Role of Notch-1 and E-Cadherin in the Differential Response to Calcium in Culturing Normal versus Malignant Prostate Cells

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Abstract

A panel of expression markers was validated and used to document that, when radical prostatectomy specimens are cultured in low (i.e., \(<260 \mu\text{mol/L})–calcium (Ca^{2+})–serum-free, growth factor–defined (SFD) medium, what grows out are not prostatic cancer cells but basally derived normal transit-amplifying prostatic epithelial cells. The selective outgrowth of the normal transit-amplifying versus prostatic cancer cells is due to the differential effect of low-Ca^{2+} medium on the structure of Notch-1 and E-cadherin signaling molecules. In low-Ca^{2+} medium, Notch-1 receptor is conformationally in a constitutively active, cell autonomous form not requiring reciprocal cell-cell (i.e., ligand) interaction for signaling. Such signaling is required for survival of transit-amplifying cells as shown by the death of transit-amplifying cells induced by treatment with a series of chemically distinct γ-secretase inhibitors to prevent Notch-1 signaling. Conversely, in low-Ca^{2+} medium, E-cadherin is conformationally inactive preventing cell-cell homotypic interaction, but low cell density nonaggregated transit-amplifying cells still survived because Notch-1 is able to signal cell autonomously. In contrast, when medium Ca^{2+} is raised to >400 \mu\text{mol/L}, Notch-1 conformationally is no longer constitutively active but requires cell-cell contact for reciprocal binding of Jagged-1 ligands and Notch-1 receptors between adjacent transit-amplifying cells to activate their survival signaling. Such cell-cell contact is enhanced by the elevated Ca^{2+} inducing an E-cadherin conformation allowing homotypic interaction between transit-amplifying cells. Such Ca^{2+}-dependent, E-cadherin-mediated interaction, however, results in cell aggregation, stratification, and inhibition of proliferation of transit-amplifying cells via contact inhibition–induced up-regulation of p27/Kip1 protein. In addition, transit-amplifying cells not contacting other cells undergo squamous differentiation into cornified (i.e., 1% SDS insoluble) envelopes and death in the elevated Ca^{2+} medium. Stratification and contact inhibition induced by elevated Ca^{2+} are dependent on E-cadherin-mediated homotypic interaction between transit-amplifying cells as shown by their prevention in the presence of a cell-impermanent, E-cadherin neutralizing antibody. In contrast to growth inhibition of normal transit-amplifying cells, supplementation of low-Ca^{2+}–SFD medium with 10% FCS and raising the Ca^{2+} to \(>600 \mu\text{mol/L}\) stimulates the growth of all prostate cancer cell lines tested.

Introduction

Human prostatic glands are composed of a simple stratified epithelium containing basal and luminal layers separated via basement membrane from a well-developed stromal compartment (1). The homeostatic maintenance of this prostatic epithelium is regulated via a hierarchical stem cell organization (2). In the prostate epithelium, stem cells are rare and are located within the basal layer (i.e., ≤1% of basal cells are stem cells (3)). Prostate stem cells proliferate rarely to replace (i.e., renew) the fraction of their progeny, which, instead of remaining as uncommitted stem cells, enter a terminal maturation process in which several sequential stages have been identified phenotypically and morphologically (3–10). The earliest stage is termed transit-amplifying cells, which has a high proliferative potential and is located in the basal layer. These transit-amplifying cells express very low to undetectable levels of androgen receptor (AR) protein and do not express prostatic differentiation marker proteins [e.g., prostate-specific antigen (PSA), human glandular kallikrein-2 (hK2), and prostate-specific membrane antigen (PSMA); refs. 4–10]. Although this subset of AR-negative transit-amplifying cells does not respond directly to androgen, these cells do require critical levels of androgen-stimulated paracrine growth factors (i.e., andromedins) for their proliferation but not survival (10). These andromedins are produced by the occupancy of the AR by its ligand within prostate stromal cells (10–12). These transit-amplifying cells express the dominant-negative, NH2-terminal truncated form of the p53-related, p63 gene (i.e., ΔNp63α isotype) within their nucleus and high levels of “basal-specific” cytokeratins (i.e., keratins 5 and 14), glutathione S-transferase a isoform (GST-Pi), standard form of CD44 (CD44s), transglutaminase type II (TGT-2), and involucrin but only low levels of luminal-specific keratins 8 and 18 (1, 4–10, 12–16). Besides proliferating, these nuclear ΔNp63α-expressing transit-amplifying cells undergo a process of maturation into “basal-intermediate” cells in the basal epithelial compartment (7–9). This maturation into basal-intermediate cells involves the loss of expression of keratin 14 while maintaining the coexpression of basal-specific keratin 5, luminal-specific keratins 8 and 18, and ΔNp63α coupled with a decrease in their growth fraction (7–9). The basal-intermediate cells continued to mature with their gain of expression of prostate stem cell antigen protein and AR mRNA but not protein during their...
migration into the luminal layer to become "luminal-intermediate" cells (7). The luminal-intermediate cells translate AR mRNA and thus express AR protein whose occupancy by androgen induces their maturing into fully differentiated, luminal secretory cells, which are nonproliferative due to their selective expression of the p27kip1 protein (5, 8). These nonproliferating luminal secretory cells express prostate-specific differentiation marker proteins like PSA, hK2, and PSMA and lack expression of basal markers keratin 5 and 14 and ΔNp63α protein (4–9). Although the engagement of nuclear AR in these luminal secretory cells does regulate PSA, hK2, and PSMA expression, it does not regulate their survival. Instead, such survival requires adequate levels of the androgen-stimulated stromally derived andromedins (11, 12).

It has been well documented that, when normal human prostate tissue is used for culturing in low (i.e., <260 μmol/L)–calcium (Ca2+)–serum-free, growth factor–defined (SFD) medium, only transit-amplifying cells have a sufficiently high rate of proliferation to allow multiple subpassaging before undergoing proliferative senescence (7, 9, 10, 14). In contrast, the low proliferation rates of prostatic stromal cells, epithelial stem cells, and luminal secretory cells in low-Ca2+-SFD medium result in the elimination of these other cell types during early serial in vitro passages (8, 9, 13, 15). Based on such selection, pure cultures of normal prostatic transit-amplifying cells (i.e., termed PrEC cells) established in and maintained with a low (i.e., 260 ± 14 μmol/L)–Ca2+-SFD medium (i.e., prostate epithelial cell basal medium (PrEBM) complete medium) are commercially available from Clonetics, Inc. (Walkersville, MD). These PrEC cells are p27kip1, AR, α-methylacetyl-CoA racemase (AMACR), PSA, hK2, and PMSA negative while expressing ΔNp63α, TGT-2, GST-Pi, CD44v6, involucrin, Notch-1, Jagged-1, and cytokeratins 5 and 14 (i.e., they are transit-amplifying cells; refs. 7, 9, 14, 19). Although not immortal, these PrEC cells can be propagated for up to 10 serial passages before becoming growth arrested (7, 9, 10).

Based on this success, many groups are using variations of low-Ca2+-SFD medium containing known growth factors (e.g., PrEBM complete medium or keratinocyte serum-free medium (K-SFM) complete medium from Invitrogen (Carlsbad, CA) in an attempt to grow sufficient numbers of prostate cancer cells from surgical material obtained at radical prostatectomy for molecular analysis and to establish new serially passagable prostate cancer cell lines (20–30). Such a serum-free approach is favored because FCS contains a variety of undefined, and thus hard to standardize, factors in addition to its potent ability to stimulate growth to unwanted prostate stromal cells (i.e., fibroblasts and smooth muscle cells). Using such a low-Ca2+-SFD medium approach, it has been claimed that pure populations of prostate cancer cells can be grown and propagated from starting prostate cancer tissue (25–28). In contrast to these low-Ca2+-SFD medium approaches, all of the presently available, serially propagated human prostate cancer cell lines (i.e., DU-145, PC-3, LNCaP, C4-2B, LAPC-4, CWR22-Rv1, CWR-R1, MDA-PCA-2A, MDA-PCA-2B, DuCap, and VCap) were originally derived andromedins (11, 12). These "in-house"–initiated epithelial cell cultures as well as normal human PrEC obtained from Clonetics were maintained routinely (up to a maximum of 10 passages) in PrEBM [i.e., a modified low-Ca2+ keratinocyte medium supplemented with bovine pituitary extract (BPE), epidermal growth factor (EGF), insulin, transferrin, hydrocortisone, retinoic acid, epinephrine, triiodothyronine, and gentamicin-amphotericin solution (Clonetics)]. The total Ca2+ level in this PrEBM complete medium was determined to be 260 ± 14 μmol/L using a colorimetric assay as described previously (31). Normal human prostatic fibroblasts were established and maintained in early-passage culture in RPMI 1640 plus 10% FCS (R-FCS) as described previously (11). Growth of cells was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (19).

The LNCaP, PC-3, and DU-145 human prostate cancer cell lines were routinely cultured in 10% FCS (Hyclone, Logan, UT) containing RPMI 1640 (Invitrogen, Carlsbad, CA) whose total Ca2+ was determined to be 650 ± 10 μmol/L. LAPC-4 human prostate cancer cells were grown in 10% FCS plus 1 mmol/L of the synthetic androgen R1881 (Perkin-Elmer, Wellesley, MA) containing Iscove's medium (Biofluid, Rockville, MD) whose total Ca2+ is 1,558 ± 10 μmol/L. MDA-PCA-2B human prostate cancer cells were grown in BRFF-HPCL (Athena ES, Baltimore, MD) complete medium containing CT, insulin, EGF, BPE, bovine serum albumin, hydrocortisone, and dihydrotestosterone plus 20% FCS whose total Ca2+ is 767 ± μmol/L in tissue culture flasks coated with FNC (i.e., fibronectin and type I collagen) coating mix (Athena ES).

957E/HETERT cells were generously provided by Dr. John S. Rhim (Center for Prostate Disease Research, Department of Surgery, Uniform Services University of Health Sciences, Bethesda, MD; ref. 26). These cells were maintained routinely in K-SFM supplemented with insulin, EGF, BPE, transferrin, hydrocortisone, triiodothyronine, and ethanalamine commercially obtained from Life Technologies (i.e., K-SFM complete medium, Invitrogen) whose total Ca2+ was determined to be 75 ± 2 μmol/L.

Western blotting. Western blots were done on cell lysates equivalent to 106 cells per lane as described previously (19). For PSA, hK2, and PSMA, the antibodies used were as described previously (32). Goat polyclonal antibodies specific for Notch-1 (C-20 diluted 1:100) and Jagged-1 (H-114 diluted 1:100) and rabbit polyclonal antibodies specific for GST-Pi (diluted 1:1,000), E-cadherin (H-108, diluted 1:200), and AR (N-20, diluted 1:200) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat polyclonal antibodies specific for the valine (1744) cleaved Notch intracellular domain (NCID) form of Notch-1 (diluted 1:1,000) was obtained from Cell Signaling (Beverly, MA).

Reverse transcription-PCR. The RNaseqy kit (Qiagen, Inc., Valencia, CA) was used to isolate RNA from cultured cells. RNA (50 ng) was subjected to reverse transcription-PCR (RT-PCR) analysis using TaqMan reverse transcription reagents and SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA). Primer sequences and conditions are as follows:
**Culture Requirements for Normal vs. Malignant Prostate Cells**

<table>
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<tr>
<th>Accession no.</th>
<th>Primer</th>
<th>Primer sequence (5'-3')</th>
<th>Localization</th>
<th>Size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Cycle no.</th>
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<td>62</td>
<td>40</td>
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<tr>
<td>v-E6-3'</td>
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<td>TATAAAACTAATAATCTACTACT</td>
<td>251-275</td>
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<td>AF486344</td>
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<tr>
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<td>CK18-3'</td>
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**Immunohistochemistry.** Immunohistochemistry was done on cells both grown to 80% confluency in four-well chamber slides (Nalge-Nunc International, Naperville, IL) and as single-cell suspensions. The binding of the primary antibody was detected using the Envision Plus kit from DAKO (Carpinteria, CA). Each of the cell lines was characterized by immunocytochemistry for the expression of (a) AR using a rabbit polyclonal N-20 at 1:500 dilution, (b) p63 using a AR-4 antibody (cocktail; NeoMarker, Fremont, CA) at 1:50 dilution, (c) p27 using kip1 mouse monoclonal antibody (BD Biosciences, San Diego, CA) at 1:50 dilution, (d) cytokeratin 5 using rabbit polyclonal MK5 (Berkeley Antibody Co., Berkeley, CA) at 1:15,000 dilution, (e) cytokeratin 8 using mouse monoclonal C51 (Imgenex, San Ramon, CA) at 1:800 dilution, (f) cytokeratin 14 using mouse monoclonal LL002 (Imgenex) at 1:100 dilution, (g) cytokeratin 18 using mouse monoclonal DC10 (Imgenex) at 1:600 dilution, (h) E-cadherin using rabbit polyclonal antibody (2 g/mL, Zymed Laboratories, South San Francisco, CA) at 10 g/mL, Zymed Laboratories, South San Francisco, CA) was used to block E-cadherin-dependent homotypic interaction. To inhibit γ-secretase, the peptide L-685,458 inhibitor (Bachem, King of Prussia, PA) and the benzodiazepine inhibitor XIX (Calbiochem, La Jolla, CA) were used at 10 μM/L as described by Shearman et al. (34) and Chrucher et al. (35), respectively. The data obtained were analyzed by one-way ANOVA. Values represent the mean ± SE. P < 0.05 was considered significant.

**Results**

**Phenotypic characteristics of normal vs. malignant prostate cells.** A panel of markers, which discriminated between normal human transit-amplifying and malignant prostatic cells, was developed and validated using commercially obtained normal transit-amplifying (i.e., PrEC) cells and a series of eight permanent human prostatic cancer cell lines. The eight cancer lines were selected to encompass the full range of clinical prostate cancers from localized cancer to soft tissue and bone metastases (Table 1). All of these malignant lines are tumorigenic in nude mice. None of these malignant lines express the prostatic basal cell characteristic markers p63 and cytokeratin 14, whereas six of eight lines express the characteristic prostatic luminal cell markers AR, PSA, hK2, and PSMA. Only the DU-145 and PC-3 cells do not express these latter luminal markers, although they and all of the eight malignant lines do express p27/kip1 and the luminal cells marker cytokeratins 8 and 18. All of the malignant cells express AMACR. In contrast, PrEC cells growing in low (i.e., 260 ± 14 μM/L)-Ca²⁺-SFD medium (i.e., PrEBM complete medium) are not tumorigenic and express the phenotypic profile characteristics of normal basal derived transit-amplifying cells (i.e., they are p63, GST-Pi, cytokeratin 5, and cytokeratin 14 positive but p27/kip1, AMACR, AR, PSA, hK2, and PSMA negative; Table 1).

**Phenotypic characteristics of cells grown from surgical material in low-calcium-serum-free, growth factor–defined medium.** Table 1 documents that there are characteristic markers that discriminate between normal transit-amplifying and prostatic cancer cells. These characteristic markers were used to determine the cell types that grow out from radical prostatectomy specimens from a series of seven patients with localized prostate cancer using low-Ca²⁺-SFD medium (i.e., PrEBM complete medium). Although specific specimens from each of these prostatectomies contained histologic cancer, four of the seven specific specimens used to initiate cultures had no histologically detectable cancer. Of the remaining three, one had >50% and the other two had >20% of the area involved with Gleason score 6 cancers in the specimen used for culture. Histologic examination (Fig. 1A) and immunocytochemical staining showed that for the cancers containing specimens the malignant cells express AR (Fig. 1B), PSA (data not shown), PSMA (data not shown), and AMACR (Fig. 1D) but not p63 (Fig. 1C), GST-Pi (data not shown), or CD44 (data not shown). When these cancer-containing specimens were cultured in the low-Ca²⁺-SFD medium, cells that grew out expressed p63 (Fig. 1E), cytokeratins 5 and 14 (data not shown), and GST-Pi and CD44 (data not shown) but not AMACR (Fig. 1F) AR (data not shown), PSA (data not shown), or PSMA (data not shown). In addition, staining for α-smooth muscle actin and type I collagen was negative, documenting the lack of stromal cell growth in the low-Ca²⁺-SFD medium. These characteristics were identical to the cells established from the patients whose specimens contained no histologically detectable cancers. Also
similar to cells from the patients whose specimens did not contain cancer, cells from the cancers containing specimens could be serially cultured in low-Ca\textsuperscript{2+}-SFD medium only for 8 to 10 passages before becoming proliferatively quiescent. Inoculation of nude mice (\(n = 5\) per specimen) with 0.2 mL Matrigel containing \(2 \times 10^6\) viable cells from the three cancer-containing specimens passaged twice in low-Ca\textsuperscript{2+}-SFD medium produced no palpable tumors even if followed for >6 months.

Low-calcium-serum-free, growth factor–defined medium selects against malignant prostate cells and normal prostatic fibroblasts. These data document that use of low-Ca\textsuperscript{2+}-SFD medium results in the growth of normal transit-amplifying cells and not prostate fibroblasts or cancer cells also present within the original surgical material. In contrast, all of the currently available permanent human prostate cancer cell lines were established with and are maintained in medium containing \(\geq 650 \mu\text{mol/L} \text{Ca}^{2+}\) and 10% to 20% FCS (18). Therefore, to test whether the inability to grow malignant cells from the cancer-containing radical prostatectomy specimens is simply a random event or whether such low-Ca\textsuperscript{2+}-SFD medium selected against growth of malignant prostate cells, the cancer lines presented in Table 1 were tested for their ability to grow continuously in two different types of low-Ca\textsuperscript{2+}-SFD medium. The first low-Ca\textsuperscript{2+}-SFD medium was the PrEBM complete medium with defined growth factors (Clonetics) used for growth of commercially obtained PrEC cells, which contains \(260 \pm 14 \mu\text{mol/L} \text{Ca}^{2+}\), and the second was K-SFM complete medium with defined growth factors (Invitrogen), which contains \(75 \pm 2 \mu\text{mol/L} \text{Ca}^{2+}\).

When FCS is not additionally provided to either of the low-Ca\textsuperscript{2+}-SFD media, all of the cancer cell lines attach poorly to the culture dishes, slow their growth, and begin to die. This process is so dramatic that for some of the lines (i.e., early-passage LNCaP, MDA-PCA-2B, and LAPC-4) all the cells die within two passages. For the other lines (i.e., late-passage LNCaP, C4-2B, PC-3, DU-145, and CWR22-Rv1), such sterilization requires a total of three passages. Likewise, when early-passage human prostatic fibroblasts, which were established initially in R-FCS and which can be passaged serially 10 to 15 times in this high (i.e., \(650 \mu\text{mol/L}\))--Ca\textsuperscript{2+} medium, are switched to low-Ca\textsuperscript{2+} PrEBM complete medium, the cells died within one passage. Thus, none of the human prostatic fibroblasts or cancer cell lines survive long-term without FCS components in low-Ca\textsuperscript{2+} medium even when they are given insulin, EGF, BPE, transferrin, hydrocortisone, triiodothyronine, and ethanolamine in their medium.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** A, histology of the radical prostatectomy specimen that contains >50% Gleason score 6 cancer used for culture in low-Ca\textsuperscript{2+}-SFD medium. Immunocytochemical staining for AR (B), p63 (C), and AMACR (D) in serial sections of specimen from (A). Asterisks, normal prostatic epithelial gland contaminating areas of malignant prostate cells. Immunocytochemical staining of fifth passage cells cultured from specimens from (A) for p63 (E) and AMACR (F).

### Table 1. Characteristics of the prostatic cell lines used in this study

<table>
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<tr>
<th>Cell line</th>
<th>Initial site of origin</th>
<th>Tumorigenic in nude mice</th>
<th>Expression of indicated phenotypic marker protein*</th>
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<td></td>
<td></td>
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<tr>
<td>PrEC</td>
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<tr>
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<td>+++</td>
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<tr>
<td>CWR22-Rv1</td>
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<tr>
<td>DU-145</td>
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*+, low; ++, medium; ++++, high level of expression; –, not detectable.
*\(^{M}\), mutated gene; WT, wild-type.
FCS and elevated calcium are required for optimal growth of malignant prostate cells but inhibits growth of normal transit-amplifying cells. The lack of growth of malignant prostate cells in the low-Ca\textsuperscript{2+}-SFD medium could be due to the low-Ca\textsuperscript{2+}-SFD medium either actively suppressing the growth of malignant prostatic cells or passively by its inability to provide critical nutrients and/or attachment growth factors. To resolve this, LNCaP cells from passage 33 were plated and exposed for 1 week to (a) their standard R-FCS medium, (b) low (i.e., 75 μmol/L)-Ca\textsuperscript{2+} K-SFM complete medium alone, (c) K-SFM complete medium plus added Ca\textsuperscript{2+} to bring its concentration 650 μmol/L (i.e., equivalent to the level in R-FCS medium), (d) K-SFM complete medium plus 10% FCS (i.e., 367 μmol/L Ca\textsuperscript{2+}), (e) K-SFM complete medium plus 10% dailyzed FCS (i.e., 100 μmol/L Ca\textsuperscript{2+}), or (f) K-SFM complete medium plus 10% FCS and added Ca\textsuperscript{2+} to bring its total concentration to 650 μmol/L. The results documented that in standard R-FCS medium there were 28,780 ± 2,478 viable cells after 1 week versus only 8,080 ± 680 viable cells (i.e., 28% of R-FCS value) when maintained for 1 week in the low-Ca\textsuperscript{2+} K-SFM complete medium. Suplementing the K-SFM complete medium to raise the Ca\textsuperscript{2+} to 650 μmol/L resulted in only a modest increase in cell number (9,650 ± 1,050). In contrast, addition of 10% FCS to the K-SFM complete medium increased the total Ca\textsuperscript{2+} from 75 ± 2 to 367 ± 5 μmol/L and supplied additional undefined growth factors resulting in a 2.5-fold increase (P < 0.05) in viable cell number at 1 week of treatment (i.e., 24,376 ± 1,229) compared with K-SFM complete medium alone or 82% of the number in standard R-FCS medium. If 10% dailyzed FCS was added to K-SFM complete medium, which supplied additional growth factors but did not increase the Ca\textsuperscript{2+} (i.e., 100 μmol/L), there was less growth at 1 week (i.e., 15,810 ± 995) compared with when this dailyzed FCS was used but twice as much growth than in unsupplemented K-SFM complete medium. When the K-SFM complete medium was supplemented with both 10% FCS plus sufficient added Ca\textsuperscript{2+} to reach a level of 650 μmol/L (i.e., equivalent with R-FCS medium), there were 29,400 ± 2,915 viable cells at 1 week (a value equivalent with that of R-FCS medium). These results document that the inability of the low-Ca\textsuperscript{2+}-SFD medium to maintain the growth of malignant prostatic cells is due to insufficient levels of Ca\textsuperscript{2+} coupled with a lack of attachment growth factors.

In contrast to the growth enhancement when prostate cancer cells are exposed to elevated Ca\textsuperscript{2+} and FCS, when early-passage (i.e., <5) PrEC cells are switched from low (i.e., 260 ± 10 μmol/L)-Ca\textsuperscript{2+}, serum-free PrEBM complete medium to such medium supplemented with either 10% FCS raising the final Ca\textsuperscript{2+} to 575 ± 5 μmol/L or with only Ca\textsuperscript{2+} to raise its level to 650 μmol/L as in R-FCS medium, the normal prostatic transit-amplifying cells become growth arrested. Time-lapse videomicroscopy documented that, in low-Ca\textsuperscript{2+}-SFD medium, transit-amplifying cells divide with a doubling time of ~48 hours. Once a transit-amplifying cell divides, the daughter cells generally do not remain in contact but migrate to fill the culture surface (Fig. 2d). If the cells are allowed to fill the surface (i.e., reach confluence) before subculture, proliferation ceases due to contact inhibition producing a continuous monolayer (Fig. 2b). If transit-amplifying cultures in low-Ca\textsuperscript{2+}-SFD medium are supple-mented with either Ca\textsuperscript{2+} to raise its level to 650 μmol/L or 10% FCS to raise Ca\textsuperscript{2+} to 575 μmol/L, the cells rapidly (i.e., within 1 hour) gain an enhanced motility. This enhanced motility allows a subset of cells to contact neighboring cells. Once this contact is made, the cells remain attached producing foci of stratified cells (Fig. 2c). Within these stratified foci, cell proliferation ceases due to contact inhibition as detected by a lack of mitotic figures. Between these stratified foci, there are large areas of unoccupied surface and scattered individual cells (Fig. 2c). These isolated individual transit-amplifying cells eventually stop their motility and undergo squamous differentiation into cornified envelopes and die. This cornification is documented by the observation that these cells are not solubilized by 1% SDS treatment. Instead, such treatment produces cornified envelopes (Fig. 2d). We have documented previously that the death of transit-amplifying cells induced by a variety of agents always results in the cross-linking of involucrin by these cells into cornified envelopes (19). In contrast, death of prostatic cancer cells induced by a variety of agents (19) or by culturing in low cell density in low-Ca\textsuperscript{2+}-SFD medium did not result in squamous differentiation into cornified envelopes.

**Culture Requirements for Normal vs. Malignant Prostate Cells**

<table>
<thead>
<tr>
<th>Cytokeratin 14</th>
<th>Cytokeratin 8</th>
<th>Cytokeratin 18</th>
<th>AR expression</th>
<th>PSA (ng/10⁷ cells/d)</th>
<th>hK2 (ng/10⁷ cells/d)</th>
<th>PSMA (pmol/min/mg protein)</th>
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<td>++ (M)</td>
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<td>12 ± 3</td>
<td>11 ± 3</td>
<td>23 ± 2</td>
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<tr>
<td>--</td>
<td>++</td>
<td>++ (WT)</td>
<td>--</td>
<td>9 ± 1</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
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<tr>
<td>--</td>
<td>++</td>
<td>++ (M)</td>
<td>--</td>
<td>2,155 ± 355</td>
<td>1 ± 1</td>
<td>19 ± 1</td>
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<td>--</td>
<td>++</td>
<td>++ (M)</td>
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<td>2,314 ± 620</td>
<td>64 ± 11</td>
<td>29 ± 3</td>
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<td>--</td>
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<td>++ (M)</td>
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<td>400 ± 35</td>
<td>22 ± 5</td>
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<td>2,100 ± 500</td>
<td>238 ± 54</td>
<td>109 ± 4</td>
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**Table 1. Characteristics of the prostatic cell lines used in this study (Cont'd)**
retrovirus encoding the human telomerase catalytic subunit of human telomerase reverse transcriptase (hTERT) to produce a line denoted as 957E/hTERT (26). Based on the results of the present studies that such low-Ca\textsuperscript{2+}-SFD medium, however, selects for normal transit-amplifying and against malignant prostate cells, the nature of this prostatic cancer line needs validation. Originally, the 957E/hTERT cells had a diploid karyotype, with the only clonal abnormality being a light staining region on the end of chromosome 4q (26). Between in vitro passages 10 and 32, this line underwent trisomy of chromosome 20 (26). On further passage in our laboratory, the line has remained stable and not gained further chromosomal changes. Because such trisomy of chromosome 20 is often characteristic of cells immortalized by E6/E7 of human papilloma virus 16 (36), the 957E/hTERT cells were tested for the presence of E6/E7 by RT-PCR (Fig. 3). Western blots confirmed that these cells were also negative for SV40 large T antigen and Ela viral protein (data not shown). Although these cells were negative for these viral antigens, they were positive for hTERT expression by RT-PCR (data not shown). 957E/hTERT cells are not tumorigenic when inoculated into nude mice as reported previously (26). These data raise the issue of whether by using low (i.e., 75 \(\mu\)mol/L)–Ca\textsuperscript{2+} K-SFM complete medium what was hTERT immortalized was not prostate cancer cells but proliferating transit-amplifying cells derived from the basal epithelium of a normal prostatic gland contaminating the starting cancer tissue.

To test this possibility, 957E/hTERT cells growing in the low (i.e., 75 \(\mu\)mol/L)–Ca\textsuperscript{2+} K-SFM complete medium (Fig. 4A) were evaluated for their expression profile for the discriminatory markers used in Table 1 and their response to adding Ca\textsuperscript{2+} to the low-Ca\textsuperscript{2+}-SFD medium. These results document that growing 957E/hTERT cells express the basal cell–specific p63 protein within their cell nuclei (Fig. 4B) and cytokeratin 5 (Fig. 4C), cytokeratin 14, and GST-Pi (data not shown) but not p27kip1, AMACR, AR, PSA, hK2, or PSMA (data not shown). In addition, using RT-PCR, these cells express mRNA for the basal cell characteristic markers CD44, TGM2, cytokeratin 5, and cytokeratin 14 while being negative for the luminal markers AR, PSMA, and PSA (Fig. 3). To test their response to Ca\textsuperscript{2+}, 957E/hTERT cells were plated and exposed to (a) their standard K-SFM complete medium with total Ca\textsuperscript{2+} (75 \(\pm\) 2 \(\mu\)mol/L), (b) K-SFM complete medium plus 10% dialyzed FCS with total Ca\textsuperscript{2+} (100 \(\pm\) 15 \(\mu\)mol/L), (c) K-SFM complete medium plus 10% undialyzed FCS with total Ca\textsuperscript{2+} (367 \(\pm\) 5 \(\mu\)mol/L), (d) K-SFM complete medium supplemented with added Ca\textsuperscript{2+} to bring level to 650 \(\mu\)mol/L, or (e) R-FCS with total Ca\textsuperscript{2+} (650 \(\pm\) 10 \(\mu\)mol/L). After 1 week, there were 47,446 \(\pm\) 2,150 viable cells in the K-SFM complete medium versus 42,510 \(\pm\) 3,150 with K-SFM medium supplemented with 10% dialyzed serum. In contrast, these were only 1,200 \(\pm\) 457 \((P < 0.05)\) viable cells in the K-SFM complete medium plus 10% FCS, 1,050 \(\pm\) 490 \((P < 0.05)\) viable cells in......

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure2.png}
\caption{Phase-contrast image of PrEC cells in low (i.e., 260 \(\pm\) 10 \(\mu\)mol/L)–Ca\textsuperscript{2+} serum-free PrEBM complete medium at 40% to 60% confluence (A) or at confluence (B). C. at \(\approx\)40% to 60% confluence, medium supplemented to 650 \(\mu\)mol/L Ca\textsuperscript{2+} for 3 days. D, such Ca\textsuperscript{2+}-supplemented cultures were exposed to 1% SDS documenting the development of insoluble cornified envelopes.}
\end{figure}
Ca$^{2+}$-supplemented K-SFM complete medium, and only 810 ± 57 (P < 0.05) viable cells in the R-FCS medium. Like the situation for the normal transit-amplifying cells, when exponentially proliferating 957E/hTERT cells are switched from their low-Ca$^{2+}$ K-SFM complete medium to medium containing >360 μmol/L Ca$^{2+}$, a subset of cells aggregate and stop proliferating, whereas scattered individual cells undergo squamous differentiation-induced death detectable as 1% SDS-insoluble cornified envelopes (Fig. 4D). These combined results document that 957E/hTERT are derived from normal transit-amplifying cells, not prostate cancer cells.

**Mechanism for the differential response to elevated calcium by normal versus malignant prostate cells.** Normal prostatic transit-amplifying cells express the plasma transmembrane receptor Notch-1 and its plasma transmembrane ligand Jagged-1 (7, 17, 37). Notch-1 is a plasma transmembrane receptor whose signaling is associated with nonterminally differentiated, proliferating progenitor cell types (38). Once synthesized, the 300-kDa full-length Notch-1 protein is cleaved by furin-like protease in the Golgi during trafficking of Notch to the plasma membrane producing two subunits, the NH$_2$-terminal 180-kDa extracellular inhibitory fragment (termed Notch-C) and the plasma transmembrane COOH-terminal 120-kDa intracellular fragment (termed Notch-CTF). These subunits remain associated as a heterodimer to form functionally inactive non-ligand-bound receptor at the plasma membrane (39, 40). The Notch-1 ligand, Jagged-1, is also a plasma transmembrane heterodimeric protein (41) composed of a NH$_2$-terminal extracellular ligand fragment bound to a plasma membrane COOH-terminal intracellular fragment (CTF) produced by proteolysis of the full-length 135-kDa Jagged protein. Reciprocal binding to the extracellular NH$_2$-terminal fragments of the Notch-1 receptor and Jagged-1 ligands between adjacent cells induces the dissociation of the two heterodimeric complexes liberating the CTF of both Notch-1 and Jagged-1. Once liberated, Notch-CTF undergoes cleavage of an extracellular piece to produce a product known as Notch extracellular truncation (NEXT; refs. 39, 40). NEXT is then proteolyzed by γ-secretase to produce a product known as NICD, which contains nuclear localization domains and is translocated to the cell nucleus where it is associated with the nuclear proteins of the RBP-Jκ family to form a transcriptional activator of gene expression (38–40).

Both PrEC and 957E/hTERT cells express the full-length, CTF, NEXT, and NICD forms of Notch-1 (Fig. 5A and B) as well as full-length and CTF forms of Jagged-1 (Fig. 5C). Dose-response studies documented that a concentration of 10 μmol/L of either of the structurally unrelated γ-secretase inhibitors L-685,458 or compound XIX completely inhibits the production of the NICD transcription factor needed for nuclear signaling by both PrEC and 957E/hTERT cells (Fig. 5D). When PrEC or 957E/hTERT cells are inoculated and allowed to attach overnight before being exposed to 10 μmol/L of either of the γ-secretase inhibitors, there were no viable cells remaining after 5 days of such exposure. This toxic effect was specific because similar exposure of the prostatic cancer cell lines growing in their standard high-Ca$^{2+}$ FCS containing medium to 10 μmol/L of either of the γ-secretase inhibitors over a 7-day period produced only a slight inhibition (P < 0.05) of proliferation and no death of any of the cancer lines tested (e.g., percentage growth inhibition was 55 ± 10% for LNCaP, 17 ± 5% for LAPC-4, 20 ± 4% for CWR22-Rv1, 34 ± 6% for PC-3, and 29 ± 6% for DU-145 cells using compound XIX). This inability to induce death of prostatic cancer cells is not due to either lack of Notch-1 and Jagged-1 expression or signaling as documented by the detection of full-length, CTF, and NICD forms of Notch-1 by these malignant cells albeit at much reduced levels (Fig. 5A-C) or the inability of 10 μmol/L γ-secretase XIX inhibitor to block NICD production by the cancer cells (Fig. 5D).

These results document that survival of normal prostatic transit-amplifying cells requires unique Notch-1-mediated signaling, which cannot be provided by signaling induced by the added growth factors in the serum-free defined medium. Such Notch-1 signaling is usually not cell autonomous but instead requires direct reciprocal cell-cell (i.e., ligand) interaction (42). This raises the issue of how such Notch-1 signaling can occur when the PrEC and 957E/hTERT cells are routinely plated at a cell density (e.g., 6,000 cells/cm$^2$) that does not allow a high degree of initial cell-cell contact. Cell autonomous, ligand-independent Notch-1 signaling can be induced by lowering the Ca$^{2+}$ concentration to prevent N$^\text{ICD}$ from initially binding with the CTF form of Notch-1 allowing its proteolysis to liberate NICD inducing nuclear signaling (43). Such low-Ca$^{2+}$-dependent, cell autonomous (i.e., ligand-independent) Notch-1 signaling could be why normal transit-amplifying cells (i.e., PrEC and 957E/hTERT) can be established selectively at initially low cell density from surgical material when using low-Ca$^{2+}$-SFD medium. To evaluate this possibility, single-cell suspensions of
PrEC or 957/hTERT cells, respectively, were plated in either low-Ca\(^{2+}\)-SFD medium or such medium supplemented to raise its Ca\(^{2+}\) to 600 \(\mu\)mol/L into 100-mm tissue culture dishes at very low cell density (1,000 cells/cm\(^2\)), so that, even by 24 hours, \(\geq\)95% of the attached cells are not in contact with other cells. These low-density cultures were harvested at 24 hours and Western blots were done. These results documented that NICD is still produced when cells are not in contact in low-Ca\(^{2+}\)-SFD medium but not produced when such low-density cultures are exposed to 600 \(\mu\)mol/L Ca\(^{2+}\) (Fig. 5E).

If these initially low-density PrEC cell cultures are allowed to grow to 50% confluence in low (i.e., 260 \(\mu\)mol/L)–Ca\(^{2+}\) PrEBM complete-SFD medium before being supplemented to raise the Ca\(^{2+}\) to 600 \(\mu\)mol/L, rapid cellular aggregation of subconfluent cultures occurs (Fig. 6A versus Fig. 6B). This results in stratification and cessation of proliferation (i.e., complete lack of mitotic figures) within 24 to 48 hours of exposure of the cell aggregates to elevated Ca\(^{2+}\). This cessation is coincident with a 2.4-fold increase in cellular E-cadherin content in the cells, exposure to 600 \(\mu\)mol/L Ca\(^{2+}\), and a 17-fold increase in the p27/kip1 (Fig. 6D). Immunocytochemical staining for E-cadherin showed weak cytoplasmic expression in PrEC cells growing in low-Ca\(^{2+}\) medium but strong expression at the plasma membrane junctions between stratified cells in culture supplemented to 600 \(\mu\)mol/L Ca\(^{2+}\). If subconfluent cultures of PrEC cells are cotreated with 2 \(\mu\)g/mL of the SHE78-7 mouse monoclonal anti-E-cadherin neutralizing antibody when the cultures are supplemented to raise Ca\(^{2+}\) to 600 \(\mu\)mol/L, stratification of the PrEC cells is completely inhibited (Fig. 6C). Likewise, the cells cotreated with neutralizing antibody continue to proliferate as detected by the presence of mitotic figures and an increase in total cell number. Thus, antibody-treated Ca\(^{2+}\)-supplemented cultures continue to proliferate beyond day 7, which is the time when non-antibody-treated cultures not supplemented with Ca\(^{2+}\) (i.e., control cells) become confluent and stopped proliferating (i.e., contact inhibited). In the low-Ca\(^{2+}\) medium, such contact inhibition is associated with an enhanced (i.e., 30-fold) expression of p27/kip1 protein but no increases in E-cadherin (Fig. 6D). Thus, by day 10, the antibody-treated cultures with 600 \(\mu\)mol/L Ca\(^{2+}\) had twice as many cells as in low-Ca\(^{2+}\) contact-inhibited control cultures (i.e., 89,000 ± 695 versus 54,890 ± 4,540; \(P < 0.05\)) and were still proliferating.

Similar experiments were done using 957E/hTERT cells in low (i.e., 75 \(\mu\)mol/L)–Ca\(^{2+}\)–KSFD complete medium supplemented with 10% FCS to raise the Ca\(^{2+}\) levels to 375 \(\mu\)mol/L or with dialyzed 10% FCS, which did not raise the Ca\(^{2+}\) (i.e., 100 ± 5 \(\mu\)mol/L). Like PrEC cells, when 957E/hTERT cells are allowed to reach confluence in low-Ca\(^{2+}\) KSFD complete medium, they up-regulate (i.e., 3.9-fold) expression of p27/kip1 and stop proliferating due to contact inhibition (Fig. 6D). Again, such up-regulation of p27/kip1 protein

**Figure 4.** A, phase-contrast image of 957E/hTERT cells growing on a chamber slide in low (i.e., 75 \(\pm\) 5 \(\mu\)mol/L)–Ca\(^{2+}\) serum-free K-SFM complete medium. B, immunocytohistochemical detection of nuclear p63 protein expression assayed on a single-cell/agar suspension of 957E/hTERT cells. C, immunocytohistochemical detection of cytokeratin 5 assayed on 957E/hTERT cells grown on a chamber slide in PrEBM complete medium. D, phase-contrast image of 957E/hTERT cells grown in PrEBM complete medium supplemented to 650 \(\mu\)mol/L Ca\(^{2+}\) and exposed to 1% SDS to detect cornified envelopes.
cells. Morphologically, human prostate cancer cell lines (e.g., CWR22-Rv1, LAPC-4, and LNCaP) form multicell clusters when growing at subconfluence in high (i.e., <600 μmol/L)-Ca²⁺ serum-containing medium. If E-cadherin neutralizing antibody is added to the high-Ca²⁺ serum-containing medium, homotypic cell clustering does not occur. Such inhibition of homotypic interaction, however, does not stimulate the overall growth of any of the tested lines as documented by identical numbers of viable cells at the end of a 7-day observation period in the antibody-treated versus control cells.

Discussion

Previous studies have documented that a critical level of Ca²⁺ is required both for converting Notch-1 from a constitutively active form allowing cell autonomous signaling to a ligand-dependent process (43) and E-cadherin from an inactive conformation to one mediating homotypic cell-cell interaction (44). Structural biology studies have documented that the mechanism for this Ca²⁺-dependence is based on cooperative effects involving multiple Ca²⁺ binding sites whose occupancy regulates the structure of Notch-1 (43) and E-cadherin (44). At the 1 to 2 mmol/L physiologic tissue Ca²⁺ level, Notch-1 signaling is not cell autonomous but involves reciprocal cell-cell interactions (42). This is because the auto-inhibitory extracellular Notch NH₂-terminal fragment (i.e., N°) must bind with the jagged-1 ligand of a neighboring cell to allow its dissociation from the Notch-CTF fragment. Once this occurs, the Notch-CTF fragment is proteolyzed to produce NICD, which translocates to the nucleus where it functions as a transcriptional coactivator (38). At low (i.e., <300 μmol/L)-Ca²⁺ level, however, the Notch-°N does not associate with the Notch-CTF fragment allowing ligand-independent (i.e., cell autonomous) signaling (43). Like Notch-1, E-cadherin functions are Ca²⁺-dependent. At 50 to 100 μmol/L Ca²⁺, E-cadherin is a monomer with a rod-like structure, which does not allow lateral (cis) dimerization at the plasma membrane (44). Cis-dimerization occurs at >500 μmol/L Ca²⁺ and is required for the trans-interaction between cis-dimerized E-cadherin molecules of adjacent cells responsible for homotypic interaction (44). Use of low (i.e., <300 μmol/L)-Ca²⁺ medium inhibits such E-cadherin-mediated homotypic interaction in culture. This is significant because such homotypic interaction is the mechanism for contact inhibition of proliferation via its ability to up-regulate expression of p27/kip1 (45).

As documented in the present study, normal human prostatic transit-amplifying cells respond dramatically to different levels of Ca²⁺. In low (i.e., <260 μmol/L)-Ca²⁺-SFD medium, Notch-1 signals cell autonomously; thus, the cells survive at low density and E-cadherin is not able to homotypically interact between transit-amplifying cells, and p27/kip1 expression is low. Therefore, these transit-amplifying cells do not aggregate and are not contact inhibited at low cell density in low-Ca²⁺ medium. This Ca²⁺-dependent behavior is consistent with why these normal transit-amplifying cells have a low rate of proliferation in vivo (46), although they are chronically supplied by sufficient levels of andromedins produced by the androgen-stimulated supporting stromal cells. Presumably, under in vivo conditions where the Ca²⁺ level is 1 to 2 mmol/L and cell density is high, E-cadherin-mediated homotypic interaction induces p27/kip1; thus, proliferation of these transit-amplifying cells is low due to contact inhibition. In contrast, when prostate tissue is dissociated and cultured in such low-Ca²⁺-SFD medium, the transit-amplifying cells (a) lose E-cadherin-mediated

Figure 5. Western blot of Notch-1 and Jagged-1 expression by normal versus prostatic cancer cells. A, detection of various Notch-1 fragments using a COOH-terminal Notch-1 antibody, FL, full length, B, detection of NICD using an antibody recognizing the valine (1744) cleaved NICD. Note values below the blot are density values normalized to PrEC cells. C, detection of various Jagged-1 fragments. D, inhibition of γ-secretase-induced NICD production. 957E/hTERT, NICD level in control 957E/hTERT cells; 957E/hTERT + inhibitor, NICD level in 957E/hTERT cells treated with 10 μmol/L γ-secretase inhibitor XIX; LAPC-4, NICD level in control LAPC-4 prostate cancer cells; LAPC-4 + inhibitor, NICD level in LAPC-4 cells treated with 10 μmol/L γ-secretase inhibitor XIX. E, PrEC-SFD, NICD in PrEC cells at low density (i.e., 1,000 cells/cm²) in PrEC complete medium; PrEC-SFD + CaCl₂, NICD in PrEC cells at low density (1,000 cells/cm²) in PrEC complete medium with 600 μmol/L Ca²⁺.

and resultant contact inhibition are not observed when the prostate cancer cell lines are grown in their standard high-Ca²⁺ medium. If subconfluent cultures are supplemented to raise the Ca²⁺ content (Fig. 6A), the increase in cellular E-cadherin and a 2.7-fold increase in p27/kip1 expression is seen (45). As documented in the present study, normal human prostatic transit-amplifying cells respond dramatically to different levels of Ca²⁺. In low (i.e., <260 μmol/L)-Ca²⁺-SFD medium, Notch-1 signals cell autonomously; thus, the cells survive at low density and E-cadherin is not able to homotypically interact between transit-amplifying cells, and p27/kip1 expression is low. Therefore, these transit-amplifying cells do not aggregate and are not contact inhibited at low cell density in low-Ca²⁺ medium. This Ca²⁺-dependent behavior is consistent with why these normal transit-amplifying cells have a low rate of proliferation in vivo (46), although they are chronically supplied by sufficient levels of andromedins produced by the androgen-stimulated supporting stromal cells. Presumably, under in vivo conditions where the Ca²⁺ level is 1 to 2 mmol/L and cell density is high, E-cadherin-mediated homotypic interaction induces p27/kip1; thus, proliferation of these transit-amplifying cells is low due to contact inhibition. In contrast, when prostate tissue is dissociated and cultured in such low-Ca²⁺-SFD medium, the transit-amplifying cells (a) lose E-cadherin-mediated interaction.
homotypic interaction and thus lack high p27/kip1 expression, (b) undergo Notch-1 cell autonomous survival signaling, and (c) proliferate due to the added growth factor in the medium even at initially low cell density. In contrast, this low-Ca\(^{2+}\)-SFD medium lacks sufficient levels of attachment/growth factors to allow malignant prostate cancer cells to adhere and grow. This would explain why using such low-Ca\(^{2+}\)-SFD medium to establish cultures results in outgrowth of normal transit-amplifying cells (e.g., 957E/hTERT) and not prostate cancer cells.

As documented by the present studies, the overgrowth of these normal transit-amplifying cells is prevented by raising the medium Ca\(^{2+}\) to >600 μmol/L. At this Ca\(^{2+}\) level, Notch-1 is not cell autonomous but dependent on cell-cell (i.e., ligand receptor) interaction. Thus, transit-amplifying cells cannot survive at low cell density if the Ca\(^{2+}\) is raised to >600 μmol/L and if the cells do not interact to activate Notch-1 signaling. Such interaction while activating Notch-1, however, allows E-cadherin to form transdimers (i.e., homotypic interaction) on the plasma membrane between transit-amplifying cells. This results in the up-regulation of p27/kip1 and thus contact inhibition of growth of these normal prostate cells. In contrast, prostate cancer cells are not growth inhibited at >600 μmol/L Ca\(^{2+}\). This is because the consequence of Notch-1 signaling and E-cadherin homotypic interaction is characteristically different in malignant versus normal prostatic epithelial cells. During prostatic carcinogenesis, Notch-1 signaling is no longer required for survival but instead is one of multiple signaling pathways stimulating proliferation of prostate cancer cells. Previous studies have documented that E-cadherin expression is decreased and its location is dysregulated in the progression of prostate cancer (47–49). In addition, there are changes in the E-cadherin signaling cascade (i.e., α-catenin and β-catenin) such that E-cadherin-mediated homotypic interaction does not induce contact inhibition of prostatic cancer cells (49, 50).

In summary, normal transit-amplifying prostatic epithelial cells activate Notch-1 signaling required for their survival by Ca\(^{2+}\)-dependent, E-cadherin-mediated homotypic interaction. Such homotypic interaction, however, restricts proliferation of transit-amplifying cells via up-regulation of p27/kip1 producing contact inhibition. In contrast, Ca\(^{2+}\)-dependent, E-cadherin-mediated homotypic interaction does not induce contact inhibition in prostate cancer cells and Notch-1 signaling is not required for survival of these malignant cells. Instead, E-cadherin enhances cancer cell attachment/motility without requiring Notch-1 signaling. These characteristic changes may help explain why prostate cancer cells can dissociate from the primary site, disseminate as single cells, and survive and establish metastases, particularly to the bone, a site of high-Ca\(^{2+}\) levels.

Acknowledgments
Received 11/15/2004; revised 5/26/2005; accepted 8/2/2005.
Grant support: NIH grant R01DK52645 and Specialized Programs of Research Excellence in Prostate Cancer grant P50CA82386.

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We thank Dorothea K. Stieff for the preparation of the article, Leslie Meszler (Cell Imaging Core, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins), Anita L. Hawkins and Dr. Constance A. Griffin (Cytogenetics Core, Department of Pathology, Johns Hopkins School of Medicine) for the karyotyping data, Jessica L. Hicks for immunocytochemical staining, and Dr. William B. Isaacs for helpful criticisms and suggestions.
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Role of Notch-1 and E-Cadherin in the Differential Response to Calcium in Culturing Normal versus Malignant Prostate Cells

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