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Involvement of Cytoskeleton in Orientation of Cell Division in Contact Guided Cells

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Single human skin fibroblasts and the skin keratinocyte cell line HaCaT show contact guidance and elongate along narrow (1-2 μm) scratches in glass substratum. During cell division these cells orientate their mitotic spindles along the long axis of the cell. Immunofluorescence staining of actin, tubulin, chromatin, and the nuclear NuMA protein complex demonstrated that cell elongation along scratches is accompanied by a corresponding rearrangement in the cytoskeleton. The results and literature suggest the following steps in the interplay between outside-in and inside-out signalling in the regulation of cell division orientation by extracellular factors. The interaction of cell surface with an anisotropy in the local environment causes changes in F-actin organization, cell elongation and alignment of stress fibres along the cell axis. This is accompanied by a corresponding reorientation of microtubules. Microtubules mediate between cell shape changes dependent upon cell interaction with substratum or other cells, the cortical actin and the position of centrosomes. Centrosomes determine the position and orientation of the mitotic spindle. The astral and central microtubules of the mitotic spindle control the localization of contraction-relaxation in the cell cortex and the position of the constriction ring and cell division plane.

Key words: Contact guidance, cell polarization, cell division, cytokinesis.

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It was recently reported that the cell division plane orients perpendicularly to the long axis of polarized cells during galvanotaxis (ZHAO *et al.* 1999), haptotaxis when spreading on oriented extracellular matrix proteins (THÉRY *et al.* 2005) or if contact guided, during spreading and movement along scratches or grooves on substratum surface (KRZYSIEK-MACZKA & KOROHODA 2008). In multicellular organisms the organization of cells in tissues and organs requires ordered cell translocation and oriented cell division (ZHAO *et al.* 1999; SEERY & WATT 2000; KALTSCHMIDT & BRAND 2002; CAYOUETTE & RAFF 2003). Some eukaryotic cells such as Protista, myxamoebae of the cellular slime mould *Dictyostelium discoideum*, cancer cells growing in semifluid media in suspension, zygotes and some embryo cells and cells of the immune system suspended in isotropic fluid medium are able to change shape and divide (EHRENGRUBER *et al.* 1996; RAPPAPORT 1999; NUMATA *et al.* 2000; UYEDA *et al.* 2000). In contrast, the majority of normal animal cells show anchorage-dependence of growth, i.e. they are able to grow, move and divide only when spread on a solid substratum. Attachment of anchorage-dependent cells to the substratum assures the development of tension due to active contraction-relaxation in cell cortical cytoplasm and influences the polarization of cell shape (THÉRY *et al.* 2006).

The regulation of orientation of cell division in multicellular animal organisms is expected to depend not only upon intrinsic and inside-out signalling (as in cells growing and dividing when suspended in isotropic fluid media) but also on extracellular, extrinsic factors causing outside-in signalling. Recent data concerning the localization and activity of numerous proteins postulated to play a role in cell division and its orientation have been reviewed by ROBINSON and SPUDICH (2004), THÉRY and BORNENS (2006), TOYOSHIMA and NISHIDA (2007), and WERNER and GLOZER (2008).

In our previous report we described the quantitative correlation of the division axis and cell shape axis in human skin fibroblasts and the HaCaT epithelial human cell line when cells were contact guided and aligned along scratches on a glass surface. In tumour cell lines this correlation was much weaker or absent (KRZYSIEK-MACZKA & KORO-

HODA 2008). The exact orientation of cell divisions in anchorage-dependent cells showing contact guidance makes them suitable for study of the interplay of outside-in and inside-out signalling pathways in the regulation of the orientation of cell shape and cell division. In this report we follow changes in F-actin, microtubules and NuMA complex displacement and localization in dividing fibroblasts and HaCaT cells when aligned on scratches in the substratum.

Material and Methods

Cells

Normal human skin fibroblasts were grown in DMEM (Sigma Chemical Co.) supplemented with 10% foetal calf serum (Gibco BRL) and antibiotics; penicillin (50 i.u./ml), streptomycin (μg/ml), neomycin (50 μg/ml) (Tarchomin S.A. Poland) in humidified 5% $C0_2$ 95% air at 37° C. Cells were grown in 25 cm^2 flasks (Sarsteadt) and passaged once a week.

The human keratinocyte cell line (HaCaT) was a kind gift from Prof. A. Klein, Kraków. Cells were grown in DMEM-g medium supplemented with 1000 mg/ml glucose, Gluta MAX-I and pyruvate (Gibco BRL, Invitrogen) with the addition of 10% foetal calf serum, penicillin, streptomycin and neomycin under the conditions described above. Cells were grown in 25 cm^2 flasks (Sarsteadt) and passaged once a week.

HaCaT cells and fibroblasts were plated at a density of 3 x 10^4 cells per 7 cm^2 and 1.5 x 10^4 cells per 7 cm^2 , respectively, onto plain and grooved glass coverslips, and placed on Petri glass dishes (Simax Czech Republic) under the conditions described above. After 24 hours HaCaT cell cultures consisted mostly of single cells which were fixed and labelled with antibodies. Fibroblasts were starved for 24 hours to obtain synchronized cell divisions, then fixed and labelled with antibodies.

Substratum patterning

The anisotropic surface was prepared with corundum powder on glass coverslips (Szklarskie Zakłady Przetwórcze 'Przełom', Poland). The scratches were 0.1-2.0 μm wide and 0.1-1 μm deep (STĘPIEŃ *et al.* 1999).

Immunofluorescence

F-actin staining. Fixed fibroblasts and HaCaT cells were extracted with 01% Tritor X -100 (Sigma Chemical Co.), washed with PBS without Ca^{2+} and Mg^{2+} (Biomed, Lublin) and labelled with rhodamin-phalloidin solution (500 ng/ml) (Sigma Chemical Co.) for 45 minutes.

Microtubule staining. Fixed fibroblasts and HaCaT cells were extracted with 0.1% Triton X-100, washed with PBS without Ca^{2+} and $Mg^{2+}$and labelled with mouse monoclonal anti-α-tubulin antibody (1:500) (Sigma Chemical Co.) for 1 hour. As a secondary antibody FITC-conjugated anti-mouse IgG (1:400) (Sigma Chemical Co.) was used.

NuMA staining. Fixed fibroblasts and HaCaT cells were extracted with 0.1% Triton X-100, washed with PBS without Ca^{2+} and Mg^{2+} and labelled with goat anti-NuMA IgG (1:100) (Santa Cruz) for 1 hour. Alexa 488-conjugated anti-goat igG (1:400) (Molecular Probes) was used as a secondary antibody. In all cases, specimens were counterstained with 1 μg/ml bis-benzimide (Hoechst 33342) (Sigma Chemical Co.) to visualize chromatin.

Microphotography. Labelled cells were photographed with an epifluorescence Leica DM IRE2 microscope.

Results

In the first series of experiments the organization of the actin cytoskeleton in cells on isotropic and anisotropic substratum was observed and changes in stress fibre shape and orientation were followed in cells approaching the scratches in substratum, extending along the scratches and later dividing with their constriction rings oriented perpendicularly to the long axes of cells. In fibroblasts, actin bundles forming the so called stress fibres follow lines of tension and extend along the long axis of polarized cells (Fig. 1A). At the leading cell front,

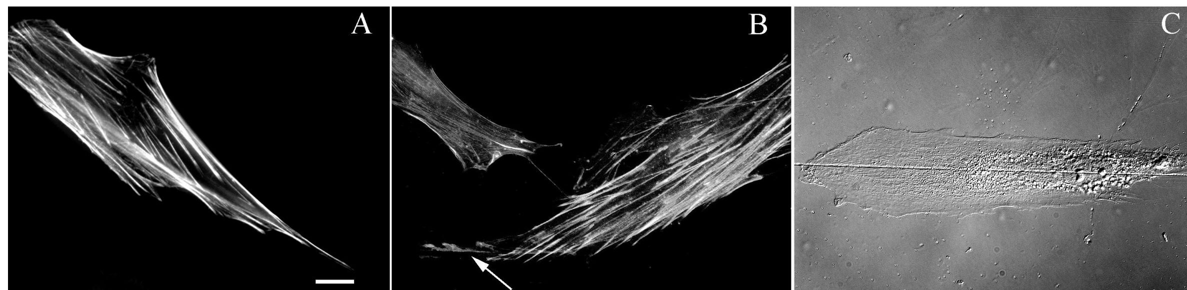

Fig. 1. The organization of the actin cytoskeleton in human fibroblasts on isotropic (A) and anisotropic substratum (B, C). The arrow shows the position of scratches in the substratum. A, B – fluorescence microscopy, C – Nomarski interference contrast (NIC). Scale bar = 10 μm.

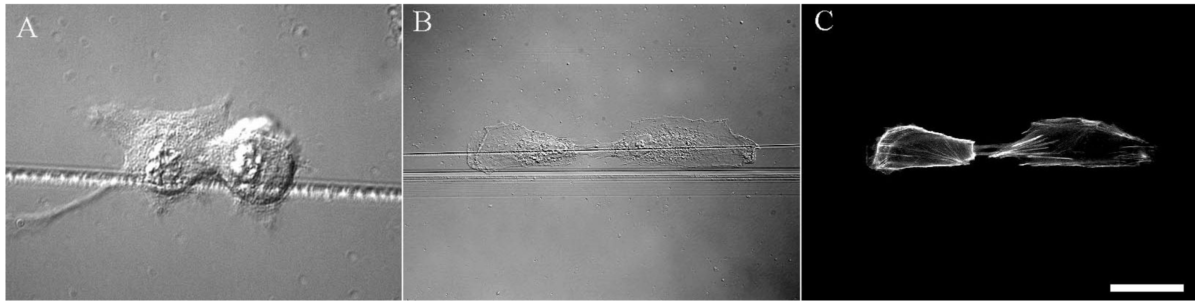

Fig. 2. The organization of actin cytoskeleton in dividing fibroblast cells on anisotropic substratum. (A). The orientation of the constriction ring of a fibroblast dividing on the scratches in substratum. (B, C) The orientation of stress fibers in telophase. A, B – Nomarski interference contrast (NIC), C – fluorescence microscopy. Scale bar = 10 μm.

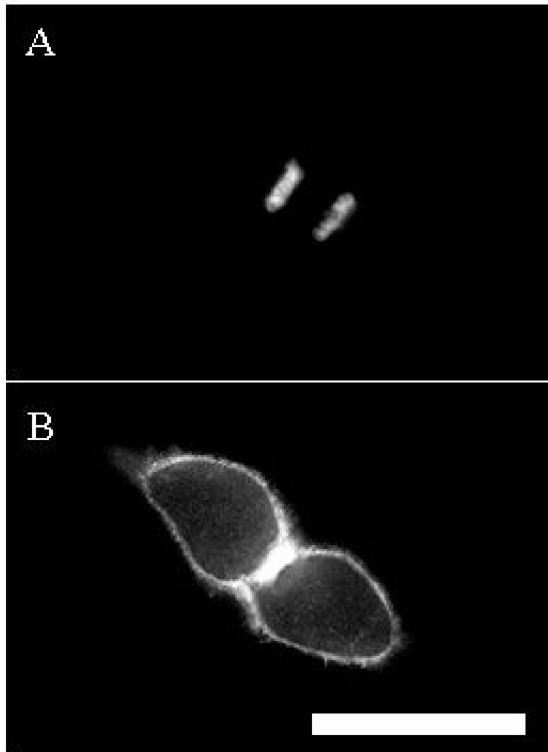

Fig. 3. The localization of actin cytoskeleton in dividing fibroblasts. During cell division, actin localizes mainly in the constriction ring and the cellcell cortex. The specimens were counterstained with 1 μg/ml bis-benzimide (Hoechst 33342) to visualize chromatin (A) and stained for actin localization (B). Scale bar = 10 μm.

stress fibres are anchored to points of adhesion with the substratum. Although filopodia and lamellipodia extend freely into fluid medium, they need to be anchored to solid substratum or other cells for stabilization and for further extension. When a cell approaches the scratch in substratum with its filopodia or leading lamellipodia, cell extension then proceeds along the long axis of the scratch. The changes in shape and bending of the lamellipodium are accompanied by a corresponding bend in stress fibres, as seen in Fig. 1B. In consequence the fibroblast becomes polarized and aligned along the scratch, even if it is very thin with a diameter less than one micrometer, as shown in Fig. 1C. Cell polarization is associated with parallel alignment of actin stress fibres in its cytoskeleton.

The polarized fibroblasts divide with constriction rings oriented perpendicularly to the long cell axes and axes of scratches (Fig. 2A) and after completion of cytokinesis they spread and extend along the scratch in the substratum (Fig. 2B & C). During cell division actin localizes mainly in the constriction ring and in the cell cortex (Fig. 3), whereas in telophase, new stress fibres appear oriented along the extending and spreading cells (Fig. 2B & C). Changes in the organization of the actin cytoskeleton in HaCaT cells moving and dividing

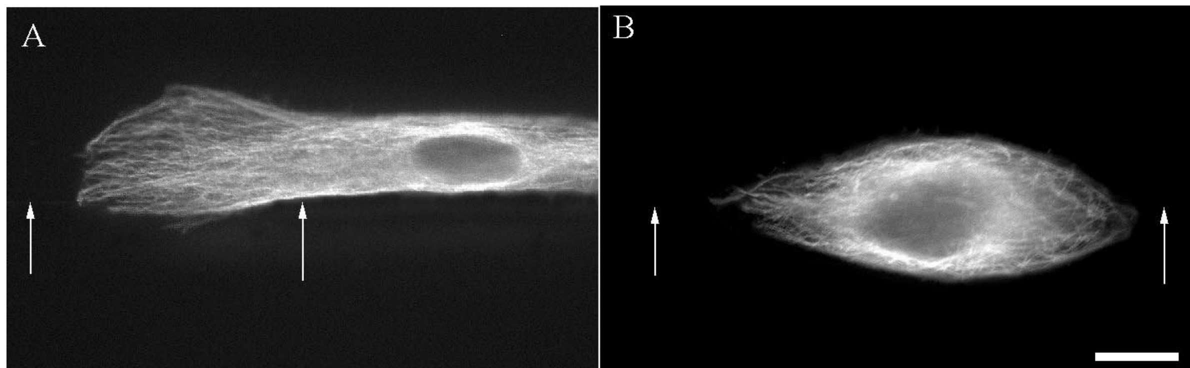

Fig. 4. The organisation and orientation of microtubules in human fibroblasts (A) and HaCaT cells (B). In these images the scratches in substratum (invisible in fluorescence microscopy) were parallel to the long axes of the cells. The arrows show the position of the scratches in substratum. Scale bar = 10 μm.

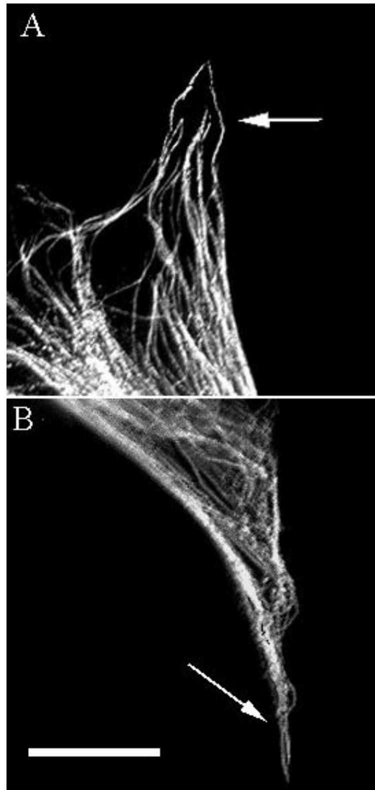

Fig. 5. The bending of microtubules in fibroblasts which approached the scratches in substratum (arrows). Scale bar = 10 μm.

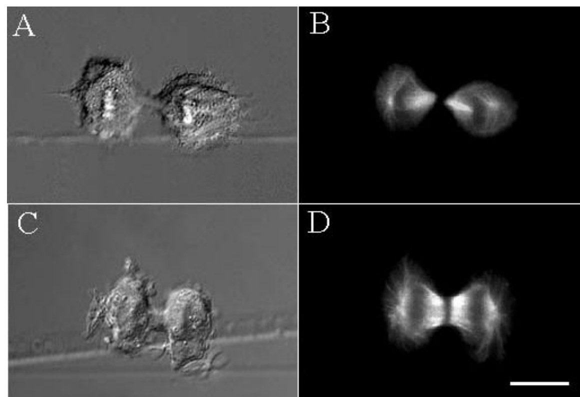

Fig. 6. The orientation of the mitotic spindle in dividing fibroblasts (A, B) and HaCaT cells (C, D) on anisotropic substratum. A, C – Nomarski interference contrast (NIC), B, D – fluorescence microscopy. Scale bar = 10 μm.

on scratches in substratum correspond to those described above for fibroblasts (data not shown).

Further experiments concentrated on the orientation of microtubules. In fibroblasts and HaCaT, cells elongate along scratches in substratum, whereas microtubules orient along the axes of the cells (Fig. 4). Cells approaching the scratches and changing their direction of extension induce the bending of not only actin stress fibres but also cytoplasmic microtubules (Fig. 5). In cells that divide on scratches, the mitotic spindle is oriented along the scratches. Visible features include the astral microtubules directed toward cell poles, the central microtubules of the spindle, as well as the position of constriction ring in anaphase cells (Fig. 6).

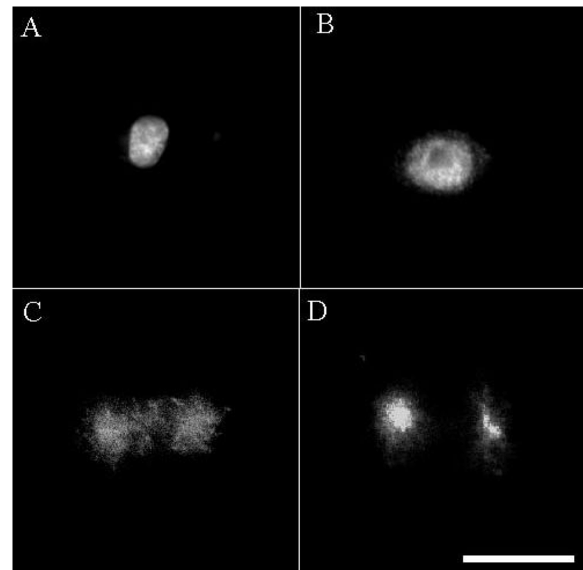

Fig. 7. The localization of NUMA protein complex on anisotropic substratum in fibroblasts (A, C) and HaCaT cells (B, D). In the cells elongated along scratches, the NuMA proteins remain dispersed within the cell nucleus before division (A, B). In dividing cells NuMA proteins moved to the poles of the mitotic spindle already in metaphase and anaphase (C, D). Fluorescence microscopy. Scale bar = 10 μm.

Subsequently the localization of the NUMA protein complex was observed, localized at the poles of the mitotic spindle of dividing cells. This protein complex has been postulated to play a role in determining the localization and orientation of mitotic spindles in dividing cells. In fibroblasts and HaCaT cells, even those elongated along scratches, the NuMA proteins remain dispersed within the cell nucleus before division (Fig. 7 A, B). In dividing cells NuMA proteins moved to the poles of the mitotic spindle already in metaphase and anaphase (Fig. 7 C, D).

Discussion

In this communication we followed the behaviour of the actin cytoskeleton, microtubules and NuMA in human skin fibroblasts and HaCaT cells as examples of mesenchymal and epithelial cells showing anchorage-dependent growth and contact

guidance. We observed a correlation between the axis of cell division and axis of cell shape, both determined by the architecture of the substratum. Our observations in many aspects correspond to the results of ZHAO *et al*. (1999) who observed division in cells oriented during galvanotaxis, and THÉRY *et al*. (2005), who induced cell shape polarization by haptotaxis on orientated ECM , but the statistically documented correlation of cell shapes and planes of cell divisions was found to be much higher in the case of contact guided cells on an anisotropic surface.

Changes in the polarization of the actin cytoskeleton architecture in the cell cortex has been observed in all studies in which cell polarization was induced by extracellular factors. In particular, WÓJCIAK-STOTHARD *et al*. (1995) demonstrated the pivotal role of actin in the reaction of fibroblasts to grooved substrata and contact guidance. Dynamic oscillatory changes in actin localization were shown to occur in dividing cells of *Dictyostelium* (YUMURA & FUKUI 1998). Considerable stress on actin organization and cell tensegrity has been shown by THÉRY and co-workers in their studies of factors determining the polarization of cell divisions (THÉRY *et al*. 2005; THÉRY *et al*. 2006; THÉRY & BORNENS 2006). Cell shape changes and cell polarization do not require the involvement of microtubules. Microtubules are absent from the filopodia of neuron growth cones. These filopodia can recognize the 0.2 μm scratches in substratum and extend along them, orienting the extension of the entire axons (STĘPIEŃ *et al*. 1999). Actomyosin activity by itself is not sufficient to regulate the orientation of the mitotic spindle. In fertilized eggs of *Xenopus laevis*, localized polar cell stimulation with AC electric fields can induce, after short lasting local cortex contractions, a relaxation of cell poles directed to electrodes. This is followed by formation of constriction rings in the cell cortex which later join together and form a deep furrow morphologically resembling the normal cell division furrow formed during cytokinesis. If this artificially induced furrow is oriented along the axis of the mitotic spindle it disappears and the ultimate cleavage furrow is formed in normal, perpendicular orientation to the long axis of the spindle (KOROHODA & RZEHAK 1972). These observations show that in fertilized eggs of *Xenopus laevis* cell cleavage orientation, as in other zygotes, depends on mitotic spindle and inside-out signalling (ROBINSON & SPUDICH 2004). This corresponds to the results of RUSAN & PEIFER (2007) and YAMASHITA and FULLER (2008) who have shown that experimentally introduced changes in the spindle position influence the plane of cell division. This suggests that in order to influence spindle orientation, the organization of actomyosin in the cell cortex must act upon the intracellular mechanisms of cell spindle formation and orientation.

Our observation of contact guided human skin fibroblasts and HaCaT cells shows that when cells approaching scratches in the substratum change the direction of extension of the lamellipodium, microtubules in their cytoplasm simultaneously bend and turn the direction of their extension. We expect that due to the association of actomyosins and microtubules in the cytoskeleton (MANDATO *et al*. 2000; ZHOU *et al*. 2002), the microtubules can intermediate between the changes in cell cortex responsible for cell shape changes and the positions of centrioles near interphase nuclei (DALBY *et al*. 2007). The NuMA complex associated with centrosomes in contact guided polarized cells is localized in the nucleus towards the two poles of the long axis of the elongated cell. Numerous data suggest that the position of centrosomes (centrioles) determines the orientation of the poles of the mitotic spindle and that microtubules play a decisive role in polarization and cytokinesis in cells dividing under isotropic conditions (RUSAN & PEIFER 2007; FOE & VON DASSOW 2008; MURTHY & WADSWORTH 2008; ODEL & FOE 2008; WERNER & GLOZER 2008). It can be safely postulated that the polar organization of NUMA and other proteins associated with the spindle microtubules play a pivotal role in the inside-out signalling related to the intrinsic mechanism of cell division orientation (NUMATA *et al*. 2000; ROBINSON & SPUDICH 2000; HAUF *et al*. 2001; THÉRY & BORNENS 2006). The astral microtubules interacting with the cell cortex influence the polar relaxation of the cell cortex, whereas the middle part of the spindle acts in anchoring the constriction ring (cleavage furrow) and membrane growth and flow in the last stages of cytokinesis (FOE & VON DASSOW 2008; MURTHY & WADSWORTH 2008; ODEL & FOE 2008). The anchorage – dependent tissue cell division plane and forces operating in cells may be dependent upon cell interaction with substratum or neighbouring cells (BURTON & TAYLOR 1997; THÉRY *et al*. 2007). In dividing fertilized eggs (KOROHODA & RZEHAK 1972; RAPPAPORT 1999) and cells remaining in suspension, inside-out signalling for localized contration-relaxation during cytokinesis dominates (NUMATA *et al*. 2000).

On the grounds of our own observations and the literature data, the following general outline can be suggested for the chain of events causing the correlation of cell shape polarization and mitotic spindle orientation in cells dividing when contact guided on anisotropic surfaces. Extracellular factors such as contact with solid surfaces (but also contact with neighbouring cells, with ECM proteins, chemoattractants, external electric fields, etc.) act

upon the cell surface. The anisotropy of these factors is recognized by receptors localized in cell membranes, such as integrins, cadherins, mechanoreceptor proteins, ion channel proteins, and other cell membrane proteins. In some cells these receptors can be localized in defined regions of the cell surface, for example in filopodia or in lamellipodia. Environmental signals are sensed, transduced and amplified by membrane integral proteins and then via signalling pathways this information is transmitted to the acto-myosin cytoskeleton. Numerous proteins are involved in these processes, including kinases, Ca-binding proteins and a variety of actin associated proteins (FOE & VON DASSOW 2008; KATO *et al.* 2008; MURTHY & WADSWORTH 2008; ODEL & FOE 2008).

The polarization of cell shape and/or cell movement does not require the involvement of microtubules. Nevertheless, the functions and architecture of the actomyosin cytoskeleton influence microtubule orientation and function. In neurons, microtubules are absent in filopodia leading the axon extension, but the microtubules present within growth cones follow the direction of extension of the filopodia. In fibroblasts approaching scratches, a concomitant turn in cells and the microtubules in their lamellipodium follows the direction of the lamellipodium extension. It remains to be determined whether the microtubules themselves can detect changes in cell membrane complex polarization as influenced by extracellular factors, without the mediating actin cytoskeleton (D'AVINO *et al.* 2005; FOE & VON DASSOW 2008; MURTHY & WADSWORTH 2008; ODEL & FOE 2008).

Microtubules remain anchored with their minus ends to gelled cytoplasm around centrosomes. The cytoplasmic microtubules and MAPs influence the localization of centrosomes (DALBY *et al.* 2007) which later determine the orientation of poles of the mitotic spindle. The position of centrosomes is associated with other co-localized proteins, including the NuMa complex (MERDES *et al.* 1996; THÉRY & BORNENS 2006). In late telophase the orientation of the spindle establishes the plane of cell division during cytokinesis (GELDMACHER-VOSS *et al.* 2003). According to this general scheme, external factors act upon cell membrane sensors, including integrins (TOYOSHIMA *et al.* 2007). This influences the acto-myosin cytoskeleton. In turn, cell shape changes dependent on the contractility of the cell cortex influence the microtubules and indirectly the positions of centrosomes. In this way the cell shape directs the orientation of the mitotic spindle, determining the plane of cell division. This scheme does not exclude that the retraction fibres anchored to aligned ECM proteins, grooves or scratches in supporting surfaces, or neighbouring cells, can influence the orientation of cell cytokinesis, as suggested by THÉRY and BORNENS (2006) and THÉRY *et al.* (2007). It also does not contradict that the astral and central spindle microtubules influence and control the contraction and relaxation processes in the cell cortex of cells dividing under isotropic conditions such as dividing zygotes, cells in suspension, etc. (ROBINSON & SPUDICH 2004; D'AVINO *et al.* 2005; FOE & VON DASSOW 2008; MURTHY & WADSWORTH 2008, ODEL & FOE 2008). The processes outlined above may be involved in the interplay of outside-in and inside -out signalling. Because they occur in a sequential manner, they can be disturbed and dissociated from one another, leading to cell multinucleation and/or aberrant cell division, as observed under pathological or experimental conditions.

For years the study of factors determining the orientation of cytokinesis concentrated on inside-out signalling and how the spindle position determines the formation of the division furrow. New experiments in which the cell shape and the positions and orientation of mitotic spindles can be exactly controlled by galvanotaxis (ZHAO *et al.* 1999), haptotaxis (THÉRY *et al.* 2005; TOYOSHIMA *et al.* 2007) or contact guidance (KRZYSIEK-MACZKA & KOROHODA 2008) can be expected to facilitate such research. Cell movement and cell division in tissues of multicellular organisms are controlled by an interplay of both intracellular and extracellular mechanisms and their significance should be equally recognized.

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