Spontaneous Neoplastic Transformation of WB-F344 Rat Liver Epithelial Cells

Michelle J. Hooth, William B. Coleman,
Sharon C. Presnell, Kristen M. Borchert,
Joe W. Grisham, and Gary J. Smith

*From the Curriculum in Toxicology and Department of
Pathology and Laboratory Medicine, University of North Carolina
School of Medicine, Chapel Hill, North Carolina*

Several studies have shown that cultured rat liver epithelial cells transform spontaneously after chronic maintenance in a confluent state *in vitro*. In the present study, multiple independent lineages of low-passage WB-F344 rat liver epithelial stem-like cells were initiated and subjected in parallel to selection for spontaneous transformation to determine whether spontaneous acquisition of tumorigenicity was the result of events (genetic or epigenetic) that occurred independently and stochastically, or reflected the expression of a pre-existing alteration within the parental WB-F344 cell line. Temporal analysis of the spontaneous acquisition of tumorigenicity by WB-F344 cells demonstrated lineage-specific differences in the time of first expression of the tumorigenic phenotype, frequencies and latencies of tumor formation, and tumor differentiations. Although spontaneously transformed WB-F344 cells produced diverse tumor types (including hepatocellular carcinomas, cholangiocarcinomas, hepatoblastomas, and osteogenic sarcomas), individual lineages yielded tumors with consistent and specific patterns of differentiation. These results provide substantial evidence that the stochastic accumulation of independent transforming events during the selection regimen *in vitro* were responsible for spontaneous neoplastic transformation of WB-F344 cells. Furthermore, cell lineage commitment to a specific differentiation program was stable with time in culture and with site of transplantation. This is the first report of a cohort of related, but independent, rat liver epithelial cell lines that collectively produce a spectrum of tumor types but individually reproduce a specific tumor type. These cell lines will provide valuable reagents for investigation of the molecular mechanisms involved in the differentiation of hepatic stem-like cells and for examination of potential causal relationships in spontaneously transformed rat liver epithelial cell lines between molecular/cellular alterations and the ability to produce tumors in syngeneic animals. (*Am J Pathol* 1998, 153:1913–1921)

Rat liver epithelial cell lines have been used extensively for investigation of the cellular stages of neoplastic transformation *in vitro*. Such cell lines have been established in culture from both normal rat livers^{1–3} and from the livers of carcinogen-treated rats.^{4,5} Previous studies have shown that neoplastic transformation of these cells *in vitro* can be achieved spontaneously,^{6–8} through carcinogen treatment,^{2,9} and by transfection with activated oncogenes.^{10–13} Neoplastic derivatives of rat liver epithelial cells produce a variety of undifferentiated and differentiated tumors after transplantation to subcutaneous or intraperitoneal sites of nude mice or syngeneic rats, including hepatocellular carcinomas, adenocarcinomas, epidermoid carcinomas, hepatoblastomas, and sarcomas.^{14–16} The differentiated types of tumors reflect the spectrum of liver neoplasms that develop in mammalian liver, suggesting that an undifferentiated stem-like cell may represent the origin of various liver tumors *in vivo*.

The WB-F344 rat liver epithelial cell line has been used to investigate the process of neoplastic transformation *in vitro* and hepatocarcinogenesis *in vivo*.^{14,17,18} We have shown previously that recovery of spontaneously transformed WB-F344 cells was selectively enhanced from a single parental population when cultures were maintained at confluence with infrequent passaging compared with cultures maintained in exponential growth.⁶ In the present study, we initiated multiple independent lineages of low-passage WB-F344 cells and subjected them in parallel to selection for spontaneous transformation to determine whether spontaneous acquisition of tumorigenicity was the result of events (genetic or epigenetic) that occurred independently and stochastically, or reflected the expression of a pre-existing alteration within the parental WB-F344 cell line. We reasoned that if the ability to produce tumors resulted from the stochastic accumulation of several spontaneous alterations, a large number of independent cultures established from the WB-F344 parental culture and grown under selection growth conditions would acquire the ability to produce tumors at different times and express different paratu-

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Address reprint requests to Dr. Gary J. Smith, Department of Pathology and Laboratory Medicine CB 7525, 414 Brinkhous-Bullitt Building, University of North Carolina, Chapel Hill, North Carolina 27599-7525. E-mail: cellsort@med.unc.edu.

morigenic and tumorigenic phenotypes. However, if a pre-existing heritable alteration, which predisposed the cells to neoplastic transformation, was present in the parental WB-F344 cell line, tumorigenicity would arise in all of the lineages at similar times and/or the tumors would be phenotypically similar.

Temporal analysis of the spontaneous acquisition of tumorigenicity by WB-F344 cells demonstrated lineage-specific differences in the time of appearance of tumorigenicity, frequency, and latency of tumor formation and histology of the tumors formed. Although spontaneously transformed WB-F344 cells produced diverse tumor types, individual lineages yielded tumors with consistent and specific patterns of differentiation. These results provide substantial evidence that the stochastic accumulation of independent transforming events during the selection regimen *in vitro* was responsible for spontaneous neoplastic transformation of WB-F344 cells. Furthermore, commitment to a specific differentiation program was stable with time in culture and with site of transplantation.

Materials and Methods

Cell Culture and Selective Growth Conditions

Diploid WB-F344 rat liver epithelial cells¹ at passage 4 served as the founder cell population for the initiation of multiple independent lineages cultured separately under the selective growth conditions. Normal WB-F344 cells are contact inhibited, do not form colonies in soft-agarose, and are nontumorigenic in neonatal Fischer-344 rats.¹ The founding wild-type WB-F344 cell population served as the control cells for the phenotypic characterization of experimental populations *in vitro* and determination of their tumorigenic potential *in vivo*. All cells were cultured in Richter's improved minimal essential medium with zinc option (Irvine Scientific, Santa Ana, CA) supplemented as described previously.⁶

The experimental design for the generation of spontaneous transformants of WB-F344 cells by maintenance under selective growth conditions (Figure 1) was modified from the protocol described previously.⁶ Eighteen separate experimental cell populations were plated at a density of 2.9×10^6 cells/150-mm tissue culture dish. The final cell density of each plate was determined at the end of each cycle of selective growth by counting trypsinized cells with a Coulter counter (Coulter Electronics, Hialeah, FL). All of the cells harvested from one plate were divided equally among four 150-mm tissue culture dishes. One of the four dishes became the experimental population for the next selection cycle and was maintained under the selective regimen. The remaining three dishes were grown to confluence to provide cells for cryopreservation, phenotypic characterization, and tumorigenicity assays. Experimental cell populations were subjected to a minimum of 10 cycles of selective growth. The nomenclature for the experimental cell populations incorporates the name of the parental WB-F344 cell line, the lineage identification number (L1 to L20), and the selection cycle number (C1 to C12).

Figure 1. Experimental design for the selection of spontaneous transformants of WB-F344 rat liver epithelial cells under conditions of selective growth. Each cycle of selective growth consisted of 4 weeks: 1 week for population expansion to confluence followed by maintenance for 3 weeks at confluent cell density with weekly feedings of fresh growth medium.

Phenotypic Characterization of Spontaneous Transformants in Vitro

Cell morphologies and growth patterns at confluence were evaluated by phase contrast microscopy. For determination of saturation densities in monolayer cultures, cells were plated at a density of 1.0×10^5 cells per 60-mm tissue culture dish and maintained in culture with a medium change every 4 days. At the end of 14 days, the cells were harvested and enumerated with a hemacytometer. For determination of saturation densities at the end of a selection cycle, cells were plated at a density of 2.0×10^4 per well on a 24-well tissue culture dish and maintained in culture with a medium change every 7 days. At the end of 28 days, the cells were harvested and counted. Anchorage-independent growth was assayed as described previously.^{19,20}

Tumorigenicity Assays and Establishment of Clonal Tumor Cell Lines

Neoplastic transformation of the independent populations was indicated by the formation of tumors after the subcutaneous transplantation of cells into neonatal syngeneic rats. Tumorigenicity assays were performed as described previously.^{17,20} WB-F344 cells of the founding cell population were transplanted similarly as controls.

Clonal populations of tumorigenic cells were established from tumors produced by the heterogeneous spontaneously transformed lineages as described previ-

Table 1. Tumorigenicity of Transplanted Spontaneous Transformants of WB-F344 Rat Liver Epithelial Cells

Lineage	Selection cycle*	Subcutaneous tumors [†]	Latency (weeks, mean ± SEM) [‡]	Range (weeks) [§]
1	8	1/7 (14%)	15	
	10	7/7 (100%)	14 ± 0.73	13–18
2	10	8/8 (100%)	22 ± 0.56	18–23
	11	10/10 (100%)	18 ± 0.92	14–23
3	11	1/7 (14%)	52	
4	8	3/6 (50%)	35 ± 4.10	27–40
	9	8/9 (89%)	30 ± 2.85	22–44
	10	9/9 (100%)	18 ± 0.07	18
6	8	2/11 (18%)	53 ± 0.36	53
	10	7/7 (100%)	16 ± 0.55	13–17
7	10	5/18 (28%)	35 ± 1.43	31–40
	11	6/8 (75%)	42 ± 4.43	29–51
8	10	3/9 (33%)	35 ± 2.67	31–40
9	10	2/19 (11%)	43 ± 10.57	32–54
11	10	2/10 (20%)	50 ± 2.21	47–52
12	10	9/9 (100%)	32 ± 1.09	30–40
14	8	5/10 (50%)	35 ± 1.29	30–37
16	8	1/10 (10%)	52	
	10	6/14 (43%)	38 ± 4.34	27–52
20	10	7/7 (100%)	19 ± 0.84	16–21
	11	1/10 (10%)	51	

Lineages and selection cycles that were negative for tumor formation are not displayed in this table. There were no differences in tumor formation for individual lineages between male and female host rats (data not shown).

*Selection cycle following which cells were transplanted into syngeneic rats.

[†]Fraction represents number of rats with tumor over total number of rats transplanted with cells.

[‡]Mean time to detection and harvest of 1 cm. diameter tumors.

[§]Range of tumor latencies.

ously.⁶ The nomenclature for the tumor cell lines incorporates the name of the heterogeneous cell population that produced the tumor (L1–20, C1–12), the tumor identification number (T1–T6), and the subclone identification number.^{1–5}

To confirm their tumorigenic potential and to evaluate their differentiation potential at a different transplantation site, 5×10^6 cells from each clonal tumor cell line were injected intraperitoneally into adult (3-month old) male Fischer-344 rats as described previously.²⁰ Studies involving the use of animals were carried out in accordance with federal and institutional guidelines put forth by the National Institutes of Health and the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Sections of tumor tissue were fixed in buffered formalin, processed for paraffin sections, and stained with hematoxylin and eosin (H&E) for histological analysis. Formalin-fixed tissues were fixed additionally in 3% glutaraldehyde in 0.15 mol/L sodium phosphate buffer and processed for transmission electron microscopy.

Results

Temporal Analysis of the Acquisition of Tumorigenicity by Spontaneous Transformants of WB-F344 Cells

Low-passage WB-F344 cells did not produce tumors during 1 year after subcutaneous transplantation into neonatal syngeneic rats. None of the lineages produced tumors before selection cycle 8, but 5/18 lineages, 7/18

lineages, and 1/18 lineages first displayed tumorigenic potential at selection cycles 8, 10, and 11, respectively (Table 1). Five lineages (L10, L13, L17, L18, and L19) were not tumorigenic at any selection cycle. Tumor incidence increased and/or latency of tumor formation decreased with increasing numbers of selection cycles in 6/7 lineages (Table 1) that were tumorigenic at more than one selection cycle. Tumorigenic potential was not related to the number of accumulated population doublings (Table 2). On average, nontumorigenic lineages accumulated 20.1 ± 1.1 ($n = 5$) population doublings, and tumorigenic lineages accumulated 20.7 ± 0.6 ($n = 13$) population doublings during the course of 10 selection cycles.

Correlation of Phenotypic Characteristics with the Acquisition of Tumorigenicity

Four cell lineages showed no evidence of morphological transformation in culture during 10 to 12 cycles of selective growth. These lineages were indistinguishable morphologically from confluent cultures of parental WB-F344 cells (pattern I, Figure 2A). The morphologies and growth patterns of the other 14 lineages could be classified into three distinct patterns of abnormal growth (Table 2 and Figure 2) that became apparent as early as selection cycle 5 and were maintained throughout subsequent selection cycles. Three of the lineages expressed a growth pattern characterized by foci of large polygonal cells separated by cords of smaller cells with scant cytoplasm that established a boundary around the foci (pattern II, Figure 2B). In more than one-third of the cell lineages, a

Table 2. Phenotypic Characteristics of Spontaneous Transformants of WB-F344 Rat Liver Epithelial Cells after 10 Cycles of Selective Growth

Lineage	Morphological pattern	Saturation density (28-day)	Anchorage-independent growth	Tumorigenic potential		Accumulated population doublings
WB control	I	2.40×10^5	No	0/8	0%	NA
L1	III	$5.80 \times 10^{5*}$	No	7/7	100%	23.0
L2	III	$6.30 \times 10^{5*}$	No	8/8	100%	19.4
L3	II	3.40×10^5	Yes	0/8	0%	15.6
L4	III	ND	No	9/9	100%	23.0
L6	III	$7.50 \times 10^{5*}$	No	7/7	100%	21.3
L7	I	$6.00 \times 10^{5*}$	No	5/18	28%	21.2
L8	II	ND	No	3/9	33%	22.0
L9	II	$3.80 \times 10^{5*}$	Yes	2/19	11%	22.1
L10	I	2.60×10^5	No	0/7	0%	20.7
L11	III	ND	No	2/10	20%	21.7
L12	IV	$4.60 \times 10^{5*}$	Yes	9/9	100%	19.2
L13	I	$5.00 \times 10^{5*}$	No	0/8	0%	20.6
L14	IV	ND	No	0/10	0%	17.4
L16	III	ND	No	6/14	43%	22.4
L17	IV	3.20×10^5	Yes	0/5	0%	20.4
L18	I	3.60×10^5	No	0/8	0%	16.2
L19	IV	ND	No	0/9	0%	22.7
L20	III	1.90×10^5	No	7/7	100%	21.3

Refer to the text for a description of each morphological pattern. Positive growth in soft agar was indicated by the formation of 0.05-mm-diameter colonies by more than 0.1% of the 1.25×10^4 cells plated under standard conditions. The number of population doublings per selection cycle = $1n[(n_t)/(n_i)]/1n2$ where n_t is the final number of cells present on the dish at the end of a selection cycle and n_i is the initial number of cells seeded at the beginning of the selection cycle. ND, not done.

*Statistically significant differences from the WB control ($n = 2$).

subpopulation of smaller densely packed cells formed a second layer on top of the attached monolayer of apparently normal cells (pattern III, Figure 2C). In four lineages, the growth pattern was characterized by the presence of free-floating aggregates of rounded cells suspended above a monolayer that resembled the parental WB-F344 cells (pattern IV, Figure 2D). In general, lineages that displayed growth pattern I were nontumorigenic, whereas lineages that displayed growth patterns II and III

were tumorigenic. Lineages that displayed growth pattern IV were variably tumorigenic. The majority of the populations did not form any colonies in soft agar (Table 2). Therefore, anchorage-independent growth was not correlated with the expression of tumorigenicity by spontaneous transformants of WB-F344 cells.

Final cell densities of the individual cell populations were evaluated at the end of each cycle of selection to detect the emergence of subpopulations that had acquired a growth advantage in the environment of confluent cell culture. The final cell density of 7/11 (64%) of the tumorigenic lineages fluctuated minimally through selection cycle 8 but increased dramatically between selection cycles 8 and 12, attaining cell numbers 4- to 14-fold above those observed at selection cycle 1 (Figure 3A). In contrast, 6/7 (86%) of the nontumorigenic lineages demonstrated only slight fluctuations in final cell density over the 12 cycles of selective growth (Figure 3B). Therefore, the progressive increase in the final cell density over the course of the late selection cycles was a good predictor of tumorigenic potential at selection cycle 10, and conversely, minimal variation in final cell density was associated with a lack of tumorigenic potential.

Saturation densities of the individual populations at selection cycle 10 after 28 days in culture provided a measure of loss of contact inhibition of the populations after prolonged maintenance under confluent conditions. A Bayesian analysis²¹ of saturation densities determined that a critical value of 3.6×10^5 cells/cm² provided the best separation of tumorigenic and nontumorigenic populations at this selection cycle (correct assignments = 83%; sensitivity = 86%; specificity = 80%). Six of seven populations that attained this density were tumorigenic, whereas four of five of the populations that did not attain

Figure 2. Morphology of cultured WB-F344 cell lineages maintained under selective growth conditions. The 18 individual lineages were characterized by four distinct growth patterns at confluence. A: Pattern I (WB-L10C9); B: Pattern II (WB-L3C9); C: Pattern III (WB-L1C12); D: Pattern IV (WB-L19C9).

Figure 3. Final cell densities of spontaneously transformed lineages. The final cell density of each individual population was determined at the end of each cycle of selection and provided evidence of the emergence of subpopulations that had acquired a growth advantage at confluence. The tumorigenic lineages demonstrated significant increases in final cell density between selection cycles 8 and 12 (A). In contrast, the nontumorigenic lineages demonstrated minimal variations in final cell density throughout the study (B).

this density were nontumorigenic (Figure 4). Although the increases in saturation density were statistically significant, none of the populations attained densities greater than threefold that of the parental WB-F344 cells.

Phenotypic Characterization in Vitro and Tumorigenicity in Vivo of Clonal Tumor Cell Lines

At least two independent clonal cell lines were isolated from tumors produced by each of seven spontaneously transformed lineages of WB-F344 cells. Almost all (14/16) of the clonal tumor cell lines exhibited a loss of contact inhibition at confluence, demonstrated by significant in-

Figure 4. The 28-day saturation density assay. Cell densities of the individual populations after 28 days in culture were used to monitor the emergence of subpopulations able to proliferate at confluence. Six of seven populations that attained cell densities greater than 3.6×10^5 cells/cm² (solid bars) after 28 days in culture were tumorigenic (filled bars), whereas four of five nontumorigenic populations (empty bars) did not attain this density.

creases in saturation densities relative to parental WB-F344 cells (Table 3). However, none of the cell lines attained densities greater than threefold that of the parental WB-F344 cells. One-half of the clonal tumor cell lines were capable of anchorage-independent growth although the average colony-forming efficiency of these lines was only 3.4% (Table 3). Despite the low capacity for anchorage-independent growth, all of the clonal tumor cell lines were tumorigenic at the intraperitoneal transplantation site (Table 3). In general, the latency of tumor formation for individual tumor cell clones derived from the same tumor was similar and was significantly shorter than that observed for the parental lineages from which they were originally derived.

Histological Analysis of the Tumors

The spontaneously transformed lineages formed a variety of tumor types including hepatocellular carcinomas, adenocarcinomas, cholangiocarcinomas, and hepatoblastomas (Figures 5 to 7). Most (65%) of the 103 tumors were classified histologically as poorly differentiated hepatocellular carcinomas. These tumors consisted of pleomorphic epithelial cells that were arranged primarily in cords or sheets interspersed with various amounts of connective tissue (Figures 5 and 6A). Some of the hepatocellular carcinomas were moderately differentiated and formed a trabecular pattern interspersed with intercellular spaces resembling sinusoids (Figure 5B). Nine tumors (9%) were classified as hepatoblastomas because the cells resembled small, primitive hepatoblasts (Figure 5C). Lineage 6 at cycle 10 (L6C10) formed a mixed epithelial-mesenchymal tumor that contained foci of osteoid tissue (Figure 5E). Twenty-seven tumors (26%) were classified as adenocarcinomas; seven of these tumors were classified more specifically as cholangiocarcinomas. The cholangiocarcinomas were composed of glands or duct-like structures with irregularly shaped lumens lined by a single layer of columnar or cuboidal cells (Figures 5D and 6B). The cholangiocarcinomas ranged from poorly differentiated to well differentiated. The twenty tumors produced by lineage 4 were similar histologically and were classified as poorly differentiated adenocarcinomas. These tumors were often hypocellular but contained prominent duct-like structures that were heterogeneous in size, shape, and density, and some contained an unidentified secretory product (Figures 5G and 6C). The duct-like structures were surrounded by a scant meshwork of epithelial cells and connective tissue.

Intraperitoneal transplantation of the 16 clonal tumor cell lines into adult syngeneic rats produced a total of 41 tumors, including poorly differentiated hepatocellular carcinomas (24/41, 59%), poorly differentiated adenocarcinomas (12/41, 26%), and osteogenic sarcomas (5/41, 9%). L6C10T5-2 and L6C10T5-3, derived from the L6C10 tumor that contained osteoid tissue, produced tumors composed almost entirely of a bony matrix and contained cells that resembled osteoblasts and osteoclasts (Figure 5F).

Ultrastructurally, the cytoplasm of the epithelial cells constituting the various tumor types contained prominent

Table 3. Phenotypic Characterization and Tumorigenicity of Transplanted Clonal Tumor Cell Lines Established from Spontaneous Transformants of WB-F344 Rat Liver Epithelial Cells

Tumor cell line	Saturation density (14-day)	Anchorage-independent growth	Intraperitoneal tumors	Latency (weeks, mean $\pm$ SEM)	Latency range (weeks)	Tumor histology
L2C10T3-2	$1.54 \times 10^{5*}$	Yes	2/3	16 ± 0.79	16–17	HCC
L2C10T3-5	$1.23 \times 10^{5*}$	Yes	3/3	15 ± 0.58	14–16	HCC
L4C8T3-2	$1.83 \times 10^{5*}$	ND	3/3	15 ± 0.57	13–15	Adenocarcinoma
L4C8T3-3	$1.63 \times 10^{5*}$	ND	3/3	13		Adenocarcinoma
L6C10T3-1	$1.47 \times 10^{5*}$	Yes	2/3	42 ± 4.50	38–47	HCC
L6C10T3-2	$1.18 \times 10^{5*}$	Yes	2/3	18 ± 3.00	15–21	HCC
L6C10T5-2	1.12×10^5	No	2/3	55		Osteogenic sarcoma
L6C10T5-3	$1.42 \times 10^{5*}$	No	3/3	55		Osteogenic sarcoma
L7C10T3-2	$1.37 \times 10^{5*}$	Yes	3/3	13 ± 0.81	12–14	HCC
L7C10T3-5	$1.59 \times 10^{5*}$	Yes	2/3	27 ± 4.07	23–31	HCC
L14C8T1-1	$1.98 \times 10^{5*}$	No	1/3	19		HCC
L14C8T1-3	7.10×10^4	No	3/3	6 ± 1.28	4–8	HCC
L16C8T1-1	1.55×10^5	ND	3/3	15 ± 1.71	12–17	HCC
L16C8T1-2	$1.30 \times 10^{5*}$	ND	3/3	15		HCC
L20C10T6-1	$1.94 \times 10^{5*}$	No	3/3	8 ± 0.10	8	Adenocarcinoma
L20C10T6-5	$2.05 \times 10^{5*}$	No	3/3	9 ± 0.43	9–10	Adenocarcinoma

Positive growth in soft agar was indicated by the formation of colonies by more than 0.1% of the cells plated under standard conditions. The fraction of intraperitoneal tumors represents the number of rats with tumors over total number of rats transplanted with cells. Latency is the mean time to detection and harvest of tumors.

*Statistically significant differences from the WB control ($n = 3$).

nuclei, abundant free polyribosomes, rough endoplasmic reticulum and lysosomes, and occasionally, lipid droplets and glycogen. Structures resembling bile canaliculi and desmosomes were apparent between adjacent cells of some tumors and numerous irregular microvilli extended from the surface of the cells into the extracellular space (Figure 6, C and D). Bundles of collagen fibers were seen in transverse and longitudinal section in the extracellular matrix surrounding the epithelial cells.

The majority of the spontaneously transformed lineages produced tumors that exhibited a lineage-specific pattern of differentiation (Figure 7). Moreover, several of the lineages that were tumorigenic at more than one selection cycle produced tumors with consistent patterns of differentiation from cycle to cycle. In addition, the histological classification of intraperitoneal tumors produced by the clonal tumor cell lines was similar or identical to that of the subcutaneous tumors produced by the corresponding parental cell lineage (Figure 5, F and H). However, tumors produced by the tumor cell lines were often less differentiated than those produced by the parental cell lineages.

Discussion

We used the selective growth condition of prolonged maintenance at confluence to potentiate the emergence of spontaneously transformed populations of WB-F344 cells to characterize the timing and independent origin of cellular changes culminating in spontaneous neoplastic transformation. We have established more than 200 related (18 lineages $\times$ 12 selection cycles), but distinct, cell populations that represent progressive steps in the pathway to spontaneous neoplastic transformation. Individual populations were characterized for the expression of multiple cellular phenotypes, including the ability to

produce tumors *in vivo*. Morphological changes in rat liver epithelial cells transformed spontaneously under the selective growth condition of maintenance at confluence have been described previously and include growth in multiple layers, decreased cell adhesion, and the loss of anchorage dependence.^{8,22,23} In this study, spontaneous transformants of WB-F344 cells displayed similar cellular traits and population growth characteristics. Montesano et al²⁴ reported that cytological characteristics were a reliable means of determining the tumorigenic potential of cultured rat liver epithelial cells. More recently, Huggett et al⁸ evaluated the tumorigenic potential of normal and morphologically transformed clones of rat liver epithelial cells established after temporary maintenance at confluence. Clones with normal morphology were unable to form tumors in nude mice, whereas all morphologically aberrant clones formed tumors.⁸ The present study demonstrated a similar relationship between morphological transformation in the individual lineages and their ability to produce tumors in syngeneic animals. The presence of a limited number of altered morphological growth patterns among the multiple lineages suggests common mechanisms of transformation among subsets of lineages or a limited number of pathways leading to neoplastic transformation.

Histological analysis of the tumors produced by the independent lineages, and cell lines established from these tumors, demonstrated that spontaneous transformants of WB-F344 cells have the capacity to differentiate along both hepatocytic and bile duct epithelial lineages. The spectrum of differentiation expressed by the spontaneous transformants differed significantly from that of chemically transformed derivatives of the WB-F344 cells. Spontaneous transformants of WB-F344 cells did not give rise to epidermoid carcinomas or undifferentiated sarco-

Figure 5. Histologies of tumors produced by parental spontaneous transformants and derived clonal tumor cell lines. Representative histologies of tumors produced by the heterogeneous independent lineages and their corresponding clonal tumor cell lines are displayed. **A:** Poorly differentiated hepatocellular carcinoma (L7C10); **B:** Moderately differentiated hepatocellular carcinoma (L14C8); **C:** Embryonal hepatoblastoma (L9C10); **D:** Cholangiocarcinoma (L20C10); **E:** Hepatoblastoma with a focus of osteoid tissue (L6C10); **F:** Osteogenic sarcoma (L6C10T5-2); **G:** Poorly differentiated adenocarcinoma (L4C8); **H:** Poorly differentiated adenocarcinoma (L4C8T3-2). The histology classification of the intraperitoneal tumors produced by the tumor cell lines was similar or identical to that of the subcutaneous tumors produced by the parental cell lineages.

Figure 6. Transmission electron microscopy of tumors produced by spontaneous transformants. **A:** Moderately differentiated hepatocellular carcinoma (L6C10) showing numerous pleomorphic epithelial cells. Magnification, $\times 2100$. **B:** Well differentiated cholangiocarcinoma (L20C10) showing a lumen lined by cuboidal cells. Magnification, $\times 3200$. **C:** Poorly differentiated adenocarcinoma (L4C8) showing two epithelial cells joined by desmosomes. Note the prominent glandular lumen surrounded by extracellular matrix components. Magnification, $\times 9600$. **D:** Poorly differentiated adenocarcinoma (L4C8) showing microvilli extending into the extracellular space. Magnification, $\times 20,800$.

mas, which constituted approximately 10% of the tumors generated by the carcinogen-transformed WB-F344 cell lines.¹⁴ Consistent with this difference, other investigators have noted an absence of mesenchymal components in tumors produced by spontaneous transformants of other rat liver epithelial cells and have suggested that mesenchymal differentiation is a rare spontaneous event.^{8,16} However, one of our spontaneous transformants produced osteogenic sarcomas that contained well differentiated bone. The spectrum of tumor types generated by the spontaneous transformants of WB-F344 rat liver epithelial cells also differed from that reported by investigators who have used the same selection protocol to neoplastically transform other rat liver epithelial cell lines.^{7,8,16} Five lineages of spontaneously transformed RL-F344 cells described by Tsao et al⁷ produced only poorly differentiated adenocarcinomas in syngeneic rats. In contrast, all of the spontaneous transformants of RLE cells described by Huggett et al⁸ and Williams et al¹⁶ produced well differentiated trabecular hepatocellular

Figure 7. Classification of tumors produced by spontaneous transformants. Paraffin-embedded sections of tumor tissue were stained with H&E. Tumors were classified histologically by light microscopy, and the distribution of tumor types produced by each tumorigenic lineage is displayed. The majority of the independent lineages of spontaneous transformants produced tumors that exhibited consistent and specific patterns of differentiation. Several of the lineages that were tumorigenic at more than one selection cycle produced tumors with consistent patterns of differentiation from cycle to cycle.

carcinomas in nude mice. In each of these studies, the spontaneous transformants gave rise consistently to a single tumor type. The more diverse spectrum of tumor types produced by the spontaneous transformants of the WB-F344 cell line may suggest that WB-F344 cells are a more primitive, multipotential stem cell with a wider differentiation potential than the rat liver epithelial cell lines used by other investigators.

The consistent and specific patterns of morphological differentiation displayed by the spontaneously transformed lineages of WB-F344 cells are particularly intriguing observations. Individual lineages of spontaneously transformed WB-F344 cells demonstrate commitment to formation of a specific tumor type in contrast to chemically transformed WB-F344 cell lines, which exhibit marked phenotypic plasticity, randomly forming any of a variety of tumor types on transplantation.¹⁴ Although the differentiation pathway of rat liver epithelial cells may be modulated by a variety of complex factors, including numerous growth factors,^{15,16,25,26} tissue microenvironment,²⁷⁻³⁰ and cell-to-cell contact,³¹⁻³³ the differentiation status also is affected by genetic programming.^{34,35} Lineage-specific patterns of differentiation probably reflect the selective outgrowth of a tumorigenic subpopulation from the heterogeneous parental populations of cells. The observation that the tumor morphology is maintained *in vivo* (in both subcutaneous and intraperitoneal sites of transplantation) in the tumor cell lines suggests that commitment to formation of a specific tumor type and level of differentiation occur early in the transformation process.

In summary, four significant observations made in this study provide substantial evidence that spontaneous transformation of individual lineages of WB-F344 cells can be attributed to unique events: 1) phenotypic diversity and differences in tumorigenic potential among lineages that have undergone the same experimental regimen (ie, the presence of nontumorigenic and tumorigenic lineages), 2) the variable temporal appearance of tumorigenic subpopulations over the course of multiple cycles of selective growth, 3) variations in frequency and latency of tumor formation among the 13 tumorigenic lineages, and 4) lineage-specific patterns of tumor cell differentiation. Furthermore, to our knowledge, this is the first report of a cohort of related, but independent, clonal tumor cell lines that collectively produce a spectrum of tumor types but individually reproduce a specific differentiated tumor type. Consequently, these cell lines represent valuable reagents for investigating the cellular and molecular mechanisms involved in the neoplastic transformation and differentiation of hepatic stem-like cells.

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