I.2

Periodicity in Shape Changes of Human Epidermal Keratinocytes

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Analysing cell motion and cell shape dynamics is of major importance for basic research and for medical application. We examined normal human epidermal keratinocytes (nHEK) and cells transfected with viral oncogenes (trHEK) (Barbosa & Schlegel 1989) to reveal typical patterns in spontaneous cell behaviour using new methods of image processing and statistical autocorrelation analysis. Actin-driven migration is necessary for normal cells to cover wounds or for carcinoma cells to invade foreign tissues. Thus, revealing the basic mechanisms of cell motion may finally lead to medical prevention of metastasis or acceleration of wound healing processes.

Under culture conditions where formation of cell-cell contacts is prevented, keratinocytes grow as isolated cells. A single keratinocyte spread on a surface consists of a cell body (Fig. 1A) containing the nucleus and cell organelles, and being surrounded by flat organelle-free lamellae. At the edges of lamellae the cell constantly protrudes small filopodia and lamellipodia which subsequently retract, lift up from the substrate and form centripetally moving ruffles (see Winklbauer *et al.* I.1 this volume).

1 One-dimensional analysis of single lamellae dynamics

The protrusive activity of single lamellae was examined using one-dimensional analysis of phase contrast image sequences of HEK along section lines transversal to the cell edge extending into the nucleus (Fig. 1A). The luminance profile of structures along the section line was stored at regular intervals (Fig. 1B) and plotted in three-dimensional diagrams where dark structures are represented as valleys (Fig. 1C). Drawing level lines of luminance values only below a certain threshold results in two-dimensional topographic plots (Fig.1D). In these plots ruffles are characterized by low luminance values and constant movement starting at the cell edge (continous line in Fig. 1D), which is detected by a new algorithm (Alt *et al.* 1995). This allows the combined analysis of cell edge and ruffle movement.

After fast protrusion of a new lamellipodium to a length of 0.8 to 1.7 μm, retraction of the cell edge follows resulting in the separation of a ruffle which is

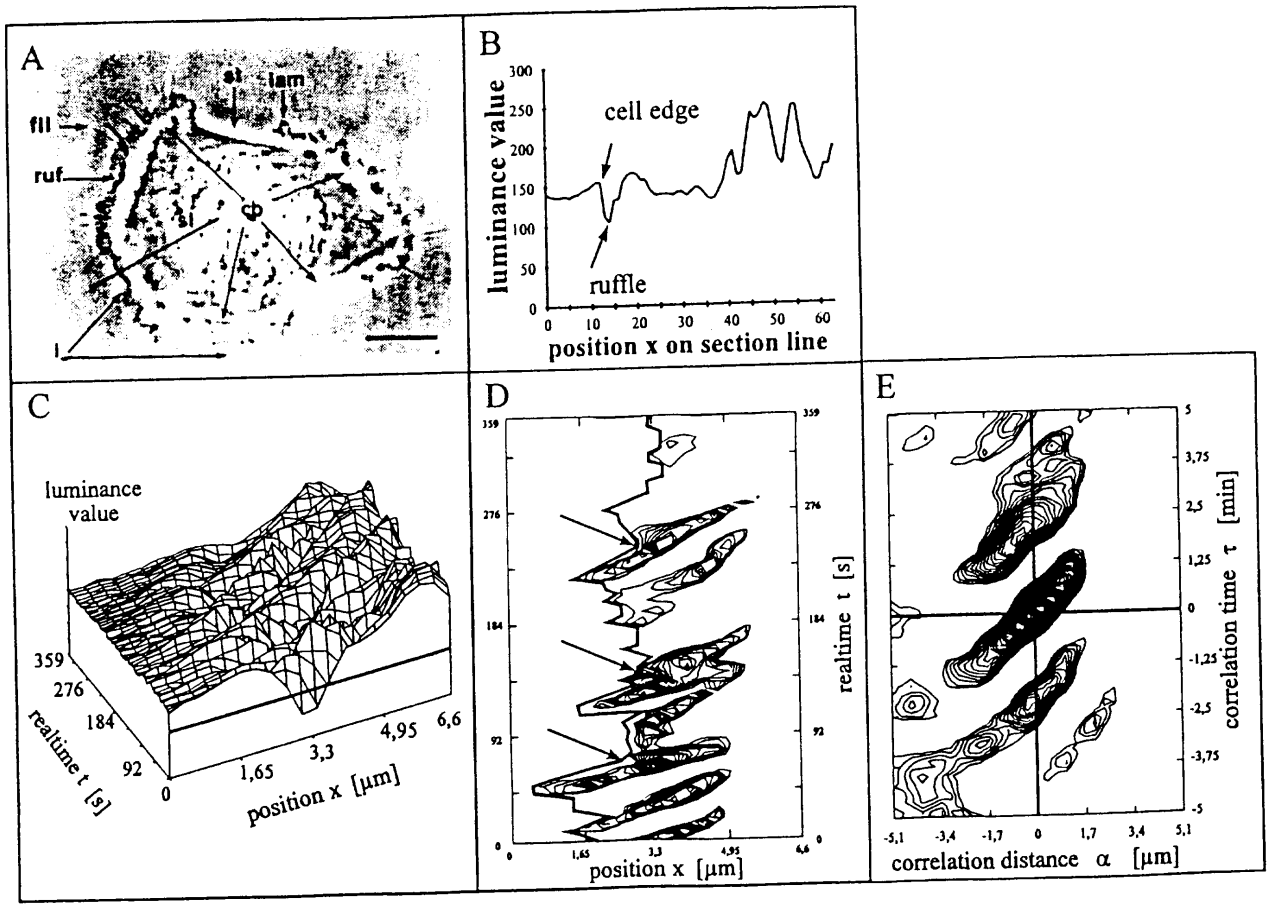

Figure 1: From digitized keratinocyte images to autocorrelation analysis of ruffle movement. (A): Phase contrast image of a typical human keratinocyte; cb: cell body, fil: filopodia, l: lamella, lam: lamellipodia, ruf: ruffles, st: stretched cell border. (B): Luminance profile of structures along a section line (sl) recorded in regular intervals and plotted over time (C). (D): Level lines of luminance values plotted below a threshold (dark line in C). (E): Autocorrelation analysis of long term observations of ruffle dynamics.

constantly moving over the cell surface towards the cell body. At the separation point of ruffle and cell edge the retraction movement stagnates for a short time (arrows in Fig. 1D) and a new lamellipodium forms at the same location on the cell periphery. Ruffle velocity decreases with increasing distance from the cell edge and the structure finally disappears at the cell body. Even in short sequences a strong periodicity in ruffle appearence and movement is obvious. To obtain quantitative results, long term sequences of up to 4 h were analysed by autocorrelation analysis (Alt *et al.* 1995). Autocorrelation was related to a reference line, and the level lines above the expectation value (Fig. 1E) were plotted. Maximum correlation between two points is represented by the center of topographical line arrangements and stands for high probability of a second ruffle appearing at distance α and correlation time τ over the whole observation period. Free areas represent none or negative correlation to the reference point. The topographical line arrangement around the reference point (line $\alpha = 0$) represents the characteristic time and space behaviour of all observed ruffles in one sequence and is therefore typical for

this cell region. Ruffles of all observed cells (15 nHEK and 15 trHEK) start with a slight acceleration, are moving most of their course with constant velocity, and finally slow down near the cell body. A new ruffle usually occurs at the same time when the former is getting slower.

Measuring ruffle velocity at different temperatures (Alt *et al.* 1995) for normal and transfected keratinocytes revealed differences between the two cell types. trHEK protrude and retract lamellipodia, transforming into ruffles, with significantly higher velocities than nHEK. Consistent with this observation, ruffles of trHEK also appear in higher frequencies (0.43 min^{-1} on average) compared to nHEK (0.24 min^{-1} on average).

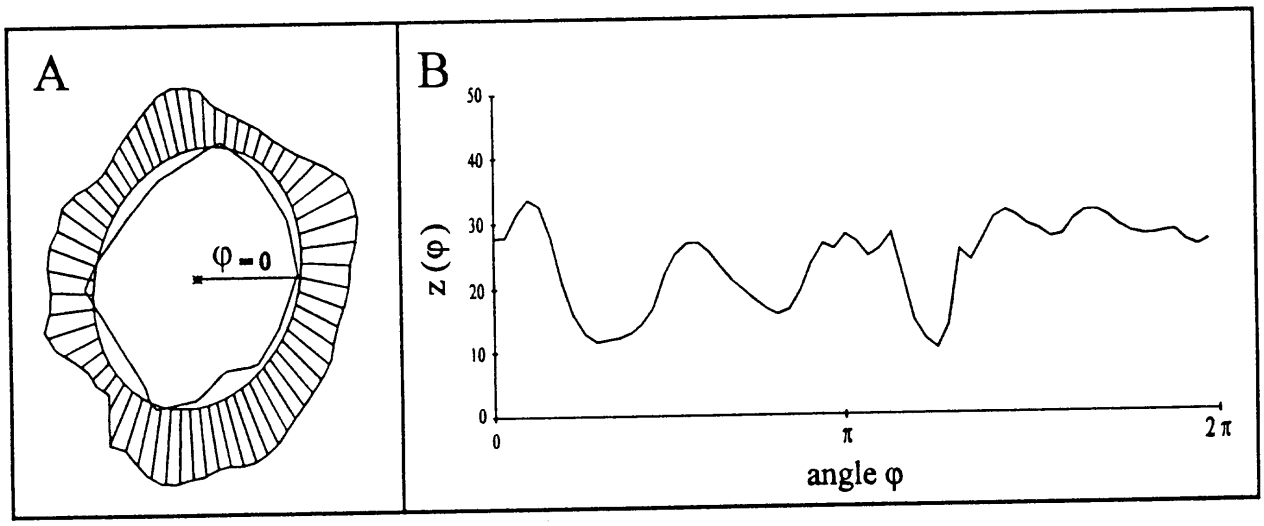

Figure 2: Determination and presentation of normal cell outline extensions. (A): Final result of automatic cell outline detection with a weighed ellipse adapted to the digitized cell body (inner line). (B): Transformation of cell outline data to normal coordinates ($z(\varphi)$) at 180 discrete angular positions.

2 Two-dimensional analysis of patterns in lamellar protrusion and retraction

The main question in analysing the cell periphery was to reveal if there are certain periodic patterns in lamellar activity around the whole cell and if spontaneous motility of keratinocytes is non-random. Differences between the spontaneous behaviour of normal and transfected cells were shown. Phase contrast video sequences of up to ten hours duration were digitized in 24 sec intervals. The cell outlines of one sequence were then detected automatically by a special image processing program (Werner Heiße, Dept. of Theoretical Biology, University of Bonn) and further processed to diminish the amount of data (Alt *et al.* 1995).

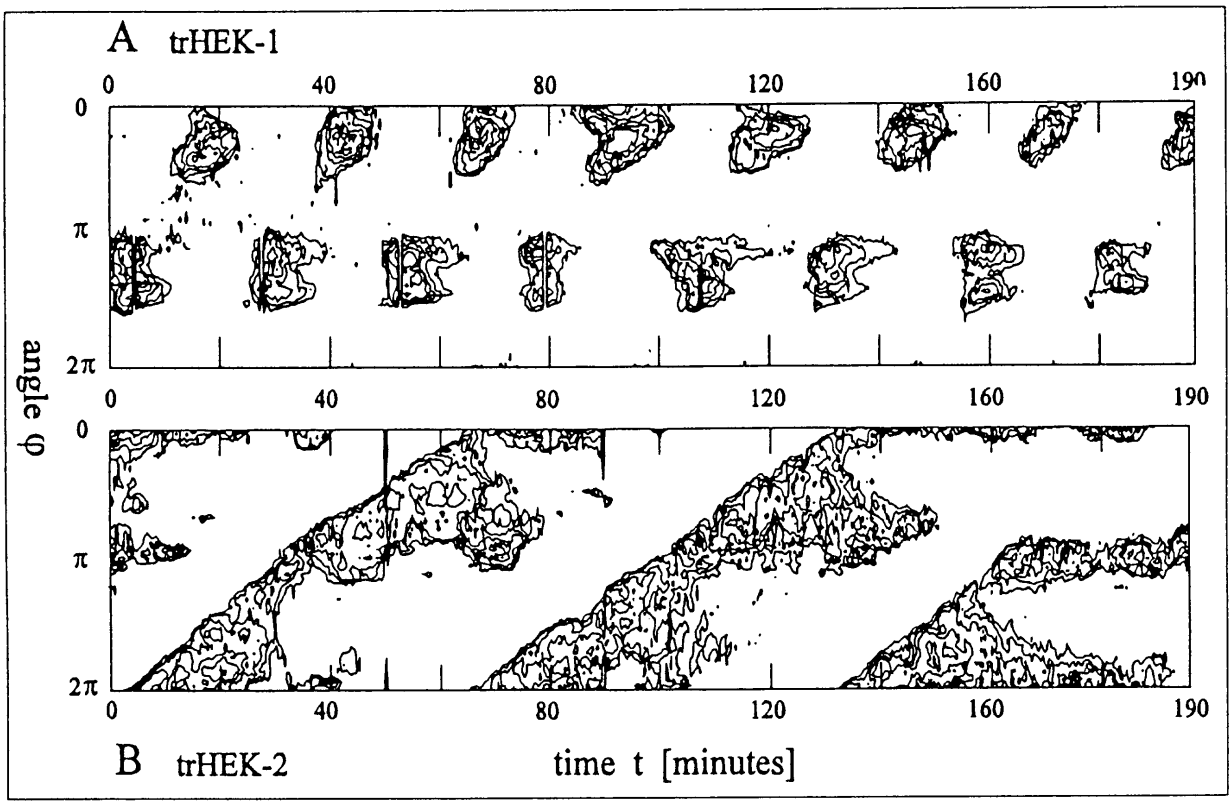

Figure 3: Topographic plot of parametrized cell outlines of keratinocytes over angle φ and time t. (A): Pulsating lamellipodial extension and retraction pattern of a trHEK. (B): trHEK with one lamella rotating counterclockwise around the cell.

To allow quantitative analysis, cell outlines were parametrized in respect to the weighed momental ellipse (Fig. 2) and presented in topographic plots (Fig. 3) analogous to Fig. 1. Angular Fourier and autocorrelation analysis was applied to detect periodic patterns in cell motility (Fig. 4). These methods are described by Brosteanu *et al.* (I.4 this volume) in detail.

All 30 observed cells formed lamellae over a long period in at least two regions of the cell periphery which were held mainly constant (Fig. 3A: lamellae at $\frac{\pi}{2}$ and $3\frac{\pi}{2}$). Lamella formation in the case of two predominant regions on the cell outline always occured in an antipodal manner. Retraction of one lamella correlated with extension of a new lamella on the other side. In the case of three or four regions of lamella formation, the largest possible distance between these regions was kept ($\frac{2}{3}\pi$ or $\frac{1}{2}\pi$) and lamellae appeared in clockwise or counterclockwise sequences around the cell. Transfected keratinocytes differed from normal keratinocytes (not shown) mainly in having more predominant regions of lamella formation and showing a higher frequency of lamella formation and retraction. Beside this typical behaviour of HEK, two special cases among the observed trHEK demonstrated an impressive regular motility pattern. In the first case (Fig. 3A, Figs. 4A-C) the cell pulsates with very high frequency between two stages of lamellar extension (15 min/extension). The path described by the first two Fourier coefficients of the cell

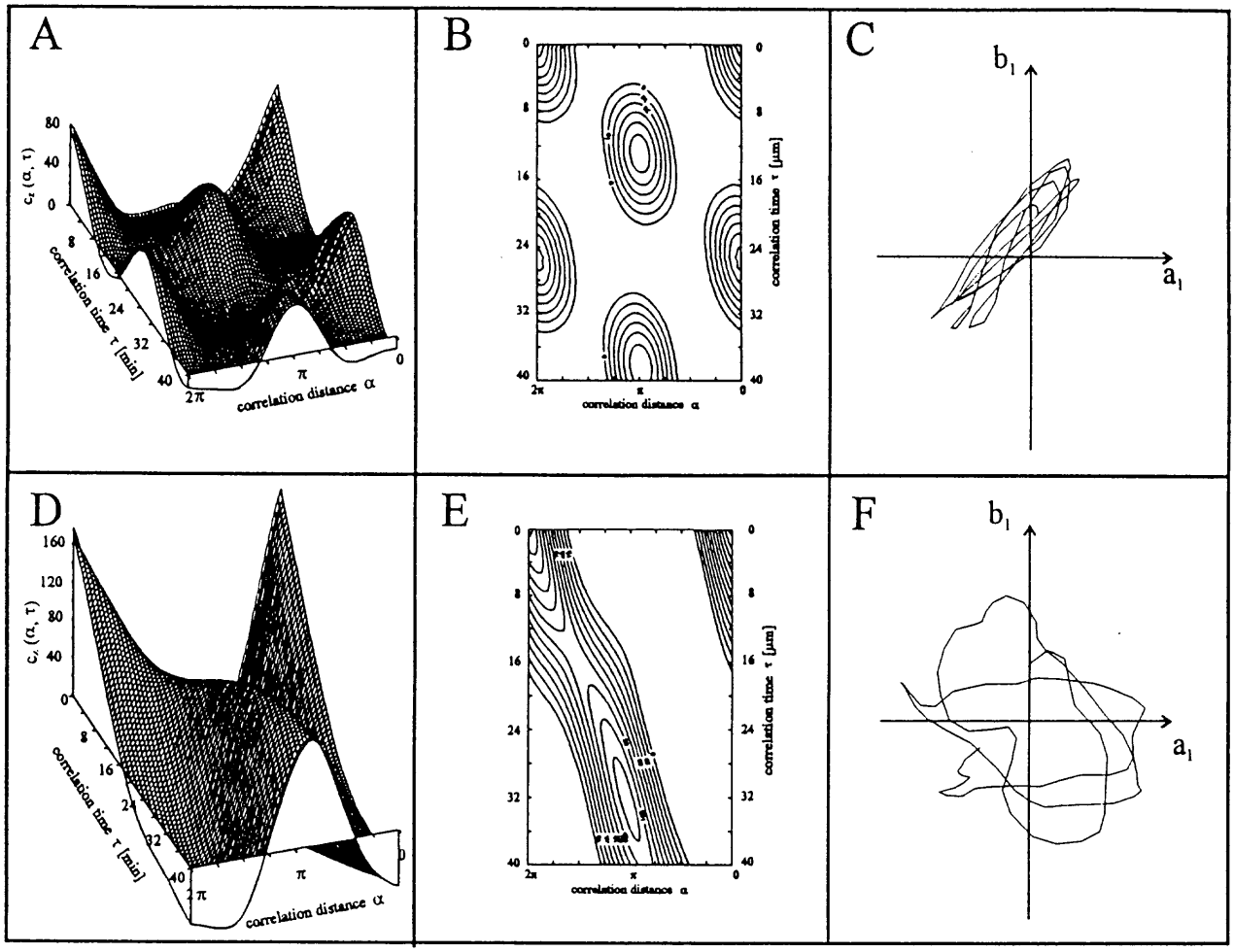

Figure 4: Autocorrelation and angular Fourier analysis of lamellar dynamics. (A), (D): Autocorrelation function $c_z(\alpha, \tau)$ for the corresponding data in Figs. 3A,B plotted in three-dimensional diagrams over correlation time τ and correlation distance α. (B), (E): Topographic plots of correlation values above zero. (C), (F): Corresponding paths described by the first two Fourier coefficients a_1 and b_1 of the cell shape during the observation period.

shape (Fig. 4C) reveals this very regular transition between the two stages, while the autocorrelation function (Fig. 4A) quantifies the mean width ($\frac{3}{8}$ cell circumference), duration (10 min) and periodicity (25 min) of the alternating antipodal lamellae. Also, a change in location of about 1/16 cell circumference per 10 min becomes visible (Fig. 4B). In the second case (Figs. 3B, 4D-F), there is no obvious region of preferred lamella formation, but one single lamella rotating counterclockwise around the cell periphery with a velocity of about one turn per hour. The rotation is reflected in the path of the first two Fourier coefficients (Fig. 4F) which describes counterclockwise cycles. The autocorrelation function (Fig. 4D) quantifies not only the mean drift, but also a cyclic increase and decrease of the width and outward extension of the lamella with a period of 32 min. The last is due to the "rear end" of the lamella, which broadens in two predominant locations (π and 2π) and persists even when the rotation stopped after 160 min (Fig. 3B).

3 Conclusion

Centripetal movement of actin containing ruffles with a velocity of 3.66 μm/min on average is similar to observations of a general backward actin flow with rates of about 6-12 μm/min in the periphery of different cell types (Symons & Mitchison 1991, Forscher *et al.* 1992, Cao *et al.* 1993). Even amoeboid cells such as *Chaos carolinensis* exhibit similar actin flow patterns (Grebecki 1990) but with much higher speed and frequency which is consistent with a generally higher migration rate of these large cells. Constant velocity of ruffles and their periodic behaviour suggest a steady intracellular retraction force, pulling the lamellar F-actin towards the cell body. Only if this force is counterbalanced by outward pressure and protrusion of new lamellipodia, can lamellae be maintained. This leads to a steady cycle of actin-assembly at the lamellar tip, actin-flow towards the cell body and disassembly of the actin network at the base of the lamella. Transduction of this steady retraction force to a substrate could lead to cell migration if the actin system is connected to the extracellular matrix by transmembrane linker proteins like integrins. In fact, migrating keratinocytes give the impression of the cell body pulling itself on a rope (the actin system) towards a pole (cell matrix adhesion sites); see also Dembo *et al.* II.2 this volume.

Our results in analysing cell shape changes clearly demonstrate that the spontaneous behaviour of HEK is in no case chaotic or random but follows regular patterns. Even the observed cancerogenic cells maintain a periodic behaviour which differs mainly by higher frequency. We propose that this periodicity is only due to cytomechanical properties of the cell including (a) contractility of the actin network presumably caused by association with myosin II, (b) intracellular hydrostatic pressure, (c) actin concentration differences with high concentration at the cell body and a more loose network in lamellae, (d) local weakening of the actin cortex by fragmentation and depolymerization, and (e) stabilization of the network by crosslinking proteins or polymerization processes. This point of view is further supported by mathematical models which reproduce the observed motility patterns using the parameters mentioned above, working with the simplifying assumption of a homogeneous distribution of actin-binding proteins (gelsolin, filamin, etc.) in sufficient concentration but excluding regulatory substances as Ca^{++}, profilin or thymosins (Alt *et al.* 1995). Every feature of the actin system in these models solely depends on the F-actin concentration distribution, and a hydrostatic pressure drop between cell body and tip is regulated by concentration and velocity of the network. In a short résumé we suggest lateral coordination of lamellar extensions as being based on the interaction of two physical properties of the dynamical cortical network: (1) the local regulation of hydrostatic pressure at the plasma membrane induced by contractile flow of the actin network and (2) local stabilization and weakening of the F-actin system at the lamellar tip by assembly/disassembly and visco-contractile tensions (compare Alt & Tranquillo I.9 this volume).