations. This simplification is justified if the time scales of epithelial transformation are much slower than the time scales of paracrine factor diffusion, which is reasonable as diffusive factor steady-state concentrations establish at fast time scales compared with cellular...
responses (17, 18). Normal (N), proliferative (P), and invasive (I) cells are labeled as $E_N$, $E_P$, and $E_I$ and their concentrations are labeled as $e_N$, $e_P$, and $e_I$ ($\mu$mol/L). For the time scales of $e_N$, $e_P$, and $e_I$, we consider $m_1$ and $m_2$ in their quasi-steady states, $m_1^*$ and $m_2^*$. For $m_1^*$ and $m_2^*$, we solve the steady-state equations [from Equation (A)] as follows:

$$D_1 \nabla^2 m_1^* = k_2 m_1^* - k_1 \sum_{i=1}^{n} \delta_{(x,y_i)}$$
$$D_2 \nabla^2 m_2^* = k_2 m_2^* - k_1 \sum_{i=1}^{n} \delta_{(x,y_i)}$$

(B)

Next, the proposed underlying mechanism for epithelial transformation can be written as follows:

$$E_N + M_1^* \rightarrow E_P$$
$$E_P + M_2^* \rightarrow E_I$$

(Ca)

for quasi-steady paracrine factor levels $M_1^*$ and $M_2^*$ that were above threshold levels $T_{M_1}$ and $T_{M_2}$. Therefore, the proposed 2-step mechanism for cancer progression was as follows:

$$E_N \rightarrow E_P \rightarrow E_I$$

(Cb)

On the other hand, for $M_1^*$ and $M_2^*$ below threshold levels:

$$E_N + M_1^* \rightarrow E_N$$
$$E_P + M_2^* \rightarrow E_P$$

(Cc)

Assuming $E_N$, $E_P$, and $E_I$ were immobile around the ducts, the kinetics of $e_N$, $e_P$, and $e_I$ can be written as follows:

$$\frac{de_N}{dt} = -k_N \cdot H(m_1^* - m_1) \cdot m_1^* \cdot e_N$$
$$\frac{de_P}{dt} = -k_P \cdot H(m_2^* - m_2) \cdot m_2^* \cdot e_P + k_N \cdot H(m_1^* - m_1) \cdot m_1^* \cdot e_N$$
$$\frac{de_I}{dt} = k_P \cdot H(m_2^* - m_2) \cdot m_2^* \cdot e_P$$

where $k_N$ and $k_P$ are transformation rate constants of $M_1$ and $M_2$ to $E_N$ and $E_P$. The Heaviside function $H(x)$ was defined as follows:

$$H(x) = \begin{cases} 0 & x < 0 \\ 1 & x \geq 0 \end{cases}$$

Because the total concentration of cells is constant ($e_N + e_P + e_I = C_0$), we may drop one of $e_N$, $e_P$, and $e_I$ equations. Here, we dropped the equation for $e_I$ ($e_I = C_0 - e_N - e_P$). For initial conditions,

$$\begin{cases} e_N(0) = C_0 \\ e_P(0) = 0 \\ e_I(0) = 0. \end{cases}$$

Additional details including a description of the model parameters (diffusion and production rates, diffusion length, and abundance of paracrine factors), steady-state analyses, and the numerical scheme are described in Supplementary Information. Quasi-steady-state paracrine factor levels were solved using the finite element method by using the MATLAB PDE (partial differential equation) toolbox (Mathworks).

continuum-based biological descriptions (9–16). Our hybrid computational model combines a discrete model for the differentiation of individual epithelial cells with a continuum model of stromally derived signaling factor diffusion. Secreted signaling factors in this study include paracrine factors (that traverse the basement membrane between the stroma and the epithelium) and heterotypic stromal signaling factors (that communicate between populations of fibroblastic cells). Herein we report that biological experiments (in vivo tissue recombination and in vitro biological models) and computational mathematical modeling together test and support hypotheses for intercellular signaling within a heterogeneous...
prostatic stromal compartment and prostatic epithelial progression to adenocarcinoma.

Materials and Methods

Immunofluorescence [for CD90 and phosphorylated Smad2 (p-Smad2)] and immunohistochemistry (for p-Smad2) of paraffin-embedded mouse or human prostate tissue sections were done as described (7, 19). A pathologist blindly evaluated immunohistochemical staining. Primary stromal cells were isolated, cultured, and recombined with prostatic epithelial organoids as described (7, 19, 20). Tissue recombinations were done as previously described (19, 21). For inhibition of DNA methylation, stromal fibroblasts were cultured for 5 days in the presence of 10 μmol/L 5-aza-deoxycytidine [(5-aza-dC); Sigma]. Tgfbr2fspKO prostate stromal cells labeled with 2.0 μmol/L 5-chloromethylfluorescein diacetate (CMFDA) dye (Molecular Probes, Invitrogen) were cocultured with Tgfbr2-Flox stromal cells in 100-mm tissue culture dishes at 2 × 10^6 cells per dish for 20 hours. Cells were sorted on a BD FACs Aria II flow cytometer into cold RNA extraction buffer. DNase I–treated total RNA was isolated using the Qiagen RNeasy Kit as instructed. Expression of SDF-1, SFRP1, –actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was evaluated by quantitative real-time PCR (qRT-PCR). Amplification specificity was confirmed by melt-curve analysis and agarose gel visualization. Expression values were normalized for relative expression by the ΔΔCt method. PCR primer sequences, numerical scheme, and steady-state analysis methods are found in Supplementary Information. All human tissue acquisition and animal procedures were approved by the Vanderbilt University Institutional Review Board and Vanderbilt Institutional Animal Care and Use Committee, respectively.

Results

Stromal heterogeneity: a 2-step mechanism hypothesized for paracrine signaling–mediated tumor progression

To extend our study of stromal TGF-β signaling, p-Smad2 expression was tested in a tissue microarray consisting of 80 human prostate cancer samples. Independent of Gleason grade, p-Smad2 was expressed homogeneously throughout the epithelial compartment of all prostate samples examined. However, the stromal fibroblastic cells had heterogeneous p-Smad2 staining in prostate cancer tissues (Fig. 1A). Corresponding tissues of benign prostate hyperplasia (BPH) had a more homogeneous p-Smad2 expression among stromal fibroblasts. Previous studies have suggested CD90 to be a marker heterogeneously expressed in prostate cancer-associated stromal cells (22). To further explore the role of stromal TGF-β signaling in prostate cancer progression, we colocalized CD90 and p-Smad2 expression by immunofluorescence in human prostate cancer-associated (N = 10 patient samples, Gleason score range: 7–9) and non–cancer-associated (N = 6 patient samples) prostatic stromal fibroblastic cells. Interestingly, CD90 and p-Smad2 expression was heterogeneous in the stromal cells of human prostate cancer tissues (Fig. 1B). CD90 expression was generally limited to cancer-associated stromal cells lacking p-Smad2 expression. Stromal compartments associated with benign epithelia expressed both CD90 and p-Smad2.

Tgfbr2fspKO mice were also found to have a heterogeneous loss of downstream Smad2 activation in stromal fibroblasts (Fig. 1C), partially attributable to the FSP1 promoter being active in only 40% to 60% of prostate fibroblasts. To determine the biological ramification of the observed stromal heterogeneity in TGF-β signaling, tissue recombination allografting experiments were conducted (N = 6 grafts per group). Prostatic stromal cells from control Tgfbr2-Flox/E2/Flox mice (Tgfbr2-Flox) and those from Tgfbr2fspKO (Tgfbr2-KO) were cultured. Although the loss of stromal heterogeneity is not observed in vivo, in the context of stromal–epithelial interactions, the culturing of prostatic stromal cells from Tgfbr2fspKO mice beyond 6 passages results in Tgfbr2 knockout in 100% of the cells (19). The recombination of WT prostatic epithelial organoids with Tgfbr2-Flox stroma under the renal capsule of syngeneic mice for 12 weeks yielded morphologically normal prostatic tissues (Fig. 2). In contrast, recombination of WT prostatic epithelial organoids with Tgfbr2-KO stromal cells resulted in PIN development in the same time period. The prostatic recombinants resulting from the equal mixture of Tgfbr2-Flox and Tgfbr2-KO stromal cells with WT prostatic epithelia resulted in prostatic adenocarcinoma. The most immediate interpretation of this striking observation is that the epithelia transform from normal to aggressive in 2 steps, where in the first step, Tgfbr2-KO stromal cells contribute to initiating epithelial transformation and, in the second step, Tgfbr2-Flox stromal cells contribute to the progression of initiated epithelia to an aggressive phenotype (invasion). Furthermore, we believe that it is likely that stromal–epithelial interactions are mediated by diffusive factors of some kind (23). Thus, we hypothesized a diffusive, 2-step mechanism for epithelial transformation dependent on stromal heterogeneity (TGF-β-responsive and nonresponsive stromal cells). In step 1 of the hypothesized model, TGF-β nonresponsive (Tgfbr2-KO) stromal cells produce a diffusive paracrine factor-1 (M1), which causes epithelial cells to transform from normal to proliferative. In step 2, TGF-β responsive stromal cells (WT and Tgfbr2-Flox) produce a diffusive paracrine factor-2 (M2), which supports the progression of proliferative epithelial cells to invade through the ductal basement membrane. For simplification, TGF-β responsive WT and Tgfbr2-Flox stromal cells will be referred to hence forward as WT.

A computational model provides proof of concept for the hypothesized 2-step mechanism of tumorigenesis

A mathematical model describing epithelial and stromal fibroblastic cell signaling interactions was developed to test the viability of this proposed mechanism for epithelial transformation. Stromal and epithelial cell positions were modeled according to a histologic section of mouse prostate (Fig. 3A), and simulations were used to investigate the predictions of a model for paracrine interaction. The model assumed epithelial...
cells transformed in response to threshold levels of stromal factors at steady-state concentrations (see Quick Guide).

Implementation of the 2-step mechanism in the computational model yielded results that qualitatively reproduced experimental observations over a wide range of simulation parameters. In simulations, the ratio of WT and Tgfbr2-KO stromal cells was varied from 0 to 1. The measured response variable for all simulations was the fraction of epithelial cells that transformed (step 1, proliferative, or step 2, invasive). The fraction of transformed epithelial cells for a typical set of simulation parameters is shown in Fig. 3B. For 100% WT stromal cells [fraction altered stroma (FAS) = 0.00], there were no associated proliferative (step 1) or invasive (step 2) epithelial cells. For 100% Tgfbr2-KO stromal cells (FAS = 1.00), there were only normal and proliferative epithelia. The computational model yielded invasion only at heterogeneous mixtures.
of stromal cells. Key model parameters [paracrine factor diffusion length (L), paracrine factor abundance (A), and paracrine factor threshold (T)] could be varied to tune the fraction of epithelial cells that became proliferative or invasive; however, all parameters yielded results that were either identically zero or qualitatively similar to those shown in Fig. 3B, in which the fraction of proliferative epithelial cells increased monotonically from zero with the fraction of Tgfbr2-KO stroma (step 1) and the fraction of invasive epithelia was nonzero only for intermediate fractions of Tgfbr2-KO stroma (step 2). For example, as a function of abundance of each paracrine factor (Fig. 3C), low abundance levels of $M_1$ (e.g., for $A_{M_1} < 200$) resulted in no epithelial transformation and at higher abundance of $M_1$ the cells became proliferative.

Figure 2. Percentage of WT and Tgfbr2-KO stroma in prostate tissue recombinant allografts promoted cancer progression. A, hematoxylin and eosin staining of grafted WT prostatic epithelial organoids recombined with 100% Tgfbr2-Flox prostate stromal cells had normal glandular architecture. B, there was observed progression to adenocarcinoma in grafts of WT prostatic epithelial organoids recombined with a 50:50 mixture of Tgfbr2-Flox and Tgfbr2-KO prostate stromal cells. C, PIN lesions developed in grafts of WT prostatic epithelial organoids recombined with 100% Tgfbr2-KO prostate stromal cells. Arrowheads (B and C) indicate transformed epithelia.

Figure 3. Establishment of a biologically informed computational model. A, hematoxylin and eosin–stained section of normal mouse prostate (left) was used to assign corresponding positions for simulated cells (right) assuming 50% Tgfbr2-KO stromal cells (black, epithelia; blue, WT stromal cells; cyan, Tgfbr2-KO stromal cells). B, simulations indicated that stromal heterogeneity altered the proliferative and invasive potential of prostate epithelial cells, where greatest invasion occurred at heterogeneous mixtures of stromal cells and the extent of epithelial proliferation and invasion depended on the ratio of paracrine factor production and threshold response. The number of cells that became proliferative (step 1, dashed line) and invasive (step 2, solid line) was based on paracrine factor diffusion lengths ($L_{M_1} = 200 \mu m$ and $L_{M_2} = 300 \mu m$), transformation response thresholds ($T_{M_1} = 0.0453$ and $T_{M_2} = 0.3432$ paracrine factor units), and fixed total paracrine factor abundance per cell source ($A_{M_1} = A_{M_2} = 10,000$ paracrine factor units). Error bars indicate the standard deviation of 100 simulations. C, phase diagram illustrating the final epithelial classifications as a function of Tgfbr2-KO cell $M_1$ abundance ($y$-axis) and WT cell $M_2$ abundance ($x$-axis) in a tissue at 50:50 mixture of WT and Tgfbr2-KO stromal cells. If the production rate of both paracrine factors was low relative to the transformation threshold, then the cells remained normal. Paracrine factor diffusion lengths and thresholds for $M_1$ and $M_2$ are as in B.
Finally, cells became invasive only if both $M_1$ and $M_2$ abundance levels were sufficiently high. In particular, the simulations showed that WT stromal cells resulted in normal tissue, Tgfbr2-KO cells induced proliferative and hyperplastic epithelia, and only the heterogeneous mix of stromal cells induced invasive epithelia. This reproduction of the qualitative features of the experimental system provided a proof of concept for the hypothesized 2-step mechanism of tumorigenesis.

Parameters for the computational model were chosen to match experimental results quantitatively, but parameters were not constrained uniquely. It was experimentally observed that at a 1:1 ratio of WT and Tgfbr2-KO stromal fibroblastic cells, the majority of epithelial cells became proliferative whereas a minority of epithelial cells became invasive. Model parameters could be tuned to match experimental epithelial transformation rates. Because increasing the diffusion length, increasing the abundance, and decreasing the threshold level all have the effect of increasing the fraction of cells that transform, this tuning was nonunique. In the Supplementary Information, we describe how we tuned the parameters to find a nonunique set that generated and matched experimental results for the fraction of proliferative and invasive epithelial cells (Supplementary Figs. S1 and S2) and that the model predicted tissue morphology and stromal cell density influence on epithelial progression toward cancer (Supplementary Fig. S3).

**Computational model predictions for the relative production of stromal factors**

In our proof-of-principle consideration of the 2-step model for tumorigenesis, we implemented the assumption that the paracrine factor produced by Tgfbr2-KO fibroblasts that supported proliferation ($M_1$) was not produced by WT fibroblasts at any level and, likewise, that the paracrine factor produced by WT fibroblasts that supported invasion ($M_2$) was not produced by Tgfbr2-KO cells. However, candidate-secreted factors dominantly expressed by fibroblastic cells that support proliferation and/or invasive cancer progression include Wnt-3a and SDF-1, respectively (7, 19, 24). Furthermore, we identified that Tgfbr2-KO stromal cells upregulated Wnt signaling by the silencing of its antagonists SFRP1 and SFRP2. Interestingly, the treatment of Tgfbr2-KO stromal cells with the DNA methylation inhibitor 5-aza-dC restored SFRP1 and SFRP2 mRNA expression nearly to that observed for WT stromal cells (Fig. 4). Similarly, Tgfbr2-KO stromal cells epigenetically downregulate SDF-1 expression, also restored by 5-aza-dC treatment. Thus, Tgfbr2-KO stroma supported paracrine Wnt signaling whereas the WT stroma promoted SDF-1 expression.

In our tissue recombination allografting experiments, it seemed that more than 80 ± 15% of epithelial cells transformed to become proliferative in the case of organoids with

![Figure 4. Epigenetic regulation of Wnt and SDF-1 signaling by TGF-β in prostate stromal cells. qRT-PCR revealed reversal of methylation-induced silencing of SFRP1, SFRP2, and SDF-1 by 5-aza-dC treatment of Tgfbr2-KO stroma, comparable with WT stroma. Data are presented as mean ± SD and normalized to GAPDH.](http://www.aacrjournals.org)
Tgfbr2-KO stromal cells. Our computational model thus predicted that the production of the factor in tissues supporting proliferation must be more than 12-fold that of tissues not supporting proliferation. However, we previously found that the production of Wnt-3a by WT stromal cells was 20% that of Tgfbr2-KO stromal cells (7), resulting in only a 3-fold difference in factor production in the tissues supporting and not supporting proliferation (Table 1). In other words, model parameters could not be found for $M_1$ (diffusion rate, decay rate, and thresholds for transformation) such that 80% of the epithelial cells would become proliferative at a 50:50 mixture of stromal cell types but 0% would become proliferative when the stromal cells are 100% normal. Indeed, we found that over a large parameter range (e.g., diffusion lengths $\leq 5000 \mu m$), the model could not accommodate this level of $M_1$ production by Tgfbr2-KO stromal cells. This was shown by the observation that for any diffusion length $L \leq 800 \mu m$, a threshold cannot be found such that 80% of epithelial cells would become proliferative at the 100% Tgfbr2-KO mixture but 0% of epithelial cells would become proliferative at 100% WT stroma (Fig. 5A).

### Consistency of SDF-1 as a $M_2$ paracrine factor

The chemokine SDF-1/CXCL12 is implicated as a promoter of tumor epithelial progression to an invasive phenotype (25, 26). Expression of SDF-1 and its receptor (CXCR4) is positively regulated by TGF-β signaling (27, 28). Previously, we have conducted microarray analysis of laser-capture microdissected (LCM) Tgfbr2$^{loxP}E2/E2$ and Tgfbr2$^{esp KO}$ prostate stromal cells (data available in GEO database record GSE22130) isolated from mice (29). The data revealed the heterogeneity of gene expression of the stroma and a 77-fold increase in SDF-1 expression by Tgfbr2$^{esp KO}$ prostate stroma. This significant induction of SDF-1 mRNA expression in a heterogeneous stromal microenvironment is consistent with SDF-1 as a candidate paracrine factor in the promotion of prostate cancer progression. Independently, SDF-1 mRNA levels in isolated cultures of WT and Tgfbr2-KO prostate stromal cells were quantified by RT-PCR. WT prostate stromal cells expressed 4-fold higher levels of SDF-1 mRNA than Tgfbr2-KO prostate stromal cells when grown alone in culture. The differential expression of SDF-1 here as compared with that measured by the microarray may be due to the presence of additional in vivo stromal cell types isolated by the LCM technique as compared with an isolated pure fibroblastic population grown in culture.

In our tissue recombination allografting experiments, it seemed that more than 20 ± 5% of epithelial cells transformed to become invasive in the case of organoids with an equal mix of both stromal cells. Our computational model predicted that the production of the paracrine factor supporting invasion by the WT cells must be more than 2-fold that produced by the Tgfbr2-KO stroma. Thus, SDF-1 was consistent with $M_2$ in the 2-step mechanism for tumorigenesis because the production of SDF-1 by Tgfbr2-KO cells is only 25% that of the WT cells. In other words, model parameters can be found for $M_2$ (diffusion rate, decay rate, and thresholds for transformation) such that 20% of the epithelial cells would become invasive at the 50:50 mixture of stromal cell types but 0% would become invasive when the stromal cells are 100% normal. For this production of SDF-1 by the Tgfbr2-KO cells, we found that the computational model predicts diffusion lengths greater than 24 μm (gray region, Fig. 5B). In this way, if a factor is consistent with being a factor in the 2-step mechanism for tumorigenesis, the computational model predicts a range for the diffusion length.

### Cooperativity of paracrine factor $M_2$

To quantify SDF-1 expression by the individual stromal fibroblastic cell, cocultured mixtures of fibroblasts were separated by fluorescence-activated cell sorting (FACS), based on CMFDA (green) dye labeling of the Tgfbr2-KO cells, and SDF-1 mRNA quantified by qRT-PCR. The cocultures were designed at the previously described ratios of Tgfbr2-KO and WT stromal cells (Fig. 3B). FACS of WT stromal cells grown in 75%/25% and 50%/50% coculture with Tgfbr2-KO stromal cells revealed an approximately 5-fold and 8-fold increase in SDF-1 mRNA expression, respectively, compared with Tgfbr2-KO

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<th>Table 1. Morphogen $M_1$ production by stromal cell type</th>
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<td><strong>Fraction altered stroma</strong></td>
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<td>Experimental outcome type A: no proliferation</td>
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<td>Experimental outcome type B: 80% proliferation</td>
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NOTE: $N$, number of stromal cells. Rate of $M_1$ production by altered stromal cells = $k_1$ ($\mu m/s$). Rate of $M_1$ production by normal stromal cells = $20\% \cdot k_1$ ($\mu m/s$).
cells grown alone (Fig. 5C). Interestingly, SDF-1 mRNA levels of the Tgfbr2-KO stromal cells were elevated approximately 2-fold as a result of coculture over Tgfbr2-KO cells grown alone. The results suggested that both WT and Tgfbr2-KO stromal cells may cooperatively induce production of 1 or more heterotypic stromal signaling factors resulting in increased SDF-1 mRNA expression. Inputting the experimentally measured relative production rates of SDF-1 for paracrine factor M2 production into the computational model resulted in the experimentally observed rate of epithelial transformation (Supplementary Fig. S5).

SDF-1 upregulation by WT stroma in the case of a mixed stromal cell population compared with 100% WT stroma showed that the presence of Tgfbr2-KO cells is communicated in some way to the WT cells, suggesting, for example, that there is an heterotypic stromal signaling factor (M3) secreted by the Tgfbr2-KO cells that WT stromal cells respond to by upregulating SDF-1 expression. Simulations tested the hypothesis that an M3 factor could result in the cooperativity of SDF-1 expression (Fig. 5D). In these simulations, the geometry of cells was assumed in which stromal cells were confluent and arranged on a 2-dimensional plate, as in the coculture experiments. We modeled the production and diffusion of an M3 expressed by Tgfbr2-KO cells that would cause the upregulation of SDF-1 in WT cells at threshold levels. We identified rates of upregulation and threshold levels that would result in experimentally measured production rates of SDF-1 for 0%, 25%, and 50% fractions of Tgfbr2-KO stroma. Although we were able to find a fit within simulation error for the lowest fractions of Tgfbr2-KO stroma (light gray line, Fig. 5D), these model assumptions could not account for the decrease in SDF-1 production for higher levels of Tgfbr2-KO stroma. (Although total production of SDF-1 by WT cells may be decreasing due to the decrease in their total number as the fraction of KO stroma increases, the production per WT cell remains upregulated.) In the same way that the increase in...
SDF-1 in the heterogeneous stromal mixtures logically argued for a threshold response to a factor produced by the Tgfbr2-KO stroma, the subsequent decreases in SDF-1 production for a higher fraction of Tgfbr2-KO cells suggested a threshold response to a factor produced by the WT stromal cells. Assuming that SDF-1 was upregulated in response to simultaneous threshold levels of an additional $M_3$ secreted by the Tgfbr2-KO stroma and an $M_4$ secreted by the WT stroma allowed us to find a set of parameters that resulted in SDF-1 production levels that exactly matched experimentally measured levels within simulation error (dashed black line, Fig. 5D).

The potential for a Tgfbr2-KO–derived $M_3$ was tested in WT stromal cells. Microarray analysis of LCM prostate stromal cells from Tgfbr2-WT and Tgfbr2-KO mice revealed altered production of multiple secreted factors including the cytokines interleukin (IL)-1α (6-fold reduction), IL-1β (4-fold reduction), and IL-6 (53-fold induction) by Tgfbr2-KO stroma in comparison with the Tgfbr2-WT stroma. Each of these factors has been implicated in the regulation of SDF-1 in the literature (30–32). Together these data imply that IL-1α, IL-1β, and IL-6 could be candidate heterotypic stromal signaling factors regulating SDF-1 expression. $M_3$ candidate factors that could upregulate SDF-1 (including IL-6, IL-1α, and IL-1β) were tested on WT stroma. IL-1β increased SDF-1 expression over untreated WT cells by 2-fold (Fig. 6A). Treatment of Tgfbr2-KO stromal cells with IL-1β increased SDF-1 mRNA levels 9-fold as evaluated by qRT-PCR. Thus, IL-1β can be a candidate $M_3$ in the cooperativity mechanism between heterogeneous TGF-β responsive prostate stromal cells. IL-1α and IL-6 had little effect on SDF-1 expression by either stromal cell type. Furthermore, the biological evidence for cooperative $M_3$ paracrine factor induction in a heterogeneous stromal microenvironment supports the computational model for a 2-step paracrine-mediated tumor initiation and progression.

**Discussion**

We describe a biological model for tumor initiation and progression on the basis of heterogeneous stromal production of diffusive factors that mediate epithelial transformation. The aim was to account for a striking set of observations in which heterogeneity (but not homogeneity) of stromal fibroblastic cells was associated with tumorigenesis. Given obtained biological data for rates of epithelial transformation, the model predicted ranges for the relative fold production of factors. Given experimental measures of factor production, the model reported whether the factor was consistent with the biological model and predicted its diffusive range.

Considering the ternary observations of the tissue recombination allografting experiments, it seemed unlikely that alternative hypotheses to the proposed mechanism (for example, that the epithelial progression does not occur in

![Figure 6. Paracrine signaling between stromal subtypes drives carcinogenesis. A. SDF-1 mRNA expression was measured in Tgfbr2-Flox and Tgfbr2-KO prostate stromal cells by qRT-PCR in response to treatment with the candidate $M_3$ heterotypic stromal signaling factor, IL-1β. B. A model of stromal TGF-β responsiveness driving prostate carcinogenesis. Loss of stromal responsiveness to TGF-β resulted in increased production of TGF-β and Wnt-3a by the stroma. Although increased Wnt paracrine signaling promoted epithelial proliferation, the increase in TGF-β in the microenvironment resulted in CXCR4 expression by the epithelium, subsequently increasing its sensitivity to SDF-1. WT and KO stroma cooperate to express elevated SDF-1, driving the progression of prostatic carcinogenesis. CAF, carcinoma-associated fibroblasts.](https://www.cancerres.aacrjournals.org/content/cr/71/10/3468/F6.large.jpg)
at least 2 steps or that these steps are not mediated by the stroma) would hold true. Certainly, the mechanism may be more complex, with added layers of interaction. Much literature supports that alterations in the stroma alone, independent of genetic events in the epithelium, can promote progression of neighboring epithelium to cancer (6, 21, 33–37). The stepwise nature of human carcinoma progression has been highlighted by the Knudson "2-hit" hypothesis (38). Paracrine-mediated cancer initiation and progression likely involve at least 2 distinct steps perpetuated by 2 or more factors produced by at least 2 independent cell types. Fibroblasts represent a heterogeneous cell population, some of which express FSP-1, and accordingly were knocked out for Tgbr2 expression in the Tgbr2\(^{2\text{apkg}}\) mice. At least 2 fibroblastic cell subpopulations could be distinguished by CD90 expression (17), and we found CD90 expression coincided with heterogeneous Smad2 activity loss in human prostate cancer-associated fibroblasts. Similar fibroblastic heterogeneity in TGF-β responsiveness occurs in Tgbr2\(^{2\text{apkg}}\) mouse prostates and is perhaps functionally essential for the ensuing cancer progression.

This biologically informed computational model was independent of specific paracrine factor production. \(M_1\) and \(M_2\) could represent individual factors or a combination of factors that result in epithelial proliferation and invasion, respectively. Thus, the model is valid to the extent that stromal interactions are dominated by diffusive signaling. On the contrary, interactions are known to occur via extracellular matrix components and cell contacts. It is also possible that one step is dominantly mediated by a diffusive factor whereas the other is not. Observing an absence of epithelial transformation within the predicted diffusive range of the factor, or the persistence of epithelial transformation after the introduction of barriers to diffusion, could invalidate the model. An experiment to simultaneously test the candidacy of an \(M_1\) or \(M_2\) factor and the model would be to apply the factor exogenously with a steady concentration and measure the radius of epithelial transformation. The model is not explicitly time resolved, as only cell response to steady-state concentrations of signaling factors is considered, which was reasonable as steady-state concentrations establish at fast time scales compared with cellular responses (17, 18). However, activities occurring at intermediate time scales, such as the gradual erosion of the basement membrane as a diffusion barrier in response to tumorigenic factors and epithelial effects on the stroma (e.g., epithelial contribution to maintaining fibroblastic heterogeneity), could not be addressed by a steady-state model. Resolving the diffusion of paracrine factors over time to follow heterogeneous stromal compartment would provide a more detailed computational model of prostatic cancer progression.

With respect to details of factor production and number, the computational model is flexible and may be modified to accommodate any set of experimental observations about factor production for a report of the viability of the model with these factors. Here, the model was modified to accommodate nonlinear, cooperative levels of \(M_2\) production as observed in SDF-1 expression. Inputting the production levels phenomenologically showed that SDF-1 was consistent with the model \(M_2\). However, modeling the experimentally observed SDF-1 levels as a function of stromal heterogeneity supported heterotypic stromal communication, for example, through additional signaling factors \(M_3\) and \(M_4\). However, it is particularly true for heterogeneous stromal interactions that this communication need not be diffusive, such as through juxtacrine signaling.

The dichotomous view of TGF-β action as a tumor suppressor in benign tissues and tumor promoter in initiated cancer can involve the fibroblasts. TGF-β is tumor suppressive in WT fibroblasts because it represses the secretion of Wnt-3a (and other Stat3-induced paracrine factors; refs. 6, 7, 18, 32). TGF-β promotes SDF-1 production by fibroblasts and the expression of its receptor, CXCR4, in epithelial cells (33). The TGF-β nonresponsive fibroblasts associated with cancerous epithelia express factors such as IL-1β (\(M_3\)) to further SDF-1 production. Such TGF-β responsive heterotypic fibroblastic and stromal–epithelial signaling would support prostate cancer onset and progression (Fig. 6B).

### Conclusions

Previous studies have emphasized the critical role of carcinoma-associated fibroblasts in cancer progression. The data suggest that the unaltered WT fibroblasts in the heterogeneous mixture of fibroblastic cells that make up the cancer stroma are not merely witnesses but also contribute to procarcinogenic cues that initiate epithelia, establishing a stromal field effect (39). Cooperativity of signaling within a heterogeneous stromal compartment would provide a mechanism whereby one could explain the multifocal and polyclonal progression of prostate cancer (39, 40). This can be envisioned where an initiating event in a particular duct-confined prostatic epithelial cell causes a change in the neighboring stroma and, in turn, potentiates initiating change(s) in the adjacent epithelia at a somewhat distant ductal site(s).

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


39. Nonn L, Ananthanarayan V, Gann PH. Evidence for field canceriza-

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