Establishment of Human Cancer Cell Clones with Different Characteristics: A Model for Screening Chemopreventive Agents

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Abstract. *Background: The present study was undertaken in order to establish phenotypically different cell clones from 10 parental lines of human breast (MCF-7 and T-47D), prostate (PC-3 and DU145), lung (A549 and A427), colon (HCT-116 and HT-29) and bladder (TCCSUP and T24) cancer cells. Materials and Methods: Sublines were established from each of the parental lines by the limiting dilution method. The derived clones were characterized in terms of plating efficiency, cell proliferation rate, saturation density and colony formation efficiency in soft agar. Results: Phenotypically different cell clones were derived from each parental human cancer cell line, with many clones having more 'normal' characteristics than the parental line from which they were derived. Conclusion: Phenotypically normal clones obtained through clonal selection from human cancer cell populations are expected to be a useful tool for the screening of cancer chemopreventive agents and the study of tumor progression.*

Studies on mechanisms of carcinogenesis have often been hindered in part by the lack of suitable *in vitro* model systems through which the continuum of phenotypic and genotypic changes associated with the malignant transformation of cells can be studied (1). Herein the method of clonal selection through fluctuation analysis of human tumor cell lines was applied to cancer chemoprevention studies (2) as a means of establishing the phenotypic continuum that underwrites the tumor cell heterogeneity found in human cancer. In these studies, identification of a phenotypically normal cell clone is particularly relevant wherein the intent is to inhibit initiation and/or progression of a presumptive normal cell towards a more malignant phenotype through chemopreventive agents. Thus, the objective of the present study was to establish and characterize phenotypically different clones from cultured human breast, prostate, lung, colon and bladder cancer cells. The newly established clones of human cancer cells were characterized in terms of plating efficiency, population doubling time, saturation density, hormone sensitivity and anchorage-independent growth. These cell clones, which share a similar etio-genetic background yet express different phenotypes, are expected to be useful for studying cancer progression and determining the efficacy of cancer preventive and therapeutic agents. Specifically, it is suggested that the phenotypically normal cell clones obtained through clonal selection represent an important tool to screen for the chemopreventive efficacy of potential agents during cancer progression.

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Materials and Methods

Cell lines and cell culture. The parental lines of human breast (MCF-7 and T-47D), prostate (DU145 and PC-3), lung (A427 and A549), colon (HCT-116 and HT-29) and bladder (TCCSUP and T24) cancer cells used in this study were obtained from American Type Culture and Collection (ATCC, Rockville, MD, USA). The descriptions of these cell lines are summarized in Table I.

MCF-7, DU145, A427 cells and clones established from these three parental cell lines were cultured in Minimum Essential Medium (EME) supplemented with 2 mM L-glutamine, Earle's Balanced Salt Solution (BSS) adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate, 0.01 mg/ml bovine insulin and 10% fetal bovine serum (FBS).

T-47D cells and clones derived from T-47D cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.2 unit/ml bovine insulin and 10% FBS.

PC-3 and A549 cells and clones established from these two parental cell lines were cultured in Ham's F12K medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate and 10% FBS.

Table I. *Description of human cancer lines used for cloning.*

Cell Line (ATCC® No)	Tissue/Organ origin	Description
MCF-7 (HTB-22)	Breast	Breast adenocarcinoma cells derived from a pleural effusion of a 69-year-old Caucasian female patient with mammary adenocarcinoma.
T-47D (HTB-133)	Breast	Breast ductal carcinoma cells isolated from a pleural effusion of a 54-year-old female patient with an infiltrating ductal carcinoma of the breast.
PC-3 (CRL-1435)	Prostate	Prostate adenocarcinoma cells initiated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old Caucasian male patient.
DU145 (HTB-81)	Prostate	Prostate carcinoma cells derived from brain metastatic site of a 69-year-old Caucasian male patient.
A549 (CCL-185)	Lung	Lung carcinoma cells initiated through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male patient.
A427 (TCCSUP3)	Lung	Lung carcinoma derived from a 52-year-old Caucasian male patient
HCT-116 (CCL-247)	Colon	Colorectal carcinoma derived from an adult male patient.
HT-29 (HTB-38)	Colon	Colorectal adenocarcinoma isolated from a primary tumor of a 44-year-old Caucasian female patient.
TCCSUP (TCCSUP)	Bladder	Isolated from an grade IV anaplastic transitional cell carcinoma in the neck of the urinary bladder of a 67-year-old female patient.
T24 (HTB-4)	Bladder	Bladder transitional cell carcinoma derived from an 81-year-old Caucasian female patient.

HCT-116, HT-29 and T24 cells and clones established from these three parental cell lines were cultured in modified McCoy's 5a medium supplemented with 1.5 mM L-glutamine, 2.2 g/L sodium bicarbonate and 10% FBS.

TCCSUP cells and clones established from TCCSUP cells were cultured in MEM supplemented with Earle's BSS, non-essential amino acids, 1 mM sodium pyruvate and 10% FBS.

The cell culturing was carried out in 37˚C humidified incubators filled with 5% CO_2 and 95% air, and the cells were subcultured when they reached confluence.

Cloning of cultured human cancer cells by the limiting dilution method. The parental MCF-7, T-47D, PC-3, DU145, A549, A427, HCT-116, HT-29, TCCSUP and T24 cells cultured in tissue culture flasks were dissociated by treatment with trypsin-EDTA, washed and re-suspended in the medium as a single cell suspension. The cells were diluted with medium to a final concentration of 8 cells per ml, distributed onto 96-well tissue culture plates at 100 μl per well, and incubated for up to four weeks with medium changes every week. Assuming a 50% plating efficiency and using the Poisson distribution approximation, it was estimated that 48.6%, 24.2%, 6.1%, 1.0% and 0.2% of the wells are expected to have 0, 1, 2, 3, 4 and 5 viable cells per well. All incubations were carried out in 37˚C humidified incubators filled with 5% CO_2 and 95% air. After a minimum of two weeks of incubation, the plates were examined under a microscope, and wells containing cell colonies were marked and counted. Cells in each well that contained a single colony, which presumably arose from a single cell, were dissociated with trypsin-EDTA, resuspended in medium and expanded sequentially into 24-well tissue culture plates, T25 and T75 tissue culture flasks as a clone. The cells of selected clones were frozen in freezing medium (8% DMSO in fetal bovine serum) and stored in liquid nitrogen as stock cultures. This process was performed twice, beginning with different flasks of cells, to obtain a total of two groups of five plates for each parental human cancer cell line. At least twenty colonies were selected for expansion into wells of 24-well plates and expanded into T-75 flasks, and six vials of each clone were frozen in liquid nitrogen for storage.

Determination of plating efficiency. To determine the plating efficiency, the cells of each selected clone were dissociated by treatment with trypsin-EDTA, re-suspended in medium at a final concentration of 80 cells per ml, and plated in 60-mm tissue dishes or T-25 flasks at 5 ml (400 cells) per dish or flask. The cells were cultured for one to two weeks and examined under an inverted phase contrast microscope every other day. When the cell colonies reached an average size of 2 mm in diameter, the plates were fixed and stained with methylene blue and crystal violet in 95% ethanol. The cell colonies in each dish were counted after the dishes were washed and dried. The plating efficiency was calculated by dividing the number of colonies by the number of cells plated and then multiplying by 100%. A minimum of 3 replicate dishes/flasks were used for each selected clone, and the results are expressed as mean±standard deviation (SD).

Determination of cell population doubling time. The cell growth rate was determined for selected clones by the sulforhodamine B (SRB) assay, which was carried out essentially as previously described (3), or by plating cells in 24-well plates followed by trypsin-EDTA treatment of cells and cell counting at different time points. To perform the experiment, the cells of each clone were plated onto seven 96-well plates at 2,000 cells per well and cultured for up to 11 days. Every 24 to 36 hours, one plate of cells was fixed with 10% trichloroacetic acid at 4˚C for 1 hour, washed five times with tap water, stained with 0.4% sulforhodamine B (dissolved in 1% acetic acid) for 15 minutes, washed 3 times with 1% acetic acid and dried in air. After all plates were fixed, stained and dried, the cells in each well were solubilized with 200 μl of 10 mM Tris base solution, and the plates were read with a PowerWave microplate reader (Bio-Tek Instruments, Winooski, VT, USA) at a wavelength of 540 nm.

Table II. *Plating efficiency of MCF-7 cells.*

Subclone	Number of replicates	Plating efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#44	6	0.2	0.1	<0.01
#16	6	0.4	0.4	<0.01
#5	6	0.5	0.7	<0.01
#48	6	1.4	0.2	<0.01
Parental	6	8.0	3.3	
#1	6	12.0	1.5	<0.01
#7	3	12.3	0.6	<0.01
#50	3	14.3	0.8	<0.01
#13	3	17.5	0.5	<0.01
#10	3	24.3	1.0	<0.01
#21	3	32.3	1.0	<0.01
#38	3	39.3	2.3	<0.01

*Compared with parental cell control.

Table III. *Plating efficiency of T-47D cells.*

Subclone	Number of replicates	Plating efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#13	3	3.1	0.4	>0.05
#27	3	3.3	0.6	>0.05
#9	3	4.1	0.3	>0.05
#23	3	5.1	0.5	>0.05
#1	6	6.7	1.6	>0.05
#12	3	7.3	0.6	>0.05
#19	3	7.1	0.8	>0.05
Parental	6	7.7	2.7	
#5	3	8.6	0.4	>0.05
#10	6	10.0	5.6	>0.05
#20	6	10.3	5.9	>0.05
#3	3	10.4	0.9	>0.05

*Compared with parental cell control.

The absorbance (A540) readings, which were proportional to the number of cells in the wells (3), were plotted over the incubation time to generate a growth curve for each clone. The cell population doubling time for each clone was calculated from the logarithmic phase of the growth curve.

Determination of cell saturation density. To determine the cell saturation density, the cultured cells of each selected clone were dissociated by treatment with trypsin-EDTA and plated in 24-well plates at 5,000 to 10,000 cells per well. The cells were incubated at 37˚C and examined under an inverted phase contrast microscope on a daily basis. When the cells reached confluence, cells in three wells for each selected clone were dissociated by treatment with trypsin-EDTA on a daily basis for four consecutive days and counted with a Coulter counter to determine the maximum cell density achieved by each selected clone. A minimum of four wells were used for each selected clone and the results (number of cells per well at saturation) are expressed as mean±SD.

Determination of hormone sensitivity for cloned cells. The hormone sensitivity of each breast and prostate cancer clone was determined by comparing population doubling times for the cells cultured in medium supplemented with regular FBS or Charcoal/Dextran treated FBS (HyClone, Logan, UT, USA) that was hormone-depleted (<5 pg/ml estrone and <3.0 ng/dl testosterone). To start the experiments, the cells were diluted in medium supplemented with 10% fetal bovine serum and plated onto 96-well plates at 2,000 cells per well. After incubation for 16 hours to allow the cells to attach, the medium was removed from the plates and fresh medium supplemented with 10% regular FBS or hormone-depleted FBS was added to the wells. The cells were incubated for up to 11 days and the growth curves were generated as described above. The cell population doubling time for the cells cultured in medium supplemented with regular FBS or hormone-depleted FBS was calculated from the logarithmic phase of the growth curve and compared to determine whether the growth of the cells was androgen sensitive.

Determination of anchorage-independent growth capability of subcloned cells. The capability of anchorage-independent growth was determined in the clones of human cancer cells by the soft agar colony formation assay, as described previously (4) with some modifications. Briefly, the cells dissociated by trypsin-EDTA treatment were resuspended in double strength cell culture medium at a concentration of $1.6x10^4$ cells per ml, mixed with an equal volume of 1.4% Methyl Cellulose 4000 CP (Sigma, St. Louis, MO, USA) and applied at 0.5 ml per well onto 24-well tissue plates that had been pre-coated with 1% agar. The cells were incubated in a CO_2 incubator for two weeks, and then stained with a 0.33% neutral red solution. The cell colonies were counted under a dissecting microscope. The colony formation efficiency was calculated by dividing the number of colonies by the number of cells (4,000 cells per well) initially plated in each well and expressed as percentages.

Data and statistical analyses. The results of plating efficiency (number of colonies/number of cells plated x 100%), cell saturation density (number of cells per cm area at plateau phase of the cell culture), and soft agar colony formation efficiency (number of colonies/number of cells plated x 100%) of the cloned cell clones and parental cell lines were analyzed by one-way ANOVA followed by a Dunnett multiple comparison that compares each clone with its parental cell line.

The cell growth curves were generated by a semi-logarithmic regression analysis using the incubation time as the independent variable and the cell number as the dependent variable. The data were fit to a growth curve equation: $N_t = N_0 \cdot 2^{(t/d)}$, where d is the cell doubling time, and N_t and N_0 are the cell numbers at time 0 and t, respectively. The cell doubling times were calculated from the logarithmic phase of the growth curves.

Results

In the present study, clones were established from parental cells of a total of 10 lines of human breast, prostate, lung, colon and bladder cancer cells. The newly established clones

Table IV. *Population doubling time of MCF-7 cells.*

Subclone	Medium supplemented with FBS			Medium supplemented with hormone depleted FBS		
	Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)		Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)	
		Lower limit	Upper limit		Lower limit	Upper limit
#50	39.8	35.4	45.3	37.2	28.7	53.0
Parental	42.1	38.4	46.5			
#13	43.5	39.1	49.0	44.6	38.4	53.2
#21	46.6	41.9	52.5	42.4	37.1	49.6
#1	47.2	42.6	52.8			
#38	49.8	44.7	56.1	38.6	33.9	44.9
#16	61.7	57.1	67.2			
#5	70.1	61.9	80.8			
#10	71.5	58.8	91.3	60.8	52.4	72.4
#48	72.2	64.7	81.8			
#44	76.2	64.0	94.2			
#7	80.7	68.8	97.6	74.8	60.9	89.8

were characterized in terms of plating efficiency, cell population doubling time, cell saturation density, hormone sensitivity (breast and prostate cancer cells) and anchorage-independent growth.

For the clones derived from the two breast cancer cell lines used in this study, the plating efficiency ranged from 0.2 to 39.3% for MCF-7 clones (Table II) and from 3.1 to 10.4% for T-47D clones (Table III), respectively. The plating efficiencies of MCF-7 clones 5, 16, 44 and 48 were significantly lower than that of the parental MCF-7 cells ($p<0.01$) whereas the plating efficiencies of MCF-7 clones 1, 7, 10, 13, 21, 38 and 50 were higher than that of the parental MCF-7 cells ($p<0.01$). The five T-47D clones were not statistically significantly different from the parental T-47D cells ($p>0.05$) in the plating efficiency.

The population doubling time of the MCF-7 and T-47D clones ranged from 39.8 to 80.7 hours (Table IV) and from 40.5 to 65.4 hours (Table V), respectively. Based on the 95% confidence interval for the mean population doubling time, the population doubling time of MCF-7 clones 1, 5, 7, 10, 16, 38, 44 and 48 were significantly longer than that of the parental MCF-7 cells whereas the population doubling times of MCF-7 clones 13, 21 and 50 were not significantly different than that of the parental MCF-7 cells. The population doubling times of T-47D clones 10, 12 and 19 were significantly shorter than that of the parental T-47D cells, the population doubling times of T-47D clones 23 and 27 were significantly longer than that of the parental T-47D cells, and the population doubling times of T-47D clones 1, 3, 5, 9, 13 and 20 were not significantly different from that of the parental T-47D cells.

The population doubling time of MCF-7 clones 7, 10, 13, 21, 38 and 50 were determined both in the cells cultured in medium supplemented with regular and hormone-depleted FBS. Based on the 95% confidence intervals for the mean population doubling time (Table IV), the cell growth rates of these clones did not decrease significantly when the cells were cultured in medium supplemented with either the regular or hormone-depleted FBS.

The cell saturation densities of MCF-7 and T-47D clones ranged from $1.39x10^5$ to $1.49x10^6$ cells per well (Table VI) and from $6.23x10^5$ to $1.16x10^6$ cells per well (Table VII), respectively. The saturation cell densities of MCF-7 clones 5, 16, 44 and 48 and T-47D clones 1, 5, 9, 12, 13, 19, 20, 23 and 27 were significantly higher than that of the respective parental cells ($p<0.05$ or 0.01) whereas the saturation cell densities of MCF-7 clone 1, T-47D clones 3 and 10 were not significantly different than those of their respective parental cells ($p>0.05$).

The soft agar colony formation efficiency of MCF-7 and T-47D clones ranged from 0.100 to 1.400% (Table VIII) and from 0.000 to 1.075% (Table IX), respectively. The soft agar colony formation efficiencies of MCF-7 clones 1, 5, 13 and 48 were significantly lower than that of the parental MCF-7 cells ($p<0.05$ or 0.01), the soft agar colony formation efficiencies of MCF-7 clones 7, 10 and 21 were significantly higher than that of the parental MCF-7 cells ($p<0.05$ or 0.01), and the soft agar colony formation efficiencies of MCF-7 clones 16, 38, 44 and 50 were not significantly different than that of the parental MCF-7 cells ($p>0.05$). The soft agar colony formation efficiencies of T-47D clones 5, 9, 13 and 27 were significantly lower than that of the

Table V. *Population doubling time of T-47D cells.*

Subclone	Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)	
		Lower limit	Upper limit
#19	40.5	36.1	46.3
#10	40.6	38.2	43.4
#12	43.6	37.3	52.4
#3	46.4	39.3	56.5
#20	50.3	47.3	53.7
#5	50.6	42.3	62.9
#9	52.9	45.0	64.3
Parental	55.3	49.6	62.6
#1	57.6	35.7	147.8
#13	57.6	49.5	68.7
#27	65.3	61.0	70.2
#23	65.4	55.0	80.5

Table VI. *Saturation density of MCF-7 cells in culture.*

Subclone	Number of replicates	Saturation density (cells/cm^2)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#1	9	139048	45964	>0.05
Parental	9	168612	9531	
#44	6	482680	19351	<0.01
#5	6	574960	129324	<0.01
#48	5	1355600	106132	<0.01
#16	6	1490680	104368	<0.01

*Compared with parental cell control.

Table VII. *Saturation density of T-47D cells in culture.*

Subclone	Number of replicates	Saturation density (cells/cm^2)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#1	10	622640	24593	<0.01
#27	4	643120	20065	<0.01
#23	12	794640	32706	<0.01
#5	6	803240	16295	<0.01
#12	4	824840	28930	<0.01
#19	4	837320	8877	<0.01
#13	8	853400	29494	<0.01
#20	4	861520	5921	<0.01
#9	4	1081280	13742	<0.05
Parental	6	1144280	32023	
#3	4	1160880	17704	>0.05
#10	4	1163200	62504	>0.05

*Compared with parental cell control.

Table VIII. *Colony formation of MCF-7 cells in soft agar.*

Subclone	Number of replicates	Colony formation efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#13	4	0.100	0.020	<0.01
#1	8	0.216	0.044	<0.01
#48	8	0.244	0.050	<0.01
#5	8	0.572	0.118	<0.05
#16	8	0.650	0.317	>0.05
#50	4	0.688	0.105	>0.05
#38	4	0.756	0.085	>0.05
Parental	8	0.784	0.128	
#44	8	0.819	0.143	>0.05
#21	4	1.069	0.113	<0.05
#7	4	1.075	0.188	<0.05
#10	4	1.400	0.137	<0.01

*Compared with parental cell control.

Table IX. *Colony formation of T-47D cells in soft agar.*

Subclone	Number of replicates	Colony formation efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#5	4	0.000	0.000	<0.01
#9	4	0.000	0.000	<0.01
#13	4	0.000	0.000	<0.01
#27	3	0.358	0.063	<0.01
#1	4	0.544	0.043	>0.05
#23	3	0.575	0.139	>0.05
Parental	4	0.625	0.035	
#12	4	0.638	0.066	>0.05
#20	4	0.719	0.097	>0.05
#10	4	0.813	0.145	<0.05
#3	4	1.069	0.055	<0.01
#19	4	1.075	0.132	<0.01

*Compared with parental cell control.

parental T-47D cells ($p<0.01$), the soft agar colony formation efficiencies of T-47D clones 3, 10 and 19 were significantly higher than that of the parental T-47D cells ($p<0.05$ or 0.01), and the soft agar colony formation efficiencies of T-47D clones 1, 12, 20 and 23 were not significantly different from that of the parental T-47D cells ($p>0.05$).

For the clones derived from the two prostate cancer lines used in this study, the plating efficiency ranged from 3.2 to 20.2% for the DU145 clones (Table X) and from 1.0 to 27.4% for the PC-3 clones (Table XI), respectively. The plating efficiencies of DU145 clones 3, 4 and 16 were significantly lower than that of the parental DU145 cells

Table X. *Plating efficiency of DU145 cells.*

Subclone	Number of replicates	Plating efficiency (%)		Dunnett multiple comparisons (*p*-value)*
		Mean	SD	
#3	6	3.2	0.6	<0.01
#4	6	7.3	1.7	<0.01
#16	6	8.9	5.0	<0.01
#14	5	11.8	0.8	>0.05
#2	6	12.0	0.4	>0.05
#15	6	13.0	3.4	>0.05
#1	6	13.3	1.6	>0.05
#13	5	15.9	3.6	>0.05
Parental	6	16.0	2.1	
#7	6	18.0	1.6	>0.05
#18	6	19.3	1.9	>0.05
#5	6	19.7	1.5	>0.05
#21	6	20.2	6.8	>0.05

*Compared with parental cell control.

Table XI. *Plating efficiency of PC-3 cells.*

Subclone	Number of replicates	Plating efficiency (%)		Dunnett multiple comparisons (*p*-value)*
		Mean	SD	
#7	3	1.0	0.3	<0.01
#6	3	5.8	0.9	<0.01
#14	3	10.0	9.1	<0.01
#3	3	11.5	3.4	<0.05
#15	3	12.1	2.3	<0.05
#20	3	16.8	7.4	>0.05
#12	3	17.8	5.4	>0.05
#18	3	18.5	5.5	>0.05
#13	3	19.0	3.3	>0.05
#8	3	24.2	11.3	>0.05
#19	3	26.9	0.6	>0.05
Parental	3	27.4	3.9	

*Compared with parental cell control.

($p<0.01$) whereas the rest of the nine DU145 clones were not significantly different from the parental DU145 cells in the plating efficiency ($p>0.05$). The plating efficiencies of PC-3 clones 3, 6, 7, 14 and 15 were significantly lower that that of the parental PC-3 cells ($p<0.05$ or 0.01) whereas the other six PC-3 clones were not significantly different from the parental PC-3 cells in the plating efficiency ($p>0.05$).

The population doubling time of the DU145 and PC-3 clones ranged from 23.8 to 40.3 hours (Table XII) and from 33.6 to 88.1 hours (Table XIII), respectively. Based on the 95% confidence interval for the mean population doubling time, the population time of DU145 clones 1, 2, 3, 4, 5, 7, 13, 14, 15 and 16 were significantly longer than that of the parental DU145 cells, whereas the population doubling times of DU145 clones 18 and 21 were not significantly different from that of the parental DU145 cells. The population doubling time of PC-3 clone 8 was significantly shorter than that of the parental PC-3 cells, the population doubling times of PC-3 clones 3, 6, 7, 12, 14, 15, 18, 19 and 20 were significantly longer than that of the parental PC-3 cells, and the population doubling time of PC-3 clone 13 was not significantly different from that of the parental PC-3 cells.

The population doubling time of the DU145 and PC-3 clones were determined both in cells cultured in medium supplemented with regular and hormone-depleted FBS. Based on the 95% confidence intervals for the mean population doubling time, the population doubling times of PC-3 clones 3, 6, 7, 12, 13, 14, 15, 18 and 19 were significantly extended when the cells were cultured in medium supplemented with hormone-depleted FBS. The population doubling times of the 12 DU145 clones, and PC-3 clones 8 and 20, were not significantly extended when the cells were cultured in medium supplemented with hormone-depleted FBS.

The cell saturation densities of the DU145 and PC-3 clones ranged from $3.69x10^5$ to $1.81x10^6$ cells per well (Table XIV) and from $8.29x10^5$ to $1.41x10^6$ cells per well (Table XV), respectively. The cell saturation densities of DU145 clones 1, 2 and 3, and PC-3 clones 3, 6, 12, 13, 14, 15, 18 and 20, were significantly lower than that of the respective parental cells ($p<0.05$ or 0.01); of DU145 clones 5, 16, and PC-3 clone 8, were significantly higher than that of the respective parental cells ($p<0.05$ or 0.01); and of DU145 clones 4, 7, 13, 14, 15, 18, 21 and PC-3 clone 19 were not significantly different than that of their respective parental cells ($p>0.05$).

The soft agar colony formation efficiency of the DU145 and PC-3 clones ranged from 0.003 to 0.903% (Table XVI) and from 0.000 to 1.375% (Table XVII), respectively. The soft agar colony formation efficiencies of DU145 clones 2, 3, 4, 7, 14, 15, 16, 21, and PC-3 clones 6, 7, 8, 13, 14, 19 and 20, were significantly lower than that of the respective parental cells ($p<0.01$), and of DU145 clones 1, 5, 13, 18, and PC-3 clones 3, 12 and 15 were not significantly different than that of the respective parental cells ($p>0.05$).

For the clones derived from the two lung cancer lines used in this study, the plating efficiency ranged from 0.1 to 18.3% for the A427 clones (Table XVIII) and from 17.6 to 40.9% for the A549 clones (Table XIX), respectively. The plating efficiencies of A427 clones 21, 23, 24, 26, 27, 30, and A549 clones 5, 10, 11, 14, 34, 42, 56 and 58, were significantly higher than that of the respective parental cells ($p<0.05$ or 0.01), whereas the plating efficiencies of the

Table XII. *Population doubling time of DU145 cells.*

Subclone	Medium supplemented with FBS			Medium supplemented with hormone depleted FBS		
	Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)		Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)	
		Lower limit	Upper limit		Lower limit	Upper limit
#21	23.8	22.1	25.8	27.3	22.9	33.8
#18	24.9	21.5	29.6	22.2	18.8	27.3
Parental	25.3	22.1	29.6	24.7	21.8	28.5
#15	30.3	26.5	35.4	29.0	24.5	35.6
#7	31.0	26.7	37.1	32.0	28.9	35.8
#5	31.3	27.0	37.2	32.7	28.8	37.8
#16	31.8	27.2	38.1	32.9	26.7	42.6
#13	32.8	26.6	43.0	29.5	25.1	35.7
#2	33.9	27.8	43.5	33.1	28.8	38.8
#14	34.3	29.3	41.3	33.0	29.0	38.4
#4	35.2	29.6	43.5	36.3	31.9	42.2
#1	35.3	31.8	39.7	39.2	34.4	45.7
#3	40.3	32.3	53.7	44.1	35.8	57.5

Table XIII. *Population doubling time of PC-3 cells.*

Subclone	Medium supplemented with FBS			Medium supplemented with hormone depleted FBS		
	Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)		Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)	
		Lower limit	Upper limit		Lower limit	Upper limit
#8	33.6	32.1	35.2	36.3	32.8	40.7
#13	33.8	31.3	36.7	39.7	37.3	42.6
Parental	35.8	33.8	38.0	43.3	40.6	46.5
#3	38.9	36.9	41.2	44.4	40.9	48.6
#15	39.2	37.7	40.9	43.4	40.9	46.3
#18	40.4	39.1	41.9	42.8	40.7	45.1
#12	40.5	37.7	43.6	47.6	43.2	53.1
#19	45.3	42.5	48.5	52.5	46.1	61.1
#14	46.8	43.7	50.4	53.3	50.2	56.8
#20	51.2	46.7	56.5	48.0	44.4	52.4
#6	56.5	51.7	62.3	73.1	66.2	81.6
#7	88.1	75.8	105.4	116.6	105.0	131.1

other seven A427 clones and the three A549 clones were not significantly different from the respective parental cells in the plating efficiency ($p>0.05$).

The population doubling time of the A427 and A549 clones ranged from 29.6 to 54.5 hours (Table XX) and from 22.0 to 33.2 hours (Table XXI), respectively. Based on the 95% confidence interval for the mean population doubling time, the population doubling times of A427 clone 18, and A549 clones 5 and 42, were significantly shorter than that of the respective parental cells, of A427 clone 27, A549 clones 10, 14 and 60 were significantly longer than that of the respective parental cells, and of the other five A427 clones and six A549 clones were not significantly different from that of the respective parental cells.

The cell saturation densities of A427 and A549 clones ranged from $1.86x10^5$ to $4.13x10^5$ cells per well (Table XXII) and from $8.99x10^5$ to $1.95x10^6$ cells per well (Table XXIII), respectively. The cell saturation densities of A427 clones 23, 24, 26, 30, 31, and A549 clones 11 and 41, were significantly lower than that of the respective parental cells

Table XIV. *Saturation density of DU145 cells in culture.*

Subclone	Number of replicates	Saturation density (cells/well)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#3	3	369432	10342	<0.01
#2	3	971320	38966	<0.01
#1	3	981280	26472	<0.01
#7	3	1182080	57504	>0.05
#4	3	1191600	186220	>0.05
#13	3	1236400	92024	>0.05
#14	3	1300040	112016	>0.05
Parental	3	1365520	181068	
#15	3	1371400	94352	>0.05
#18	3	1500040	217084	>0.05
#21	3	1580360	18604	>0.05
#5	3	1749960	163364	<0.01
#16	3	1814520	178852	<0.01

*Compared with parental cell control.

Table XV. *Saturation density of PC-3 cells in culture.*

Subclone	Number of replicates	Saturation density (cells/cm^2)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#6	4	828760	4208	<0.01
#3	8	900080	16012	<0.01
#12	4	908160	20655	<0.01
#15	8	957080	16987	<0.01
#20	4	1016680	6719	<0.01
#13	4	1112760	9018	<0.01
#18	4	1124080	353	<0.01
#14	4	1151120	11006	<0.05
#19	4	1183320	40868	>0.05
Parental	4	1201080	50644	
#8	4	1412000	40052	<0.01

*Compared with parental cell control.

Table XVI. *Colony formation of DU145 cells in soft agar.*

Subclone	Number of replicates	Colony formation efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#3	8	0.003	0.009	<0.01
#16	8	0.100	0.023	<0.01
#15	8	0.225	0.038	<0.01
#2	8	0.228	0.056	<0.01
#7	8	0.528	0.183	<0.01
#14	8	0.531	0.094	<0.01
#21	8	0.531	0.130	<0.01
#4	8	0.538	0.093	<0.01
#5	8	0.734	0.164	>0.05
#1	8	0.741	0.191	>0.05
#13	8	0.772	0.271	>0.05
Parental	8	0.850	0.231	
#18	8	0.903	0.300	>0.05

*Compared with parental cell control.

Table XVII. *Colony formation of PC-3 cells in soft agar.*

Subclone	Number of replicates	Colony formation efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#6	4	0.000	0.000	<0.01
#7	4	0.000	0.000	<0.01
#13	4	0.000	0.000	<0.01
#14	4	0.000	0.000	<0.01
#8	4	0.06250	0.1250	<0.01
#19	4	0.06250	0.1250	<0.01
#20	4	0.1250	0.2500	<0.01
#18	4	0.4375	0.2394	>0.05
#15	4	0.6250	0.3227	>0.05
#3	4	0.6875	0.2394	>0.05
Parental	4	0.8125	0.2394	
#12	4	1.375	0.5951	<0.05

*Compared with parental cell control.

($p<0.05$ or 0.01); of A427 clones 15, 21, 27, and A549 clones 5, 10, 34, 51, 56 and 58, were significantly higher than that of the respective parental cells ($p<0.05$ or 0.01); and of A427 clones 12, 13, 18, 19, 29, and PC-3 clones 14 and 42, were not significantly different than that of their respective parental cells ($p>0.05$).

The soft agar colony formation efficiency of A427 and A549 clones ranged from 0.000 to 1.488% (Table XXIV) and from 0.072 to 0.800% (Table XXV), respectively. The soft agar colony formation efficiency of A549 clone 5 was significantly lower than that of the parental A549 cells ($p<0.01$), of A427 clones 13, 21, 23, 24, 30, 31, and A549 clones 42 and 51, were significantly higher than that of the respective parental cells ($p<0.005$ or 0.01), and of A427 clones 12, 15, 18, 19, 26, 27, 29, A549 clones 11 and 41 were not significantly different than that of the respective parental cells ($p>0.05$).

For the clones derived from the two colon cancer lines used in this study, the plating efficiency ranged from 11.0 to 30.3% for HCT-116 clones (Table XXVI) and from 26.2 to 48.3% for HT-29 clones (Table XXVII), respectively. The plating efficiencies of HCT-116 clones 8, 13, 16, 17, 18, 22, 31, 36 and HT-29 clone 27 were significantly lower than that of the respective parental cells ($p<0.01$), of HT-29 clone 10 was significantly higher than that of the parental HT-29 cells ($p<0.01$), and HCT-116 clones 9, 30 and 37 were not

Table XVIII. *Plating efficiency of A427 cells.*

Subclone	Number of replicates	Plating efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#29	5	0.1	0.1	>0.05
#19	5	0.4	0.3	>0.05
Parental	6	1.1	0.5	
#15	5	1.2	1.1	>0.05
#18	3	1.6	0.1	>0.05
#31	3	3.8	0.9	>0.05
#13	5	4.0	2.6	<0.05
#12	3	4.3	1.0	>0.05
#24	5	4.4	2.7	<0.05
#23	3	5.5	0.7	<0.01
#27	3	6.5	0.3	<0.01
#21	3	11.4	1.3	<0.01
#30	3	13.8	1.5	<0.01
#26	3	18.3	3.5	<0.01

*Compared with parental cell control.

Table XIX. *Plating efficiency of A549 cells.*

Subclone	Number of replicates	Plating efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
Parental	3	15.6	1.1	
#41	3	17.6	1.0	>0.05
#60	3	19.0	0.4	>0.05
#51	3	19.3	0.9	>0.05
#11	3	20.9	4.4	<0.05
#42	3	22.7	2.8	<0.01
#14	3	26.6	2.7	<0.01
#34	3	30.0	2.4	<0.01
#56	3	31.6	1.2	<0.01
#58	3	32.3	1.0	<0.01
#5	3	32.4	1.9	<0.01
#10	3	40.9	0.9	<0.01

*Compared with parental cell control.

significantly different from the parental HCT-116 cells ($p>0.05$).

The population doubling time of the HCT-116 and HT-29 clones ranged from 19.2 to 25.2 hours (Table XXVIII) and from 24.5 to 29.8 hours (Table XXIX), respectively. Based on the 95% confidence interval for the mean population doubling time, the population doubling time of HT-29 clone 10 was significantly shorter than that of the parental HT29 cells, of HCT-116 clones 16 and HT-29 clone 27 were significantly longer than that of the respective parental cells, and of HCT-116 clone 22 was not significantly different than that of the parental HCT-116 cells.

Table XX. *Population doubling time of A427 cells.*

Subclone	Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)	
		Lower limit	Upper limit
#18	29.6	27.9	31.6
#31	35.8	34.1	37.8
#23	36.0	34.2	38.1
Parental	38.6	34.1	44.4
#26	40.5	27.3	78.4
#30	42.7	37.9	49.0
#21	43.4	40.5	46.7
#27	54.5	49.7	60.5

Table XXI. *Population doubling time of A549 cells.*

Subclone	Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)	
		Lower limit	Upper limit
#5	22.0	20.3	23.9
#42	24.9	23.0	27.2
#51	25.8	24.4	27.2
#11	26.3	24.4	28.5
Parental	26.8	25.6	28.1
#41	26.9	25.9	28.0
#56	27.0	25.0	29.4
#58	27.0	25.1	29.2
#34	27.2	24.6	30.3
#10	30.3	28.4	32.5
#60	30.8	27.9	34.4
#14	33.2	26.6	44.1

The cell saturation densities of HCT-116 and HT-29 clones ranged from $5.77x10^5$ to $9.37x10^5$ cells per well (Table XXX) and from $9.45x10^5$ to $2.05x10^6$ cells per well (Table XXXI), respectively. The cell saturation density of HT-29 clone 27 was significantly lower than that of the parental HT-29 cells ($p<0.01$), of HCT-116 clone 16 was significantly higher than that of the parental HCT-116 cells ($p<0.01$), and of HCT-116 clone 22 and HT-29 clone 10 were not significantly different than that of their respective parental cells ($p>0.05$).

The soft agar colony formation efficiency of HCT-116 and HT-29 clones ranged from 0.006 to 0.792% (Table XXXII) and from 0.113 to 1.575% (Table XXXIII), respectively. The soft agar colony formation efficiency of HT-29 clone 27 was significantly lower than that of the parental HT-29 cells ($p<0.01$), of HCT-116 clones 12, 13 and 30 were significantly higher than that of the parental HCT-116 cells ($p<0.005$ or

Table XXII. *Saturation density of A427 cells in culture.*

Subclone	Number of replicates	Saturation density (cells/well)		Dunnett multiple comparisons (*p*-value)*
		Mean	SD	
#23	8	186440	9764	<0.01
#31	4	263680	1438	<0.01
#26	8	269292	6544	<0.01
#30	4	285132	2749	<0.01
#24	4	307940	3920	<0.01
#12	4	342012	6814	>0.05
#19	4	342520	7536	>0.05
#29	4	348440	3581	>0.05
Parental	4	349760	9906	
#18	4	361012	5570	>0.05
#13	8	362776	22028	>0.05
#15	4	382380	9724	<0.01
#21	4	390292	1997	<0.01
#27	4	413000	13795	<0.01

*Compared with parental cell control.

Table XXIII. *Saturation density of A549 cells in culture.*

Subclone	Number of replicates	Saturation density (cells/well)		Dunnett multiple comparisons (*p*-value)*
		Mean	SD	
#11	4	898840	15244	<0.01
#41	8	922120	35769	<0.05
Parental	4	1066040	4910	
#42	4	1162000	102832	>0.05
#14	4	1201400	15140	>0.05
#51	4	1204440	44828	<0.05
#5	4	1474880	23341	<0.01
#56	4	1622920	193780	<0.01
#34	4	1702600	35876	<0.01
#58	4	1709520	54520	<0.01
#10	4	1952920	25954	<0.01

*Compared with parental cell control.

0.01), and of HCT-116 clones 15, 18, 19, 21, 23, 26, 27, 31 and HT-29 clone 10 were not significantly different than that of the respective parental cells ($p>0.05$).

For the clones derived from the two bladder cancer lines used in this study, the plating efficiency ranged from 7.3 to 30.4% for TCCSUP clones (Table XXXIV) and from 9.7 to 35.7% for T24 clones (Table XXXV), respectively. The plating efficiencies of TCCSUP clones 1, 2, 4, 19, and T24 clones 1, 5, 8 and 16 were significantly lower than that of the respective parental cells ($p<0.05$ or 0.01), of TCCSUP clones 5, 11, 12, 14, 24, and T24 clones 3 and 9 were significantly higher than that of the respective parental cells

Table XXIV. *Colony formation of A427 cells in soft agar.*

Subclone	Number of replicates	Colony formation efficiency (%)		Dunnett multiple comparisons (*p*-value)*
		Mean	SD	
#12	4	0.000	0.000	>0.05
#15	4	0.000	0.000	>0.05
#18	4	0.000	0.000	>0.05
#27	4	0.000	0.000	>0.05
#29	4	0.000	0.000	>0.05
#19	4	0.125	0.054	>0.05
Parental	8	0.156	0.069	
#26	4	0.194	0.055	>0.05
#31	4	0.281	0.083	<0.05
#23	4	0.488	0.377	<0.05
#30	4	0.494	0.171	<0.05
#21	4	0.663	0.344	<0.01
#13	4	1.319	0.298	<0.01
#24	4	1.488	0.156	<0.01

*Compared with parental cell control.

Table XXV. *Colony formation of A549 cells in soft agar.*

Subclone	Number of replicates	Colony formation efficiency (%)		Dunnett multiple comparisons (*p*-value)*
		Mean	SD	
#5	8	0.072	0.025	<0.01
#41	8	0.241	0.087	>0.05
#11	8	0.350	0.125	>0.05
Parental	8	0.466	0.052	
#51	8	0.769	0.266	<0.05
#42	8	0.800	0.354	<0.01

*Compared with parental cell control.

Table XXVI. *Plating efficiency of HCT-116 cells.*

Subclone	Number of replicates	Plating efficiency (%)		Dunnett multiple comparisons (*p*-value)*
		Mean	SD	
#31	3	11.0	0.9	<0.01
#16	3	11.5	0.9	<0.01
#17	3	12.9	0.6	<0.01
#22	3	13.1	1.1	<0.01
#18	3	15.9	0.8	<0.01
#36	3	17.7	0.7	<0.01
#13	3	18.3	2.3	<0.01
#8	3	19.5	0.7	<0.01
#30	3	26.1	2.5	>0.05
#9	3	27.0	1.6	>0.05
Parental	3	28.4	1.0	
#37	3	30.3	2.6	>0.05

*Compared with parental cell control.

Table XXVII. *Plating efficiency of HT-29 cells.*

Subclone	Number of replicates	Plating efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#27	6	26.2	2.7	<0.01
Parental	6	41.4	3.6	
#10	6	48.3	3.9	<0.01

*Compared with parental cell control.

Table XXVIII. *Population doubling time of HCT-116 cells.*

Subclone	Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)	
		Lower limit	Upper limit
Parental	19.2	16.2	23.7
#22	19.8	16.3	20.9
#16	25.2	20.6	32.4

Table XXIX. *Population doubling time of HT-29 cells.*

Subclone	Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)	
		Lower limit	Upper limit
#10	24.5	23.0	26.3
Parental	27.4	25.9	29.2
#27	29.8	28.4	31.2

Table XXX. *Saturation density of HCT-116 cells in culture.*

Subclone	Number of replicates	Saturation density (cells/cm^2)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
Parental	4	577040	10738	
#22	4	598600	22011	>0.05
#16	4	937440	102048	<0.01

*Compared with parental cell control.

Table XXXI. *Saturation density of HT-29 cells in culture.*

Subclone	Number of replicates	Saturation density (cells/cm^2)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#27	4	944800	30090	<0.01
Parental	4	1920120	185480	
#10	4	2054040	23328	>0.05

*Compared with parental cell control.

Table XXXII. *Colony formation of HCT-116 cells in soft agar.*

Subclone	Number of replicates	Colony formation efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#23	4	0.006	0.013	>0.05
#26	4	0.006	0.013	>0.05
#21	4	0.013	0.025	>0.05
#27	4	0.038	0.043	>0.05
#19	4	0.075	0.035	>0.05
Parental	4	0.081	0.024	
#15	4	0.100	0.020	>0.05
#31	4	0.225	0.121	>0.05
#18	4	0.238	0.207	>0.05
#13	4	0.369	0.204	<0.05
#12	4	0.606	0.334	<0.01
#30	3	0.792	0.126	<0.01

*Compared with parental cell control.

Table XXXIII. *Colony formation of HT-29 cells in soft agar.*

Subclone	Number of replicates	Colony formation efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#27	4	0.113	0.032	<0.01
Parental	4	1.438	0.161	
#10	4	1.575	0.084	>0.05

*Compared with parental cell control.

($p<0.05$ or 0.01), and TCCSUP clones 3, 9, and T24 clones 2, 4, 6, 7 and 14, were not significantly different from the respective parental cells ($p>0.05$).

The population doubling time of the TCCSUP and T24 clones ranged from 24.6 to 88.7 hours (Table XXXVI) and from 25.1 to 34.0 hours (Table XXXVII), respectively. Based on the 95% confidence interval for the mean population doubling time, the population doubling times of TCCSUP clones 11, 12, 14, 19, 24, and T24 clones 3, and 5, were significantly shorter than that of the respectively parental cells, of TCCSUP clones 2, 5, and T24 clone 16, were significantly longer than that of the respective parental cells, and of TCCSUP clones 1, 3, 4, 9, and T24 clones 1, 2,

Table XXXIV. *Plating efficiency of TCCSUP cells.*

Subclone	Number of replicates	Plating efficiency (%)		Dunnett multiple comparisons (*p*-value)*
		Mean	SD	
#2	3	7.3	1.5	<0.01
#4	3	8.6	0.3	<0.01
#19	3	8.8	0.8	<0.01
#1	3	10.8	1.3	<0.01
#3	3	12.8	0.5	>0.05
Parental	3	15.3	0.4	
#9	3	17.3	0.5	>0.05
#14	3	18.3	0.3	<0.05
#5	3	19.8	1.3	<0.01
#12	3	20.3	0.5	<0.01
#24	3	24.7	2.5	<0.01
#11	3	30.4	0.8	<0.01

*Compared with parental cell control.

Table XXXV. *Plating efficiency of T24 cells.*

Subclone	Number of replicates	Plating efficiency (%)		Dunnett multiple comparisons (*p*-value)*
		Mean	SD	
#16	3	9.7	1.5	<0.01
#8	3	22.7	1.5	<0.01
#5	3	26.3	2.1	<0.01
#1	3	27.7	1.2	<0.05
#7	3	28.7	1.2	>0.05
#4	3	28.7	2.5	>0.05
#14	3	29.3	1.5	>0.05
Parental	3	31.3	1.2	
#2	3	31.3	1.5	>0.05
#6	3	32.3	0.6	>0.05
#9	3	35.0	0.0	<0.05
#3	3	35.7	1.5	<0.05

*Compared with parental cell control.

Table XXXVI. *Population doubling time of TCCSUP cells.*

Subclone	Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)	
		Lower limit	Upper limit
#14	24.6	21.9	28.1
#12	28.4	26.6	30.5
#19	28.6	26.5	31.0
#11	29.1	27.5	30.9
#24	33.6	29.7	38.7
#1	41.5	33.2	55.3
#9	43.4	40.4	46.9
#4	44.5	42.7	46.3
Parental	46.8	38.9	58.5
#3	47.5	42.0	54.8
#5	60.3	54.4	67.7
#2	88.7	80.7	98.3

Table XXXVII. *Population doubling time of T24 cells.*

Subclone	Mean doubling time (hours)	95% Confidence interval of the mean doubling time (hours)	
		Lower limit	Upper limit
#5	25.1	22.9	27.7
#3	25.5	23.3	28.2
#9	26.3	23.8	29.2
#4	26.7	23.8	30.5
#6	28.5	25.6	32.1
#14	28.6	26.5	31.1
Parental	28.6	27.4	29.8
#1	30.0	28.0	32.2
#7	30.5	27.0	35.0
#2	30.9	27.8	34.8
#8	32.3	28.4	37.5
#16	34.0	32.2	36.1

4, 6, 7, 8, 9 and 14, were not significantly different than that of the respective parental cells.

The cell saturation densities of TCCSUP and T24 clones ranged from $4.72x10^5$ to $2.72x10^6$ cells per well (Table XXXVIII) and from $7.60x10^5$ to $1.67x10^6$ cells per well (Table XXXIX), respectively. The saturation cell densities of all eleven TCCSUP clones and T24 clones 2, 5, 8 and 14 were significantly lower than that of the respective parental cells ($p<0.01$), of T24 clones 1, 3, 4, 9 and 16 were significantly higher than that of the parental T24 cells ($p<0.01$), and of T24 clones 6 and 7 were not significantly different than that of the parental T24 cells ($p>0.05$).

The soft agar colony formation efficiency of TCCSUP and T24 clones ranged from 0.000 to 0.713% (Table XL) and from 0.000 to 0.244% (Table XLI), respectively. The soft agar colony formation efficiencies of TCCSUP clones 1, 2, 4, 5, 9, 11, 12, 14, 19 and 24 were significantly lower than that of the parental TCCSUP cells ($p<0.01$), of T24 clones 3, 4, 8, 9, 14 and 16 were significantly higher than that of the parental T24 cells ($p<0.005$ or 0.01), and of TCCSUP clone 3, and T24 clones 1, 2, 5, 6 and 7 were not significantly different than that of the respective parental cells ($p>0.05$).

Discussion

The present study was undertaken in order to establish and characterize phenotypically different clones from 10 lines of

Table XXXVIII. *Saturation density of TCCSUP cells in culture.*

Subclone	Number of replicates	Saturation density (cells/cm^2)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#2	8	472480	49344	<0.01
#5	4	662720	17775	<0.01
#1	8	899360	26111	<0.01
#9	4	1092760	8838	<0.01
#4	6	1419200	51096	<0.01
#3	8	1446880	47520	<0.01
#24	4	1536040	36167	<0.01
#19	4	1566120	18842	<0.01
#14	4	1844400	70988	<0.01
#11	4	1935400	48264	<0.01
#12	12	1994080	43132	<0.01
Parental	4	2719400	72776	

*Compared with parental cell control.

Table XXXIX. *Saturation density of T24 cells in culture.*

Subclone	Number of replicates	Saturation density (cells/cm^2)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#8	16	760280	15263	<0.01
#2	4	942880	16392	<0.01
#5	8	942920	21920	<0.01
#14	12	1024960	14439	<0.01
#6	8	1033160	32851	>0.05
Parental	8	1053160	15726	
#7	8	1072880	24779	>0.05
#1	4	1104960	25656	<0.01
#16	4	1284760	40220	<0.01
#3	4	1415480	43520	<0.01
#9	6	1630240	2696	<0.01
#4	4	1674680	29332	<0.01

*Compared with parental cell control.

Table XL. Colony formation of TCCSUP cells in soft agar.

Subclone	Number of replicates	Colony formation efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#1	4	0.000	0.000	<0.01
#2	4	0.000	0.000	<0.01
#5	4	0.000	0.000	<0.01
#14	4	0.000	0.000	<0.01
#24	3	0.000	0.000	<0.01
#19	4	0.006	0.013	<0.01
#11	4	0.025	0.020	<0.01
#12	4	0.081	0.066	<0.01
#4	4	0.094	0.072	<0.01
#9	4	0.281	0.189	<0.01
#3	4	0.688	0.468	>0.05
Parental	4	0.713	0.226	

*Compared with parental cell control.

Table XLI. *Colony formation of T24 cells in soft agar.*

Subclone	Number of replicates	Colony formation efficiency (%)		Dunnett multiple comparisons (p-value)*
		Mean	SD	
#5	4	0.000	0.000	>0.05
#6	4	0.000	0.000	>0.05
#1	4	0.013	0.014	>0.05
#2	4	0.019	0.024	>0.05
#7	4	0.019	0.024	>0.05
Parental	4	0.056	0.013	
#4	4	0.144	0.069	<0.05
#8	3	0.150	0.025	<0.05
#14	3	0.175	0.025	<0.01
#3	3	0.217	0.038	<0.01
#16	4	0.219	0.072	<0.01
#9	4	0.244	0.043	<0.01

*Compared with parental cell control.

human breast, prostate, lung, colon and bladder cancer cells. MCF-7 is a breast adenocarcinoma cell line which retains several characteristics of the differentiated mammary epithelium, including the ability to process estradiol *via* cytoplasmic estrogen receptor (5) and the capability of forming domes (6-8). MCF-7 cells are estrogen receptor positive and estrogen-dependent (9, 10) with a low level of HER2/c-erbB-2 oncogene expression (11). T-47D, a mammary ductal carcinoma cell line, is also estrogen receptor positive (12). PC-3 is a prostate adenocarcinoma cell line, originally derived from a bone metastasis of a grade IV prostatic adenocarcinoma (13). DU145, a prostate carcinoma cell line, was isolated from a lesion in the brain of a patient with metastatic prostate carcinoma (14). Unlike LNCaP prostate carcinoma cells, which are androgen-sensitive, capable of producing PSA and weakly tumorigenic (15-18), DU145 and PC-3 cells are androgen-insensitive, incapable of producing prostate specific antigen (PSA) and readily form tumors when inoculated subcutaneously in nude mice or ICR-SCID mice (13, 19, 20). A549 is a non-small cell lung carcinoma cell line derived through explant culture of lung carcinomatous tissue (21). A427 is a lung adenocarcinoma cell line derived from a solid tumor (21). HCT-116 is a colorectal carcinoma cell line established from a primary cell culture of a single human colonic carcinoma (22) with inducible COX-2 expression (23). HT-29 is a colorectal adenocarcinoma cell line with constitutive COX-2 expression (23). TCCSUP is a bladder carcinoma cell line

that was derived from an undifferentiated, Grade IV transitional cell carcinoma (24). T24 is a bladder carcinoma cell line derived from transitional cell carcinoma (25). These cell lines represent major human epithelial cancers with clinical relevance.

The clones of human breast, prostate, lung, colon and bladder cancer cells established in this study were characterized in terms of plating efficiency, cell proliferation rate, saturation density and colony formation efficiency in soft agar. These criteria were believed to be indicative of whether the different clones are potentially more malignant or more normal in phenotype. It has been well established that plating efficiency in tissue culture dishes and the ability of cells to form colonies in soft agar are well correlated with the ability of the cells to produce tumors in nude mice (26-31). Decreased plating efficiency has been associated with a low efficiency of colony formation in soft agar and/or inhibition or delayed onset of tumor formation in nude mice in U118-9 human glioma cells transfected with the copper zinc superoxide dismutase gene (28) and MCF-7 human breast cells transfected with the manganese superoxide dismutase gene (29, 31). In human colon cancer cells that were fused with normal human colon mucosa cells and histologically normal cells derived from tissues near the cancer lesion, the colony formation efficiency in soft agar was decreased significantly and the low colony formation efficiency was associated with longer latency and slower growth rate of the hybrid cells in nude mice (30). High cell proliferation rate and/or saturation density have also been linked to increased malignant potential in several *in vitro* and *in vivo* systems. As examples, subclones of MGC-803 human stomach cancer cells with low growth rate have a very low efficiency of colony formation in soft agar whereas the subclones with high growth rate are highly efficient at forming colonies in soft agar (32). In human urothelial cell lines propagated in culture, mortal and non-tumorigenic cell lines have low cell growth rates and saturation densities whereas tumorigenic cell lines have high cell growth rates and saturation densities (33). In two human squamous carcinoma cell lines (SCC-15G and SCC-25) maintained in culture, treatment with a potent carcinogen, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin significantly increased cell proliferation rates and saturation densities (34). In contrast, treatment with retinoic acid, an anticarcinogenic agent, significantly decreased the saturation density of Dunning R-3327 rat prostatic adenocarcinoma cells, although the cell proliferation rate was not affected (35).

The clones established from the human breast (MCF-7) and prostate cancer cell lines were also characterized in terms of hormone-sensitivity. Breast cancers often progress from hormone-dependent and non-metastatic phenotypes to hormone-independent phenotypes with highly invasive and metastatic growth properties (36, 37). The loss of hormone-dependency is also associated with more malignant phenotypes in prostate cancer cells. LNCaP cells, which are androgen-sensitive, do not form tumors when injected subcutaneously in nude mice (15, 16, 18) unless a large number of cells are injected (17) or the cells are orthotopically implanted into the prostate glands of nude mice (18, 38). However, LNCaP clones that have lost hormone-dependency are highly tumorigenic in nude mice (39, 40). DU145, PC-3 and TSU-Pr1 human prostate cancer cell lines, which are androgen-insensitive, also readily form tumors when inoculated subcutaneously in nude mice or ICR-SCID mice (13, 15, 19, 20, 41, 42).

The growth of six MCF-7 cell clones evaluated for hormone-dependency in this study all appeared to be insensitive to hormone deprivation. This is surprising since the parental MCF-7 cells are known to be estrogen receptor positive and estrogen-dependent (9, 10). Three of the six hormone insensitive MCF-7 clones (clones 7, 10 and 21) were shown to have significantly higher colony formation efficiency in soft agar as compared to the parental MCF-7 cells, indicating that these clones might be more malignant than the parental MCF-7 cells. In contrary, nine of the 11 PC-3 clones established in this study were found to be hormone sensitive whereas the parental PC-3 cells are known to be hormone insensitive (20). Five of the nine hormone sensitive PC-3 clones (clones 6, 7, 13, 14 and 19) had significantly lower colony formation efficiency in soft agar as compared to the parental PC-3 cells, indicating that these clones might be more normal than the parental PC-3 cells.

The clones established in this study are expected to be useful for studying the progression of human breast, prostate, lung, colon and bladder cancers as they reflect the naturally occurring heterogeneity of cells in these human cancers and are likely to represent different stages of carcinogenesis. The use of cell clones representing different stages of carcinogenesis is important, since carcinogenesis is believed to be a multistep process (43, 44). The existence of multiple intermediate stages in carcinogenesis is supported by statistical and epidemiological evidence (45-49), as well as by the discoveries that multiple genes are involved during cancer development (50, 51). Comparison of the gene expression patterns among these cell clones by new technologies, such as microarray analysis (52, 53), may yield clues about the mechanisms that are important for the cancer development and progression, as they pertain to the heterogenous nature of human cancer.

The clones of human breast, prostate, lung, colon and bladder cancer cells established in this study are also expected to be useful evaluation agents for cancer chemoprevention and/or treatment. It is expected that cells at different pre-neoplastic or neoplastic intermediate stages may possess different biological properties and respond differently to treatments with various cancer preventive

and/or therapeutic agents, which may inhibit the growth of cells at certain stages but not affect the growth of cells at other stages. Unlike the parental cell population, the use of cell clones at different stages of cancer progression is likely to reduce the probability that any given chemopreventive or therapeutic agent with the desired effects on cells at one stage of cancer progression would be considered ineffective, simply because the cell lines utilized for the assay were at a different stage of cancer progression. The use of multiple cell clones that represent different intermediate stages of carcinogenesis will also help to determine whether the potential cancer preventive and/or therapeutic agents inhibit cell growth to a greater extent in more malignant cancer cells than in non-malignant or pre-malignant epithelial cells. Specifically, it is suggested that the phenotypically normal cell clones obtained through clonal selection represent an important tool to screen for the chemopreventive efficacy of potential agents during cancer progression.

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