# COMMENTARY

## Keratinocyte cultures: an experimental model for studying how proliferation and terminal differentiation are co-ordinated in the epidermis

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### Introduction: the organization of keratinocytes in epidermis

The epidermis is the outer covering of the skin. It consists of multiple layers of epithelial cells, keratinocytes, that are renewed throughout adult life. Proliferating keratinocytes are generally confined to the basal (deepest) layer of the epidermis. Cells that leave the basal layer have lost the ability to divide and they undergo terminal differentiation as they move upwards towards the tissue surface. During terminal differentiation the cells change in size, shape and ultrastructural appearance, and synthesize a range of new proteins. Keratinocytes at the final stage of terminal differentiation, in the outermost epidermal layers, have no nucleus or cytoplasmic organelles; they are full of keratin filaments and have an insoluble protein envelope (the cornified envelope) closely apposed to the inner surface of the plasma membrane. The accumulated layers of terminally differentiated cells at the surface of the epidermis protect the underlying living layers from desiccation and mechanical damage.

If we ignore certain important complications (hair, sweat and sebaceous glands, melanocytes, Langerhans and Merkel cells) the epidermis is a simple structure and an ideal tissue in which to investigate a number of important questions of relevance to all renewing tissues. These are: (1) What signals control proliferation? (2) What signals trigger the intiation of terminal differentiation? (3) What mechanism ensures that there is a balance between the rate of production of new cells in the basal layer and the rate of loss of terminally differentiated cells from the outermost layers?

The development of methods for re-creating human epidermis in culture (see Watt, 1987, for review) makes these questions readily amenable to investigation. In this article I shall briefly review some of the answers that are emerging from studies of keratinocytes cultures.

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### Regulation of proliferation

Cell kinetic analysis of murine epidermis suggests that, by analogy with the haemopoietic system, there may be two different types of dividing keratinocytes: stem cells and transit amplifying cells (Potten, 1981). Stem cells would have the capacity for unlimited proliferation, whereas transit amplifying cells would be committed to undergo terminal differentiation after a finite number of rounds of division. The daughters of each stem cell division could either be stem cells themselves, or else transit amplifying cells. If the epidermis is in a steady state, where the rates of proliferation and terminal differentiation are balanced, then, on average, one daughter of each type would be produced for each stem cell division.

The main problem encountered when exploring this model experimentally is to find criteria for identifying the different types of dividing cell, other than the fate of their progeny. There is some evidence that stem cells can be distinguished from transit amplifying cells by a longer cell cycle time and shorter S phase (Potten *et al.* 1982). It is also possible that stem and transit amplifying cells occupy different positions ('niches') within the epidermis (Schofield, 1978; Lavker & Sun, 1983). So far, no molecular markers that distinguish the two types of dividing cell have been identified.

In cultures of keratinocytes there is good evidence that the dividing cells are heterogeneous, and different subpopulations may correspond to stem and transit amplifying cells. Thus, discrete subpopulations with different cell cycle times have been identified (Dover & Potten, 1983; Albers *et al.* 1986) and the proliferative potential of individual keratinocytes has been found to vary, with the smallest cells having the greatest clone-forming ability (Barrandon & Green, 1985). In addition, three different types of clone derived from the progeny of individual cells have been identified: holoclones, with high growth potential, in which the

majority of cells do not undergo terminal differentiation; paraclones of low proliferative potential, in which all of the progeny terminally differentiate; and meroclones, which are intermediate between holoclones and paraclones (Barrandon & Green, 1987*a*). Cells in holoclones may be stem cells, while it is possible that those in paraclones are transit amplifying cells. The identification of different subpopulations of dividing cells *in vitro* offers the potential for detailed analysis of their fate and inter-relationships in ways that are not possible in intact epidermis.

Proliferation in the epidermis depends not only on the nature of the dividing cells, but also on external signals to which they respond. Agents that stimulate growth of keratinocytes have been identified during attempts to optimize the composition of culture medium, both serum-containing (Allen-Hoffman & Rheinwald, 1984) and serum-free (Boyce & Ham, 1983). Undefined growth stimulators are found in bovine pituitary extract (Boyce & Ham, 1983), in foetal calf serum and in medium conditioned by feeder layers of 3T3 cells (Rheinwald & Green, 1975). Growth is also stimulated by transferrin, insulin and triiodothyronine (Watt & Green, 1981) and by hydrocortisone (Rheinwald & Green, 1975) and cholera toxin (Green, 1978). Cholera toxin and hydrocortisone have marked effects on colony morphology, with cholera toxin increasing the proportion of small cells in the colonies (Green, 1978). Epidermal growth factor (EGF) and the structurally related polypeptide, transforming growth factor $\alpha$ (TGF$\alpha$), increase the life-span of keratinocytes (i.e. number of generations before senescence) without affecting growth rate: they act by stimulating lateral migration of the peripheral zone of dividing cells in expanding colonies (Barrandon & Green, 1987*b*). In addition to growth stimulators, a number of growth inhibitors have been identified, including transforming growth factor $\beta$ (TGF$\beta$), which causes keratinocytes to accumulate in $G_1$ (Shipley *et al.* 1986).

Keratinocytes not only respond to growth regulating molecules; they also express them. Cultures of normal human keratinocytes synthesize TGF$\alpha$ and addition of EGF or TGF$\alpha$ to the medium induces TGF$\alpha$ gene expression (Coffey *et al.* 1987). Normal keratinocytes in culture also secrete TGF$\beta$-like molecules (Shipley *et al.* 1986). Expression of these molecules is not confined to cells in culture, because TGF$\alpha$ has been detected in all the living layers of normal human epidermis (Coffey *et al.* 1987); TGF$\beta$ is expressed at low levels in normal mouse epidermis and at high levels after treatment with the tumour promoter 12-*O*-tetradeconyl-phorbol-13-acetate (TPA) (Akhurst *et al.* 1988).

The conclusion to be drawn from these studies is that regulation of keratinocyte proliferation is complex and involves a number of different mechanisms. The dividing cell population is heterogeneous and a range of exogenous factors influence growth rate. Different subpopulations may differ in responsiveness to growth stimulators and inhibitors, and growth stimulators potentially may act either by reducing the cell cycle time or by increasing the proportion of cycling cells. Finally, since keratinocytes can produce their own growth regulatory molecules there may be an element of autocrine regulation of proliferation (Sporn & Todaro, 1980).

## Induction of terminal differentiation

The earliest event in terminal differentiation is irreversible withdrawal from the cell cycle: this occurs within a specific subset of basal cells, which may correspond to the transit amplifying population (Albers *et al.* 1987). The time interval between the final S phase and the onset of expression of involucrin, the major precursor of the cornified envelope, is not fixed: with increasing time after S there is an increasing probability that a cell will start to synthesize the protein and it seems possible that cells at any stage in the cell cycle can initiate terminal differentiation (Dover & Watt, 1987). In low-calcium monolayers of keratinocytes the differentiation rate has been calculated: approximately four cells per 5000 cells begin to express involucrin every hour (Dover & Watt, 1987).

Although terminal differentiation is normally coupled to cell cycle withdrawal, inhibition of proliferation may not be a sufficient signal for terminal differentiation, since inhibition is not always irreversible: cells arrested by serum starvation or treatment with TGF$\beta$ are able to re-enter the cycle after addition of serum or removal of TGF$\beta$ (Wille *et al.* 1984; Shipley *et al.* 1986). Furthermore, the boundary between the dividing and differentiating cell compartments is not rigid, because a small number of S phase keratinocytes express terminal differentiation markers, both *in vivo* (Régnier *et al.* 1986) and *in vitro* (Dover & Watt, 1987).

What, then, are the signals that induce a cell to undergo terminal differentiation? Two potential signals that have been identified are cell size and substratum contact area. The onset of involucrin synthesis is correlated with cell enlargement (Watt & Green, 1981) and restricted substratum contact induces involucrin expression (Watt, 1987). These two observations can be linked by the following model (Watt, 1988). The longer that a keratinocyte has been postmitotic, the more likely it is to increase in size. If there is no more space available on the substratum to accommodate the enlarged cell, then the proportion of its surface area in contact with the substratum will be reduced, triggering terminal differentiation. The adhesive affinity of keratinocytes for the substratum is

reduced during terminal differentiation (Watt, 1987) and there may therefore be a positive feedback system in which cells are at the same time induced to differentiate and to move upwards from the basal layer.

A useful approach for identifying terminal differentiation signals is to study conditions that induce premature terminal differentiation or that modulate the differentiated phenotype itself. Growth of keratinocytes in suspension or exposure of the cells to TPA both cause rapid inhibition of DNA synthesis, followed within 24 h by stimulation of involucrin synthesis and 1–4 days later by assembly of cornified envelopes (Rice & Green, 1978; Rheinwald, 1979; Parkinson & Emmerson, 1982; Watt & Dover, unpublished data). A minority of keratinocytes do not terminally differentiate under these conditions and it is possible that such cells are stem cells (Parkinson *et al.* 1983; Hall & Watt, unpublished data).

Agents that influence the nature of the differentiated phenotype include vitamin A and hydrocortisone. An excess of vitamin A *in vivo* causes mucous metaplasia and a deficiency results in squamous metaplasia. In culture the concentration of vitamin A in the medium affects cornified envelope formation (Green & Watt, 1982) and keratin expression (Fuchs & Green, 1981). Whereas high concentrations of vitamin A suppress envelope formation, hydrocortisone promotes envelope assembly and thus may antagonize the effects of vitamin A (Cline & Rice, 1983).

## Epidermal homeostasis

Although I have argued that there are probably distinct signals controlling keratinocyte proliferation and terminal differentiation, the two processes must be tightly co-ordinated, so that epidermal homeostasis, i.e. the balance between the rates of proliferation and terminal differentiation, is maintained. *In vivo* some mechanism ensures that the epidermis remains a constant thickness in spite of continual cell turnover; and also allows homeostasis to be re-established following injury. This mechanism is retained *in vitro*, because when the suprabasal cell layers are stripped from a culture, the remaining basal cells are able to regenerate them (Jensen & Bolund, 1988; Read & Watt, 1988).

One mechanism that has been proposed for epidermal homeostasis is that terminally differentiating cells produce a substance that normally suppresses proliferation of cells in the basal layer; if cells in the upper layers are lost through injury, the inhibition is relieved, resulting in increased proliferation until the upper layers are regenerated and homeostasis re-established. The proposed inhibitors have been named chalones (Bullough, 1962) and there have been many attempts to purify them. Recently, a pentapeptide that inhibits proliferation of mouse and human keratinocytes has been purified from mouse epidermis (Elgjo *et al.* 1986; Watt & Elgjo, unpublished data). TGF$\beta$ is also a stong candidate for a role in homeostasis, because it inhibits proliferation (Shipley *et al.* 1986) and is strongly induced in the upper layers of mouse epidermis that have been treated with TPA, a drug that causes transient hyperplasia (Akhurst *et al.* 1988).

Cellular communication *via* TGF$\beta$ presumably involves secretion and binding to cell surface receptors. Another mechanism of cell–cell communication is *via* gap junctions and there has also been speculation that they may be involved in homeostasis. During terminal differentiation keratinocytes lose the ability to transfer Lucifer Yellow dye *via* gap junctions, both *in vivo* (Kam *et al.* 1986) and *in vitro* (Kam *et al.* 1987). If the equilibrium concentration of some low molecular weight molecule transmitted through gap junctions were to determine what proportion of cells in the basal layer divide, then loss of cells from that communication compartment as a result of terminal differentiation would regulate proliferation of the remaining basal cells (Kam *et al.* 1987). This idea remains to be tested experimentally by using antibodies to junctional proteins to block communication.

## Conclusions

The control of proliferation and terminal differentiation in the epidermis must be co-ordinated in order for homeostasis to be maintained. Although withdrawal from the cell cycle is normally the first stage in terminal differentiation, the signals controlling proliferation and differentiation may be distinct. The proliferative status of the epidermis is influenced by heterogeneity within the dividing cell population and by the ability of keratinocytes to produce and respond to a range of growth stimulators and inhibitors. Cultures of human epidermal keratinocytes provide a useful experimental model for analysing how proliferation and terminal differentiation are controlled.

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