Peculiarities of Proliferation and Differentiation of Cambial and Daughter Cells of Epidermal-Dermal Morphofunctional Zone in Normal Epithelium and in Cancer

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Translated from *Kletochnye Tehnologii v Biologii i Medicine*, No. 2, pp. 88-94, April, 2010
Original article submitted March 5, 2009.

The cambial and daughter cells of normal epithelium function in the morphofunctional zone consisting of two subunits with 12 cambial cells in each. Daughter cells are differentiated in an electrical field created by 12 pairs of maternal and daughter cells, products of division of cambial cells located in the same subunit. The differentiation requires relaxation of the cortex of daughter cells via expression of SH3 domain of Src kinase by dermal daughter cells, which leads to a decrease in activity of RhoA in epidermal cells, their stretching, and activation of SH2 domain of Src responsible for differentiation. Reduction of the number of cambial cells to 6 and, consequently, weakening of electrical field produced by them to a threshold value corresponding to very weak stretching of daughter epithelial cells results in a decrease in SH2 domain expression in these cells and its kinase contribution in Src. This leads to an increase in RhoA relative to Src, enhances cell contraction, impairs formation of stress fibrils and focal contacts, reduces cell flattening, and increases cell mobility. The decrease in the number of microtubules, intermediate filaments, and stress-fibrils changes the major cell axis direction, which, in turn, sharply reduces nucleus stretching and leads to impaired chromosome looping out near the centromeres and telomeres; the cells acquires signs of an epitheliocyte and a fibroblast, protein transcription is impaired, and daughter cells are transformed into malignant cell.

Key Words: *morphofunctional zone; proportion between PhoA and Src kinase; major cell axis; cancer*

Processes of cell proliferation and differentiation are closely related to structural organization of the tissue [4]. There are published reports that normal epithelium and benign and malignant tumors are structurally organized as epidermal proliferative units with a cambial cell in the center [15,31]. However, possible differentiation of these cells in the system of tissue units and the relationships between tissue structuring and molecular mechanisms are poorly understood.

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Here we summarize the results of our experimental studies of spatial organization of different epithelia (anterior corneal epithelium of the eye, ear skin epidermis) in 350 mature male mice BALB/cj and in 15 breast cancer patients (BC) aging 40-60 years. We revealed some morphofunctional peculiarities of cambial cells under normal and pathological conditions and differentiation of the closest descendants of these cells, as well as the relationships between these processes and transduction pathways and tissue structuring.

The basal layer of the epidermis and epithelium consist of rosette-like structures, where one cambial cell is surrounded by 6-7 peripheral cells. The long

0007-4888/10/1494-0521

axis of the cambial cells is perpendicular to the basal membrane, but not parallel to it as in other cells. Therefore, after its division one cells (maternal) is still adjacent to the basal membrane, while the other (daughter) cell is located above it [9,10]. These cells remain in close contact, which blocks polarization of the maternal cell along its major axis, because it is mechanically squeezed between the daughter cell and basal membrane. Flattening of the maternal cells and a sharp increase in density of the negatively charged chromatin in it, compared to daughter cell, lead to redistribution of the surface charge between the pair of these cells and create a certain electrical field [9,12]. However, the functioning and maturation of daughter cells occur in not one rosette, but in a morphofunctional zone consisting of 24 rosettes [8]. The zone is functionally divided into two equal subunits (12 rosettes and cambial cells in each) working alternately. First, cambial cells of the first subunit divide and form 12 pairs of maternal and daughter cells, then the same occurs in the second subunit. When the number of daughter cells in both subunits of the zone attains 24, the earlier formed 12 and then the other 12 are transformed into reserve cells (30%) providing physiological regeneration in the morphofunctional zone [13]. In each proliferating subunit, not all 12 cambial cells enter mitosis simultaneously: first 6 cells divide and then the other 6 do the same, because not more than a half of all cells can divide simultaneously. The daughter cells in the first 6 pairs of maternal and daughter cells slightly lengthen along the major axis, *i.e.* the field created by 6 pairs is insufficient for differentiation. Only after the number of these pairs increases to 12 after division of the other 6 cambial cells of this subunit, the daughter cells lengthen along the field intensity lines and simultaneously are attracted to negatively charged maternal cells; this results in stretching of daughter cells parallel to the basal membrane. Then, the maternal and daughter cells connect to each other and form a ring structure; upon breaking-up of this structure, the daughter cell undergoes differentiation [9,12]. Hence, daughter cells are differentiated in an electrical field created by 12 pairs of maternal and daughter cells located in the same subunit; 6 pairs of maternal and daughter cells create a threshold field insufficient for differentiation of the daughter cells. However, epidermal daughter cells cannot differentiate without dermal cells.

It was shown that the same morphofunctional zones exist in the derma [12]. In the papillary layer, on the other side of the basal membrane, cambial fibroblast cells synchronously with epidermal cells enter into mitosis and undergo identical transformation stages. When the number of pairs of maternal and daughter cells in one subunit of the derma attains 12, the daughter cells in them differentiate. Hence, the epidermal and dermal zones work according to the same principle. They function together and form a united zone. Thus, in the first subunit of the united zone, simultaneous division of cambial cells of the epidermis and derma yields 12 pairs of epidermal and dermal maternal and daughter cells. The daughter cells of the epidermis and derma produce mutual opposite effects due to regulatory factors produced by them: epidermal cells induce shrinkage of cells sensitive to them, while dermal cells induce lengthening. Thus, 12 formed epidermal daughter cells shrunk due to the action of their own grown factors can stretch in the electrical field (*i.e.* can differentiate) only when growth factors of 12 dermal cells relax their cortex.

It is known that cell shrinkage is a result of contraction of the cortical actin–myosin complex [33]. The major role in this process is played by RhoA enzyme, one of minor G-proteins (Cdc42,Rac, RhoA) regulating the actin cytoskeleton. It activates Rho kinase phosphorylating the light myosin chain, which potentiates myosin interaction with actin threads. Inhibition of RhoA is required for relaxation of the cortex of epidermal daughter cells. The central role in this process is played by Src tyrosine kinase, one of the major regulators of cell proliferation, differentiation, and migration. The enzyme has a catalytic domain and two other in the N-terminal domains: SH2 domain binds to specific phosphotyrosine sites and SH3 domains binds to poly-proline sites. Phosphorylation of a specific tyrosine residue in the C-terminal fragment by C-terminal Src kinase (Csk) leads to its interaction with SH2 domain and inactivation of Src. Activation of Src is possible upon dephosphorylation of this tyrosine residue or binding of SH2 and SH3 domains with the corresponding ligands. In active state Src phosphorylates p190 RhoGap protein inactivating RhoA [14,16]. Hence, growth factors of dermal daughter cells should activate Src kinase in epidermal cells. Since epidermal daughter cells are influenced by EGF produced by themselves and a growth factor produced by dermal daughter cells (HGF/SF and FGF type), stimulation with these factors leads to activation of the corresponding receptors in membranes of epidermal cells and their autophosphorylation. This leads to activation of p85 protein, an adapter subunit of phosphatidylinositol 3-kinase (PI3K), an important regulator of cell proliferation and mobility [19,23,38] not only in the receptors, but also in the central compartment of the cells via a series of transmitters [37]. P85 is activated at the expense of its module domains: CH3, BCR, poly-proline PRD, two SH2 domains (cSH2 and nSH2), and intermediate iSH2 domain located between them and to which the catalytic subunit, p110 protein, is attached. nSH2 domains in inactive p85 is posi-

tioned in such a way that it inhibits p110 activity [34]. Despite the fact that nSH2 domain plays the major role in inhibition of p110, stimulation of both nSH2 and cSH2 leads to complete disinhibition of p110 [26]. It should be noted that epidermal receptors activated by EGF primarily bind to nSH2 domain and therefore directly modulate activity of p110, while Met receptors (through HGF/SF) primarily bind cSH2 domain and induce conformation changes in SH3, BCR, and PRD domains [35]. This breaks the intramolecular interaction between SH3 and PRD domains and enables PRD interaction with other proteins carrying SH3 domains [26], *e.g.* inactive Src located in the perinuclear space near the centrosome. Src binds via its SH3 domain to PRD of active p85 subunit, which results in activation of Sh3 domain and the corresponding kinase part of Src kinase. Hence, activation of Met receptors or other receptors characterized by high affinity to cSH2 domain of PI3K results in enhanced expression of SH3 domain of Src kinase in cells. Thus, activation of both domains (cSH2 and nSH2) in PI3K abolished the inhibition of p100 protein and enables binding of RAS, a G protein playing an important role in activation of ERK (extracellular signal-regulated kinase), kinase activity of PI3K is enhanced under these conditions. Hence, primary activation of inactive Src in the central compartment of the cell is effected via interaction of its SH3 domains with active PI3K. Then activated Src kinase reduced the expression of RhoA by phosphorylating p190 RhoGap protein. However, RhoA has two effectors, Rho kinase and formins [16]. Therefore inactivation of RhoA leads to inhibition of Rho kinase, which in turn, weakens the interaction between actin and myosin and to relaxation of the cortex. Cortical layers primarily contain non-branching actin filaments formed under the effect of another RhoA effector, formins, large multidomain proteins, which are inactivated due to binding of inhibitory N-terminal domain (diaphanous inhibitory domain, DID) with autoregulatory C-terminal domain (diaphanous auto-regulatory domain, DAD) [20]; FH1 and FH2 domains being located between them. RhoA activates formins by binding to GBD (GTPase-binding domain) and DID of the formin molecules and promotes dissociation of DID and DAD, which leads to activation of FH2 domain necessary and sufficient for nucleation of actin filaments. Binding of polyproline-rich FH1 domain to FH2 domain leads to more effective nucleation of actin. However, the FH1–FH2 constriction is insufficient for rapid generation of non-branching filaments assembled from the barbed end. This requires binding of FH1 domain with profilin–actin, because the FH1–FH2 construct without profilin acts as a classical capping protein or nucleates actin from a new fixed point [25,28]. On average, the higher is the number of active profilin–actin binding sites in FH1 domain, the higher is the rate of elongation of actin filament from the barbed end [24]. However, FH1 is characterized by low affinity to profilin and should be preactivated with Src expressed in the central compartment of the cell enriched also with formins. Src kinase binds to polyproline sites of FH1 domain via SH3 domain and phosphorylates them, thus promoting binding of profilin–actin to these sites. Bearing in mind that the content of active formins decreases with decreasing RhoA, while the level of active Src kinase remains unchanged, activation of greater number of polyproline sites in FH1 domain should be expected. Profilin-actin binding to active FH1 domain increases the concentration of actin and facilitates its appropriate orientation near the barbed end, which leads to rapid formation of long non-branching filaments in central compartments of the cell. Their interaction with Rho kinase leads to the formation of stress-fibrils

Thus, the action of growth factors of 12 dermal daughter cells on 12 epidermal cells leads to relaxation of the cortex in the latter due to activation of SH3 domain of Src kinase in them, which inhibits the expression of RhoA in these cells and initiates redistribution of actin from the cortical layers to the central compartments, thus providing primary polarization of the cell.

Not until this moment the epidermal daughter cells are stretched in the electrical field. However, stretching of the nucleus becomes possible only when microtubules bind to intermediate filaments and stretch the nucleus [1]. Hence, stretching of relaxed cortex of epidermal cells in the electrical field is accompanied by the formation of the system of microtubule and intermediate filaments, whose formation and assembly depends on microtubules [18,22]. Cell stretching in the electrical field leads to enhanced separation of exocytotic vesicles from membranes of the Golgi complex for their incorporation into the plasma membrane and to the release of a new portion of inactive Src located in these membranes. Inactive Src in the central compartment of the cell undergoes activation by not SH3, but its own SH2 domain, which plays an important role in nucleation and polymerization of microtubules. We previously wrote that PI3K can be completely activated due to binding of RAS. PI3K, Src, and γ-tubulin in centrosome membranes form large protein complexes responsible for microtubule nucleation [36]. However, inactive Src is characterized by low affinity to inactive γ-tubulin and weakly interacts with it. But active PI3K can interact with inactive γ-tubulin via its SH2 domain that binds and phosphorylates some tyrosine residues in γ-tubulin, thereby activating it [27]. Not until this moment inactive Src binds to active phosphotyrosine site of γ-tubulin via its SH2 domain essential for stable binding of tubulin monomers via

SH2-group. The kinase part of SH2 domain is also activated. Moreover, Src kinase provides binding of γ-tubulin to membranes, because it has no sites for membrane binding. This leads to fixation of minus-ends of microtubules on centrosomes, which prevents dissociation of tubulin dimers and promotes nucleation. Hence, stretching of epidermal daughter cell in the electrical field leads to activation of SH2 domain of Src, which induces the synthesis of microtubules promoting the formation of filaments and stretching of the nucleus.

When the cell enters into mitosis and exits from it, the major axis of the cambial call is perpendicular to the basal membrane. This orientation of the axis is determined by the absence of expression of SH2 domain of Src (because its stretching was mechanically blocked by the daughter cells) and sharp activation of RhoA compared to normal. Contraction of cambial cells increases and stress-fibrils, microtubules, and intermediate filaments are not formed, which prevents cell flattening on the basal membrane and determines vertical axis of the cell. It is known that chromosomes by the end of mitosis have regular orientation, because they are pulled apart with microtubules bound to the centromeres (anaphase-telophase orientation) [7], that is why in daughter cells in case of vertical division axis the centromeres are located in the upper pole and telomeres in the lower pole of the cell and in maternal cells visa-versa. Hence, the major axis of cambial cell and daughter cells before its stretching coincide with chromosome direction. Then, the greater stretching force of electrical fields acts on the bottom parts of daughter cells adjacent to maternal cells, that is why the formed microtubules and intermediate filaments turn the lower pole of daughter cells and stretch them parallel to the basal membrane. Hence, telomeres fixed to the nuclear membrane and the centrosome also turn from vertical to the horizontal position and are attracted to the leading edge of the epidermal cell. The chromosomes having special sites for binding with nucleus periphery and packed in the form of loop rosette-like structures [6,7] are stretched near the telomeres, which leads to loop untwisting and decondensation of chromatin in these regions enabling its transcription. In dermal daughter cells with pronounced activation of SH3 domain of Src, activity of RhoA decreases to a greater extent than in the epidermis, which leads to considerable relaxation of the cortex in these cells and potentiates their stretching in the electrical field; this increases the expression of SH2 domain and the number of microtubules. The number of stress-fibrils decreases due to sharp decrease in the number of active formins and profilin-actin binding sites. Enhanced polymerization of microtubules stretches the nucleus of the dermal daughter cells along its major axis vertically, *i.e.* towards the centromeres, and shifts the centrosome in the same direction. Therefore, in cells differentiated in the electrical field, the centrosome (with Src on its membranes) is located not along the major axis, but near the leading edge of the cell and near the nucleus pole where the chromosomes are loped out and where the epitheliocytes and fibroblast-like cells are formed. This manifests in the formation of active cell pole accumulating Src kinase, the ratio of Src to RhoA expression determines the degree of cell polarization.

After stretching and resultant differentiation, the daughter cells are separated from the maternal ones and are flattened on the basal membrane in accordance with the acquired polarity. In this cell, the stress-fibrils, microtubules, and intermediate filaments providing this process are already formed due to activation of SH2 and SH3 domains of Src. Src activated at the expense of its SH3 domain goes down to focal contacts from the centrosome area, *i.e.* from the leading edge of the cell [21]. But focal contacts should be prepared for this. Indeed, stimulation of cell receptors by growth factors induces activation of phospholipase C (PLC) in focal contacts, which in turn leads to activation of phosphatidylinositol-4,5-biphosphate (PIP2), cofilin, Cdc42, and WASP (Wiskott-Aldrich syndrome protein) regulating actin polymerization [29,30]. WASP proteins expressed with participation of PIP2 and Cdc42 interacts with Src and directs it to these contacts. Then, signal molecules (including Ras involved into the formation of lamellopodias) are stimulated with participation of Src kinase. Cell flattening is accompanied by actin redistribution from cortical layers to stress-fibrils. Indeed, flattening increases cell surface and facilitate the release of a new portion of Src and activation of its SH2 domain, which increases its total kinase activity and moderately decreases RhoA and active formins. This increases the number of active binding sites for profilin–actin in FH1 domain and promotes the formation of long actin threads in the central compartment of the cell. Their interaction with Rho kinase results in the formation of stress-fibrils pulling apart the focal contacts and transforming them into focal adhesions. The activated Src stimulates different transduction pathways, the most important of them are RAS/ERK cascade and PI3K/AKT pathway (serine/threonine protein kinase B) [16]. ERK enters the nucleus, amplifies many genes, and induces two events: transcription of cyclin D and induction of inhibitors of cyclin-dependent kinases (p21/p27) arresting the cell cycle. AKT inactivates inhibitors and induces their transport from the nucleus to the cytoplasm, where they degrade in proteosomes [17]. Under conditions of increasing activity of RAS/ERK and inhibitors, AKT cannot completely inacti-

vate them, because it participates in inactivation of both inhibitors. DNA synthesis in the cell cannot be started in the presence of these inhibitors, but other conditions are also required. Thus, one subunit of the united zone in the interphase contains the following cells: separated epidermal maternal and daughter cell and thk corresponding dermal cells. When dermal daughter cells leave the zone, the area of remaining cells shrinks by 1.3-1.5 times and then DNA synthesis starts. These cells leave the zone first, because their major axis, in contrast to the axis of epidermal cell, is oriented towards the tissue depth, but not along the basal membrane. Since epidermal daughter cells are organized as a stratum, while dermal cells that activated SH3 domain of Src in them are displaced to the depth of the tissue, epidermal daughter cells in which only SH2 domain is activated start to predominate in the subunit. This leads to a decrease in Src expression in these cells and predominance of RhoA. RhoA predominance is even more pronounced in cambial maternal cells, because SH2 domain is not expressed in them. Thus, the cells shrink and the expression of RAS/ERK cascade in them decreases, which leads to a decrease in the synthesis of inhibitors. However, RAS activates not only ERK, but also PI3K, therefore the decrease in RAS activity reduces its binding with PI3K. Under these conditions, PI3K is activated primarily by expressed RhoA, which can interact with p110 [32], therefore AKT activity does not decrease. Due to reduced synthesis of inhibitors and unchanged AKT activity, all inhibitors are inactivated. This results in disinhibition of cyclin E/cdk 2 complex and synthesis of DNA (but not RNA). Indeed, cell shrinkage increases compactness of DNA loop packing in the nuclei, *i.e.* leads to partial chromatin condensation, which in turn, interferes the work of transcription factors [3], leads to inhibition of RNA synthesis in epithelial daughter cells, and decelerates utilization of ribonucleotides (constitutively formed in the organism) in this process. Non-utilized ribonucleotides are converted into deoxyribonucleotides [2], the latter accumulate in the cell, which leads to induction of potentially active genes of the corresponding enzyme complex. Thus, the synthesis of nucleic acids on DNA template is still going on, but now with the use of deoxyribonucleotides. Active chromatin is replicated first of all, because the mechanism of the synthesis of nucleic acids (both DNA and RNA) on DNA template is the same. Due to the involvement of deoxyribonucleotides principally differing by their chemical structure from ribonucleotides, double-strand DNA molecules are formed [5].

During the formation of malignant epithelial tumor, primary changes occur in one subunit of the epithelium and not involve the stroma [11]. The number of cambial cells in this subunit decreases to 6, while the corresponding stromal subunit still contains 12 cells (normal). Therefore, simultaneous proliferation of cambial cells of the epithelium and stroma leads to predominance of 6 pairs of stromal maternal and daughter cells over epithelial cells in the first subunit. When cambial cells of the second pathologically changed subunit enter mitosis, growth factors of 6 "extra" stromal daughter cells of the first subunit influence the 6 pairs of newly formed epithelial maternal and daughter cells of the second subunit, *i.e.* activate SH3 domain of Src kinase in them and relax the cortex. However, the field of each subunit is formed by only 6 pairs of maternal and daughter cells and has a threshold value insufficient for the interaction of fields of different subunits, therefore stretching of daughter cells is negligible. The lesser is the number of cambial cells in epithelial subunit (<6), and consequently, the lesser in the number of pairs of maternal and daughter cells, the weaker is the electrical field and the stretching force produced by them. Epithelial daughter cells are characterized by normal expression of SH3 domain of Src and very weak expression of SH2 domain, which enhanced RhoA activity and increases the number of active formins. However, expression of SH3 domains does not change and, hence the number of profilin–actin binding sites in FH1 domain decreases. Therefore, the growth of long actin filaments leading to the formation of stress-fibrils decreases. FH1–FH2 construct induces actin nucleation from new start points, which leads to accumulation of short actin threads in cell cortex and a cell spasm. The decrease in the number of stress-fibrils, microtubules, intermediate filaments due to low activity of SH2 domain results in reduction of the number and size of focal adhesions and disturbances in cell flattening, while the direction of the major axis in daughter cells relatively to the basal membrane becomes oblique, but not horizontal. DNA loops near the telomeres are less stretched and decondensed compared to normal and less extensive DNA fragments are available for transcription. The signs of epithelial cells are less pronounced. Further decrease in the expression of SH2 domain leads to a sharp impairment of cell flattening, while predominance of RhoA leads to vertical stretching of the cell and its nucleus and rotation of the major axis of the cell. Under these conditions, the chromosomes are incompletely looped out near the centromeres and signs of fibroblasts appear. Hence, signs not only epithelial cells, but also fibroblasts appear in tumor cells during aggravation of the process. Indeed, one and the same tumor cell can simultaneously express cytokeratin and vimentin microfilaments and growth factors with contraction and relaxation activities essential for cell growth and division, due to which the cells become independent on the

presence of serum growth factors. The rotation of the major call axis angularly to the basal membrane directs the growth of tumor cells along the same vector, which determines tumor growth in the depth of the tissue or on its surface in the form of a node.

Reduced expression of SH2 domain of Src does not decrease RhoA activity to normal, therefore RAS/ERK and RhoA/PI3K/AKT pathways are simultaneously activated in this cell during the interphase. Hyperexpression of these pathways stimulates the formation of proliferation inhibitors by reducing the number of stress-fibrils and microtubules decelerating the transport of endocytotic vesicles; however, high activity of RhoA stimulates p110 PI3K/AKT pathway, which leads to their inactivation. Due to these changes the cell acquires the capacity to grow irrespective of attachment to the substrate.

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