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Human Squamous Carcinoma Cells Express Complex Defects in the Control of Proliferation and Differentiation

ROBERT E. SCOTT, MD, MARK S. WILKE, MD, JOHN J. WILLE, Jr., PhD, MARK R. PITTELKOW, MD,* BETH M. HSU, BA, and JAN L. KASPERBAUER, MD†

From the Section of Experimental Pathology and the Departments of Dermatology and Otorhinolaryngology,† Mayo Clinic/Foundation, Rochester, Minnesota*

Four human squamous carcinoma cell (SCC) lines (SCC-9, SCC-13, SCC-15, and SCC-25) were studied to characterize their relative ability to control proliferation and differentiation. These experiments were based on previous data that established that in normal human keratinocytes three distinct and sequential steps are involved in the integrated control of proliferation and differentiation: 1) reversible growth-arrest at a predifferentiation state, 2) irreversible loss of proliferative potential, and 3) terminal differentiation. The current results show that SCC can show changes in the culture conditions required to undergo reversible growth-arrest and SCC can express partial or complete defects in their ability to irreversibly growth-arrest or terminally differentiate. For example, SCC-9 and SCC-25 cannot irreversibly growth-arrest or terminally differentiate, SCC-13 can irreversibly growth-arrest but cannot terminally differentiate, and SCC-15 can irreversibly growth-arrest and terminally differentiate to a moderate extent. These results therefore extend previous data by establishing that the malignant transformation of human epithelial cells does not simply result from defects in the control of terminal differentiation but rather from a combination of complex defects in the regulation of proliferation and differentiation. (Am J Pathol 1988, 133:374–380)

THE PROCESS OF carcinogenesis has been established in previous studies to be associated with the development of defects in the control of both cellular proliferation and differentiation. Studies on murine cells demonstrated that neoplastic transformation correlates well with loss of the ability of cells to undergo density-dependent growth inhibition and with a reduced requirement for specific growth factors.[1-3] In addition, transformed murine cells commonly acquire the ability to grow in soft agar in an anchorage-independent manner.[4] Some of these phenotypic changes have been implicated to result from an autocrine mechanism whereby the transformed cells produce their own growth factors.[5] Murine cells that undergo neoplastic transformation also commonly show a decreased potential to differentiate both nonterminally and terminally. The latter observations are supported by studies on transformed murine 3T3 T mesenchymal stem cells,[6] murine leukemia cells,[7] and murine embryonal carcinoma cells.[8]

More limited studies have been performed on human cancer cells. Some reports suggest that human squamous carcinoma cells (SCC) can differentiate well both *in vitro* and *in vivo*,[9,10] whereas other studies suggest that SCC are defective in their ability to terminally differentiate.[11]

This controversy serves as the basis for the current experiments. Of most interest for the current studies is the report of Rheinwald and Beckett[11] that SCC lines that are cultured in an anchorage-independent microenvironment show a decreased rate of commitment to terminal differentiation compared with normal cells and therefore possess at least a partial defect in the mechanisms that trigger terminal differentiation.

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Address reprint requests to Robert E. Scott, 511B Guggenheim Building, Mayo Clinic/Foundation, Rochester, MN 55905.

These results raised a series of important questions that have not been answered; they concern the underlying biologic defects that may exist in human squamous carcinoma cells. Why do SCC spontaneously differentiate poorly compared with normal human keratinocytes? Why do SCC show a decreased rate of terminal differentiation? Why do many SCC lose their potential to proliferate when cultured in suspension? Can SCC irreversibly arrest their growth whether they differentiate or not? Do SCC express defects in their potential to reversibly arrest their proliferation?

The current studies were performed to directly investigate some of these questions specifically with reference to recent data[12] that establish that three specific sequential processes are involved in the control of proliferation and differentiation leading to the state of terminal differentiation of normal human keratinocytes. These are: 1) reversible growth arrest at a predifferentiation state, 2) irreversible loss of proliferative potential, and 3) expression of the terminally differentiated phenotype.

The authors now report that human squamous carcinoma cell lines can be defective in many of these regulatory events and not just in their ability to terminally differentiate. SCC-25 specifically show distinct requirements to undergo reversible growth-arrest compared with normal keratinocytes and show near complete defects in their ability to irreversibly growth-arrest and terminally differentiate. SCC-9 is similar to SCC-25. In contrast, SCC-13 and SCC-15 both undergo reversible and irreversible growth-arrest but only SCC-15 are competent to terminally differentiate to a significant extent.

The authors interpret these results to support the conclusion that human epithelial carcinogenesis is associated with the expression of multiple defects in the integrated control of proliferation and differentiation and not simply defects in rate of commitment to terminal differentiation.

Materials and Methods

Cells and Cell Culture

Four human squamous carcinoma cell lines were studied: SCC-9, SCC-13, SCC-15, and SCC-25. Cells were obtained from the American Type Culture Collection or J. G. Rheinwald and all are reported to be highly tumorigenic in nude mice.[11] SCC-9, SCC-15, and SCC-25 were originally derived from squamous cell carcinomas of the tongue whereas SCC-13 was derived from a squamous cell carcinoma of the skin.[11]

In the current studies, SCC-25 was grown in complete MCDB 153 medium, which is composed of nutrient medium MCDB 153 supplemented with ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), hydrocortisone (5×10^{-7} M), insulin (5 μg/ml), epidermal growth factor (10 ng/ml), and bovine pituitary extract (140 μg/ml) in 0.1 mM Ca^{++} unless otherwise stated. This serum-free medium is identical to that used to grow normal human keratinocytes.[13] SCC-9, SCC-13, and SCC-15 could not be successfully adapted to grow in this serum-free medium for undetermined reasons. Therefore, SCC-15 was grown in DMEM:MCDB 153 complete (1:1) containing 10% FBS, and SCC-9 and SCC-13 were grown in a similar medium containing 2.5% FBS.

Stock cultures of these cell lines were typically passaged and plated at approximately 2×10^3 cells/sq cm. All stock cultures were grown on standard tissue culture plastic dishes/flasks unless otherwise stated in a humidified atmosphere containing 5% CO_2 and 95% air at 37 C. All SCC lines were proven to be free of mycoplasma contamination by the method of Chen.[13]

Cell Proliferation Assays

Characterization of cell growth parameters by cell density analysis was performed as described previously.[12,14–18] Cell cycle characteristics were determined by cytofluorometry on a FACS IV apparatus as described.[13,18] The extent of cell proliferation, ie, traverse of the "S" phase of the cell cycle, was determined by evaluation of [^{3}H]thymidine incorporation into DNA by either 1-hour pulse incubation followed by DNA precipitation with quantitation by scintillation spectroscopy or 48-hour incubation with [^{3}H]-thymidine followed by autoradiographic analysis of nuclear labeling. These procedures have been described previously.[12,14–18] Cell proliferation potential was also determined by clonogenic assay as described[13–18] wherein cells before and after various treatments were plated at 500 cells/dish in optimum growth medium and cultured therein for 10–14 days, after which time the percentage of cells that formed colonies was quantitated.

Cell Differentiation

Three different assays were used to evaluate differentiation. First, a polyclonal anti-involucrin antibody was employed to stain differentiated cells containing involucrin and such cells were detected using indirect immunofluorescent techniques. The method used to detect this antigen was comparable with that described previously.[12,14–18] Second, microscopic quantitation of cell stratification and so-called "crown" formation was also used in some assays as an indica-

Table 1—Effect of Ethionine Containing Medium on the Proliferation of Squamous Carcinoma Cells (SCC-25)

Culture condition	Cell density (cells/sq cm × 10^{-3})	DNA synthesis (cpm)	Labeled nuclei (%)	Cell cycle states (G_1 S G_2/M (% cell populations)			Colony forming efficiency* (% control)
Complete MCDB 153							
Day 0	5.7	–	–	–	–	–	–
Day 1	8.3	9200	–	–	–	–	–
Day 2	13.5	23,900	86	54	30	16	–
Ethionine containing MCDB 153							
Day 1	6.0	400	–	–	–	–	–
Day 2	5.9	115	2	70	8	22	85

* Plating efficiency for untreated, rapidly growing control SCC-25 was ≥50% and the colony forming efficiency varied from 60–80%. Colony forming efficiency was calculated on the basis of the actual number of cells that plated in each assay.

tion of whether or not morphologic differentiation occurred. Finally, the Ayoub and Shklar histochemical stain was employed; this procedure stains undifferentiated keratinocytes blue and differentiated keratinocytes red as reported by several other investigators who have used this stain for similar purposes.[14,18,19]

SCC-25 were evaluated for their differentiation response following culture in isoleucine-deficient medium, in ethionine (8 mM) containing medium, or in growth factor-deficient medium containing 2 mM calcium. Differentiation assays were also performed on SCC-9, SCC-13, SCC-15, and SCC-25 that had been cultured in suspension on agar coated dishes. More specifically, in these assays 60 mm petri dishes were coated with 1.6% Sea Plaque agarose (FMC Corporation, Rockland, ME). After the agarose solidified, suspension of cells at approximately 1×10^5/dish was plated in appropriate medium and cultured thereon as described.

Results

SCC-25 in Monolayer Culture Microenvironment

The initial studies sought to determine the biologic characteristics of SCC-25 that were grown in a serum-free medium designated complete MCDB 153, which is the same medium in which normal human keratinocytes are grown.[12,14] In this medium, SCC-25 express an epithelial morphology and they show a population doubling time of 43 ± 7 hours with a >95% labeling index of DNA synthesis and S-phase traverse. In addition, they grow to a saturation density of ≥8 × 10^4 cells/sq cm.

Studies were next performed to determine if SCC-25 could be induced to growth-arrest and differentiate in a manner similar to normal human keratinocytes. To induce growth-arrest, rapidly growing SCC-25 were refed medium containing one of the potential growth arrest inducing agents described below. The cells were then evaluated to determine if growth-arrest occurred and whether it was reversible. The cell cycle characteristics of the growth-arrested cells were also assayed. Studies were performed subsequently to evaluate the differentiation potential of SCC-25. Because ethionine treatment or isoleucine deficiency induced reversible growth-arrest in normal human keratinocytes,[12,16] the current studies tested the effect of these agents on SCC-25. Because razoxane treatment or culture in high calcium-containing medium devoid of growth factors induced irreversible growth-arrest in normal human keratinocytes,[12,16] the effect of these agents on SCC-25 also was tested.

Table 2—Effect of Isoleucine-Deficient Medium on the Proliferation of Squamous Carcinoma Cells (SCC-25)

Culture condition	Cell density (cells/sq cm × 10^{-3})	DNA synthesis (cpm)	Labeled nuclei (%)	Cell cycle states (G_1 S G_2/M (% cell populations)			Colony forming efficiency* (% control)
Complete MCDB 153							
Day 0	4.8	–	–	–	–	–	–
Day 1	7.0	13,500	–	–	–	–	–
Day 2	11.0	20,600	96	53	31	16	–
Isoleucine-deficient MCDB 153							
Day 1	4.6	700	–	–	–	–	–
Day 2	4.8	150	4	62	7	31	50

* Plating efficiency for untreated, rapidly growing control SCC-25 was ≥50% and the colony forming efficiency varied from 60–80%. Colony forming efficiency was calculated on the basis of the actual number of cells that plated in each assay.

Table 3—Effect of High Calcium Medium Devoid of Growth Factors (GF-2 Medium) on Squamous Carcinoma Cells (SCC-25)

Culture condition	Cell density (cells/sq cm × 10^{-3})	DNA synthesis (cpm)	Labeled nuclei (%)	Cell cycle states (% cell populations) G_1	S	G_2/M	Colony forming efficiency* (% control)
Complete MCDB 153							
Day 0	3.6	–	96	–	–	–	–
Day 2	7.0	–	–	55	28	17	–
Day 4	12.7	18,200	–	–	–	–	–
GF-2 medium							
Day 0	3.6	–	–	–	–	–	–
Day 2	4.0	100	61	–	–	–	–
Day 4	3.6	100	6	75	8	17	–
Day 6	3.3	–	2	–	–	–	95

* Plating efficiency for untreated, rapidly growing control SCC-25 was ≥50% and the colony forming efficiency varied from 60–80%. Colony forming efficiency was calculated on the basis of the actual number of cells that plated in each assay.

Effect of Ethionine Treatment

The data in Table 1 show that SCC-25 growth-arrest in ethionine-containing medium because after ethionine treatment the cell density does not increase and DNA synthesis is markedly inhibited. Under these culture conditions, SCC-25 also accumulate predominately in the G_1 phase of the cell cycle although arrest of some cells in G_2 is also evident.

Analysis of the clonogenic potential of SCC-25 induced to growth arrest by ethionine treatment also shows that growth arrest in such cells is reversible because SCC-25 retain their proliferation potential under these culture conditions. Even after 6 days of culture in ethionine containing medium SCC-25 show a clonogenic potential of ≥50% of untreated cells.

Effect of Isoleucine-Deficient Medium

To determine if SCC-25 reversibly arrest their growth in isoleucine-deficient medium as do normal keratinocytes,[12,16] the studies reported in Table 2 were performed. The results show that SCC-25 stop growing within 2 days after culture in isoleucine-deficient medium and that such cells accumulate predominately in the G_1 and G_2 phases of the cell cycle. The growth-arrest induced under this culture condition is also reversible because SCC-25 growth-arrested in isoleucine-deficient medium show a high clonogenic potential when refed complete MCDB 153. This result was observed even when clonogenic assays were performed after the cells were maintained in isoleucine-deficient medium for 6 days (data not shown).

Effect of High Calcium–Low Growth Factor Medium

When rapidly growing SCC-25 are fed high calcium-containing medium devoid of growth factors, Table 3 shows that they also rapidly arrest their growth. Evidence that they stop growing is that the cell density does not increase significantly and DNA synthesis is markedly inhibited as assayed both by autoradiography and scintillation spectroscopy. Growth arrest that occurs under these culture conditions is associated with the predominant accumulation of cells in the G_1 phase of the cell cycle, although some cells also accumulate in G_2.

In normal keratinocytes, growth arrest induced by high calcium-growth factor-deficient medium results in the irreversible loss of proliferative potential.[12,16] To determine if growth-arrest of SCC-25 induced by this medium is also irreversible, growth-arrested SCC-25 were passaged into complete MCDB 153 medium and their clonogenic potential was assayed. The results show that their proliferative potential is not lost because the majority of the SCC-25 retain their clonogenic potential after 6 days of culture in this medium. Even if SCC-25 are cultured in high calcium–growth-factor-deficient medium for 9 days, their clonogenic potential is 90% of control levels. These results establish a significant difference between normal keratinocytes and SCC-25. In SCC-25, growth-arrest induced by high calcium–growth-factor-deficient medium does not result in the irreversible loss of proliferative potential as it does in normal keratinocytes.

Effect of Razoxane Treatment

In normal keratinocytes, 10 μg/ml razoxane also induces irreversible growth-arrest in the G_2 phase of the cell cycle.[12,16] Such arrested cells then can be induced to differentiate if incubated in medium containing 2 mM calcium. SCC-25, in contrast, show a much more complex and variable response to razoxane. For example, 10 μg/ml razoxane is nontoxic to SCC-25, yet it partially inhibits cytokinesis and increases the percentage of cells in G_2 without completely inhibiting DNA synthesis.

Razoxane at 75 μg/ml, however, irreversibly growth-inhibits SCC-25 but 75 μg/ml razoxane is also

Table 4—Differentiation Potential of Squamous Carcinoma Cells (SCC-25)

Treatment	Cell viability (%)	Differentiation assays*		
		Ayoub and Shklar histochemistry	Cell stratification and crown formation	Involucrin expression
Ethionine†	80–95	0	0	0
Isoleucine deficiency‡	>75	0	0	0
High calcium-growth-factor deficiency	95	0	0	0

* Percent positive cells or colonies. The designation of 0% actually signifies a <1% response.

† Differentiation assays were performed in complete MCDB 153 + ethionine + 2 mM Ca^{++} and in growth-factor-deficient MCDB 153 + ethionine + 2 mM Ca^{++}.

‡ Differentiation assays were performed in isoleucine deficiency complete MCDB 153 in 2 mM Ca^{++}. Assays were also performed in isoleucine-deficient and growth-factor-deficient MCDB 153 in 2 mM Ca^{++}.

highly toxic and can cause the death of 50–85% of treated cells within 2–8 days. Therefore, razoxane was not studied further to evaluate its effect in the control of SCC-25 proliferation and differentiation.

Differentiation Potential of Growth Arrested SCC-25

Normal keratinocytes that have been growth-arrested at either reversible or irreversible states possess the potential to differentiate when cultured for prolonged intervals, especially in high (2 mM) calcium-containing medium, such as growth-factor-deficient MCDB 153 containing 2 mM Ca^{++}.[12] SCC-25 that had been growth-arrested by the conditions described above were treated with similar medium or with the growth-arrest-inducing medium to which 2 mM Ca^{++} had been added. Differentiation was then sequentially assayed over a 9–12-day interval and viability studies were also performed at the time of assay to determine the relative health of the cells.

The data in Table 4 show that SCC-25 are markedly defective in their ability to differentiate. This defect in cellular differentiation was evident when either of three differentiation assays were employed. These included: 1) morphologic evaluation of cell stratification and so-called "crown" formation, 2) histochemical analysis using the Ayoub and Shklar stain, and 3) immunologic evaluation of differentiation using the anti-involucrin antibody. Representative studies also were performed using a physicochemical method to assay differentiation by the formation of cross-linked envelopes[11] and therein SCC-25 also showed minimal differentiation.

These results establish that SCC-25 differ from normal human keratinocytes in a significant way as summarized in Table 5. More specifically, SCC-25 cannot be induced to irreversibly growth-arrest when cultured in high calcium–growth-factor-deficient medium and they fail to differentiate under a variety of culture conditions. This is true even though SCC-25 can be induced to reversibly growth arrest under certain culture conditions. To continue these studies, the authors next employed the culture of SCC in a suspension microenvironment to evaluate its effect on SCC proliferation and differentiation characteristics. These studies used SCC-25 together with three additional squamous carcinoma cell lines: SCC-9, SCC-13, and SCC-15. For these experiments, cells were cultured in tissue culture medium on top of a nonadhesion agar surface to eliminate the technical difficulty of recovering cells from methylcellulose as reported in previous studies.[11]

SCC-9, SCC-13, SCC-15, and SCC-25 in Suspension Culture Microenvironments

SCC-9, SCC-13, SCC-15, and SCC-25 were studied after culture in a highly stringent growth inhibiting microenvironment, ie, culture on a nonadherent surface of agar with or without other growth inhibiting agents such as ethionine, 2 mM Ca^{++}, or growth factor deficiency. The results of these studies, summarized in Table 6, show that all four SCC lines growth-arrested after culture in suspension and all lines except SCC-13 showed excellent viability. A pronounced difference was specifically observed in the proliferative potential of these SCC lines after growth arrest

Table 5—Proliferation and Differentiation Control Defects Expressed by Human Squamous Carcinoma Cells (SCC-25) Relative to the Characteristics of Normal Human Keratinocytes

Growth-arrest inducing agent/ condition	Growth-arrest characteristics*		Differentiation characteristics*	
	Normal keratinocytes	SCC-25	Normal keratinocytes	SCC-25
Ethionine	Reversible	Reversible	+	[−]
Isoleucine deficiency	Reversible	Reversible	+	[−]
Growth factor deficiency-high calcium	Irreversible	[Reversible]	+	[−]

* The defects evident in SCC-25 are boxed. This summary reviews data presented in this and two previously published papers.[15,17]

Table 6—Growth-Arrest and Differentiation Characteristics of Human Squamous Carcinoma Cell Lines SCC-9, SCC-13, SCC-15, and SCC-25 When Cultured in a Suspension Microenvironment

Cell line	Culture condition	Duration of culture (days)	Growth-arrest (% control [^{3}H]thymidine/cell)	Viability (%)	Colony forming efficiency (% control)	Differentiation (% involucrin positive cells)
SCC-9*	A†	3	<10	83	83	0
SCC-25	A	3	<10	86	82	0
SCC-13	B‡	4	<10	26	30	4
SCC-15	B	3	<10	70	20	20

* SCC-9 appears to contain two or more morphologically distinct cell populations.
† A: On agar in growth factor-deficient MCDB 153 containing 8 mM ethionine + 2 mM Ca^{++}.
‡ B: On agar in DMEM:complete MCDB 153 (1:1) containing 2.5 to 10.0% FBS.
The most significant data are enclosed in a box.

on agar. SCC-9 and SCC-25 retained their clonogenic potential and did not differentiate, whereas SCC-13 and SCC-15 showed a marked decrease in their clonogenic potential and showed some evidence of differentiation. Similar results were reproducible in repeated experiments.

These observations significantly expand on previously published data[11] and support the conclusion that human squamous carcinoma cells can show a variety of distinct biologic defects in the control of proliferation and differentiation with respect to the three steps defined in normal human keratinocytes. More specifically, SCC lines can show changes in the culture conditions that induce reversible growth-arrest, SCC lines can fail to undergo irreversible growth-arrest, and SCC lines can show defects in their ability to terminally differentiate.

Discussion

Rheinwald and Beckett[11] have reported that human squamous carcinoma cell lines show a reduced rate of terminal differentiation and suggested that such defects may represent a necessary step in the neoplastic transformation of keratinocytes. In contrast, Fusenig and coworkers[9] reported that murine squamous carcinoma cell lines differentiate as efficiently as normal keratinocytes even though malignant keratinocytes do show significant changes in the environmental conditions required to induce terminal differentiation.

These two reports typify the conflicting concepts that exist concerning what specific defects squamous carcinoma cells express in their ability to regulate their proliferation and differentiation. Because it has been established recently that three sequential steps are involved in the process of terminal differentiation of normal human keratinocytes, the current studies were performed to determine if SCC lines show defects in one or more of these biologic regulatory processes. These include the ability to: 1) reversibly growth-arrest, 2) irreversibly growth-arrest, and 3) terminally differentiate.[12,16,17]

This and previous studies[15,17] have demonstrated that the human squamous carcinoma cell line SCC-25 expresses numerous biologic defects. In contrast to normal human keratinocytes, SCC-25 show changes in the culture conditions required to induce reversible growth-arrest and SCC-25 also nearly completely lack the ability to irreversibly growth-arrest or terminally differentiate. The human squamous carcinoma cell line SCC-9 resembles SCC-25. In contrast, SCC-13 and SCC-15 can both be induced to reversibly and irreversibly arrest their proliferation but only SCC-15 expresses significant evidence of terminal differentiation.

Table 7 summarizes the results of the current experiments and thereby serves as an interface with the previous studies by other investigators on the characteristics of human squamous carcinoma cells.[9,10,19–22] The authors suggest that these observations support the conclusion that squamous cell carcinogenesis is a complex biologic process involving the expression of multiple defects in the control of cellular proliferation and differentiation and not simply defects in their ability to terminally differentiate. These conclusions are compatible with the findings that multiple onco-

Table 7—Summary of the Proliferation and Differentiation Characteristics of Normal Human Keratinocytes Compared with Four Human Squamous Carcinoma Cell Lines

	Reversible growth-arrest	Irreversible growth-arrest	Terminal differentiation
Normal keratinocytes	++++	++++	++++
SCC-9	++++	–	–
SCC-13	++++	++	±
SCC-15	++++	++	++
SCC-25	++++	–	–

genes must be expressed in normal human keratinocytes before they undergo complete neoplastic transformation.[23] In summary, the authors propose that the available data can best be interpreted by suggesting that squamous cell carcinogenesis can result from a variety of different combinations of biologic and molecular defects.

References

1. Pardee AB: Principles of cancer biology: Cell biology and biochemistry of cancer, Cancer Principles and Practice of Oncology. Edited by VJ DeVitta Jr, S Hellman, SA Rosenberg. Philadelphia, JB Lippincott, 1982, pp 59–72
2. Holley RW, Kiernan JA: "Contact-inhibition" of cell division in 3T3 cells. Proc Natl Acad Sci USA 1968, 60: 300–304
3. Ruddon RW: Cancer Biology. New York, Oxford University Press, 1981, pp 175–190
4. Todaro GJ, DeLarco JE, Marquardt H, Bryant ML, Sherwin SA, Sliski AH: Polypeptide growth factors produced by tumor cells: A possible growth advantage for the producer cells, Cold Harbor Conference on Cell Proliferation. VI. Hormones and Cell Culture. Edited by S Sato, R Ross. Cold Spring Harbor, Cold Spring Harbor Press, 1979, pp 113–127
5. Sporn MB, Roberts AB: Autocrine growth factors and cancer. Nature 1985, 313:745–747
6. Wille JJ Jr, Scott RE: Suppression of carcinogenesis by the cell-cycle-dependent control of cellular differentiation and proliferation. Int J Cancer 1986, 37:875–881
7. Sachs L: Constitutive uncoupling of pathways of gene expression that control growth and differentiation in myeloid leukemia: A model for the origin and progression of malignancy. Proc Natl Acad Sci USA 1980, 77: 6152–6156
8. Pierce GB: Differentiation of normal and malignant cells. Fed Proc 1970, 29:1248–1254
9. Breitkreutz D, Hornung J, Pöhlmann J, Brown-Bierman L, Bohnert A, Bowden PE, Fusenig NE: Environmental induction of differentiation-specific keratins in malignant mouse keratinocyte lines. Eur J Cell Biol 1986, 42:255–267
10. Pierce GB, Shikes R, Fink LM: Cancer: A Problem of Developmental Biology. Englewood Cliffs, Prentice-Hall, 1978, pp 35–39
11. Rheinwald JG, Beckett MA: Defective terminal differentiation in culture as a consistent and selectable character of malignant human keratinocytes. Cell 1980, 22: 629–632
12. Wilke MS, Hsu BM, Wille JJ Jr, Pittelkow MR, Scott RE: Biologic mechanisms for the regulation of normal human keratinocyte proliferation and differentiation. Am J Pathol 1988, 131:171–181
13. Chen TR: In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp Cell Res 1977, 104:255–262
14. Wille JJ Jr, Pittelkow MR, Shipley GD, Scott RE: Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum-free medium: Clonal analyses, growth kinetics, and cell cycle studies. J Cell Physiol 1984, 121:31–44
15. Wille JJ Jr, Pittelkow MR, Scott RE: Normal and transformed human prokeratinocytes express divergent effects of a tumor promoter on cell cycle-mediated control of proliferation and differentiation. Carcinogenesis 1985, 6:1181–1187
16. Pittelkow MR, Wille JJ Jr, Scott RE: Two functionally distinct classes of growth arrest states in human prokeratinocytes that regulate clonogenic potential. J Invest Dermatol 1986, 86:410–417
17. Shipley GD, Pittelkow MR, Wille JJ Jr, Scott RE, Moses HL: Reversible inhibition of normal human prokeratinocyte proliferation by type β transforming growth factor-growth inhibitor in serum-free medium. Cancer Res 1986, 46:2068–2071
18. Pittelkow MR, Scott RE: New techniques for the *in vitro* culture of human skin keratinocytes and perspectives on their use for grafting of patients with extensive burns. Mayo Clin Proc 1986, 62:771–777
19. Steinberg ML, Defendi V: Altered pattern of growth and differentiation in human keratinocytes infected by simian virus 40. Proc Natl Acad Sci USA 1979, 76:801–805
20. Green H: Terminal differentiation of cultured human epidermal cells. Cell 1977, 11:405–416
21. Lechner JF, McClendon IA, LaVeck MA, Shamsuddin AM, Harris CC: Differential control by platelet factors of squamous differentiation in normal and malignant human bronchial epithelial cells. Cancer Res 1983, 43: 5915–5921
22. Yuspa SH, Morgan DL: Mouse skin cells resistant to terminal differentiation associated with initiation of carcinogenesis. Nature 1981, 293:72–74
23. Rhim JS, Jay B, Arnstein P, Price FM, Sanford KK, Aaronson SA: Neoplastic transformation of human epidermal keratinocytes by AD12-SV40 and Kirsten sarcoma viruses. Science 1985, 227:1250–1252

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