ROMANIAN ACADEMY **INSTITUTE OF BIOLOGY BUCHAREST**

SUMMARY OF THE PhD THESIS

"Investigations on ultrastructural and molecular alterations of the tumor- peritumoral stroma interface in basal cell carcinoma and squamous cell carcinoma"

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INTRODUCTION

The normal human skin represented by (1) a pavement stratified epithelium called epidermis of ectodermal origin, attached to (2) the dermis of mesenchymal origin and, more deeply, to (3) hypoderm, is considered the second organ as size of the human body. Skin is a vital organ. Epiderm is the first line of defense against possible injuries and rests on the underlying dermis. Between the epidermis and the dermis of a normal skin there is a so-called dermal-epidermal junction zone, at which a continuous anchor infrastructure, known as the basement membrane, appears as a specialized extracellular matrix, well organized at the molecular level, which separates itself and the same time joins the epidermis to the dermis. The depletion or possible functional deficiencies of any component of the basement membrane may be lethal at certain stages of embryonic development or during delivery, or may induce skin damage later in life. The skin can be involved in inborn or acquired skin diseases. There are two main categories of skin cancer: (1) malignant melanoma and (2) non-melanoma. Basal cell carcinoma accounts for approximately 80% of all non-melanoma skin cancers.

In recent years, the incidence of skin cancers has greatly increased due to exposure to environmental risk factors and the negligence of the first signs, which are often premalignant lesions. Cancer is considered an irreversible disease, mainly due to the gradual accumulation of one or several mutations and / or the disruption of oncogenes and tumor suppressor genes and chromosomal anomalies. It develops as a progressive process in several stages where the cells involved undergo consecutive genetic alterations and in collaboration with stromal cells gradually acquire phenotypic changes, so that transformed cells will grow rapidly and uncontrollably. However, changes in tumor cells are not sufficient to generate a tumor, requiring a suitable stromal micromedium.

Most human cancers have an epithelial origin, accounting for about 80%. Although there was a particular interest in knowing the morphological aspects of the tumor cells *per se*, there are still other unknown infrastructure changes of both the tumor micromedium and the extracellular micromedium that make up the tumor ecosystem. The purpose of this paper is to know such particular abnormalities that accompany tumor development and tumor cell behavior. Here are some of the original observations made by the electron microscopic investigations of the various human tumors involved in premalignant skin lesions and in the development of basal cell carcinoma (BCC) and squamous cell carcinoma (CSC).

THE PURPOSE AND OBJECTIVES OF THE DOCTORAL THESIS

The last decade was marked by a statistically significant increase in the incidence of skin cancers (both melanoma and nomelanomic - including basal cell carcinoma and squamous cell carcinoma), which motivated the development of new studies to later understand the behavior of these

pathologies developing new therapeutic approaches. The multitude of premalignant lesions as well as the complex classification of the carcinomas required a more accurate differentiation of the differential diagnosis, the aspect in which the present thesis contributes significantly through the techniques approached. Thus, the developed researches have both theoretical and practical aspect, differential diagnosis, prognostic and even a stage assessment.

The aim of this study was to discover and understand new aspects of ultrastructure that occur in basal cell carcinoma and squamous cell carcinoma cases investigated by us based on clinical data provided.

The main objectives of this doctoral thesis were:

1) to elaborate a comparative study regarding the possible ultrastructural abnormalities found in the cases of benign lesions studied (in the transition to the premalignant phenotype), allowing to decipher the subtle infrastructure changes accompanying the malignant transformation;

2) observing how microenvironmental changes exert a direct influence on adjacent epithelia and contribute to a better understanding of malignant transformation by observing the alterations occurring at the level of the dermal-epidermal junction zone (DEJZ) in the cases of premalignant lesions examined;

3) electronomicroscopic examination of skin samples from patients diagnosed with basal cell carcinoma and squamous cell carcinoma in order to follow the way in which the specific phenotype of keratinocytes is retained;

4) tracking the distribution of molecular components of the various infrastructures in the tumor cells from the tumor-peritumoral stroma interface;

5) detection of the presence of telocytes as a distinct interstitial cell phenotype within the BCC tumor stroma and SCC, respectively, following the establishment of homo- and hetero-cellular junctions and the transfer of microvesicles in cell signaling;

6) observing a possible involvement of telocytes in inducing altered cell-cell communications in the peritumoral stroma and subsequently throughout the tumor mass;

7) finding possible polymorphic ultrastructural aspects of altering cell-cell junctions and cell-extracellular matrix in basal cell carcinoma that can prepare tumor cells for their invasive growth;

8) comparing the results of TEM and IEM investigations in maintaining / losing epithelial cell polarity;

9) observation of the localization of some hemidesmosomal molecules connectors for the cytoskeleton;

10) to determine the relevance of all these changes in determining the invasive capacity of tumor cells within the peritumoral stroma.

STRUCTURE OF THE DOCTORAL THESIS

The Ph.D. thesis titled *"Investigations on ultrastructural and molecular alterations of the tumor- peritumoral stroma interface in basal cell carcinoma and squamous cell carcinoma"* contains 298 pages and is divided into two parts: "Theoretical Founding of Research" and "Personal Research Results". The paper is structured in six chapters, of which the "Theoretical Founding of the Research" contains 97 pages and contains generalities regarding the normal histology and ultrastructure of the skin, the pathology of the skin with epidemiology, etiology, genetics and classification for both non-malignant and non-melanoma carcinomas, as well as therapeutic aspects in skin tumors. The "Personal Research Results" extends to 160 pages, and is made up of the chapters describing the materials and methods used, the results and the discussions based on them, the conclusions of the investigations. The paper contains 158 Figures, of which 139 original, to which are added 9 schemes and 2 tables. Finally, the bibliography and the list of scientific papers published in the subject of the PhD thesis are added.

MATERIAL AND METHODS

The design and development of this doctoral thesis involved the extensive experience of the doctoral supervisor as well as the personal experience accumulated in the scientific researches (including the consultation of the biomedical scientific literature which is in a continuous dynamics and development).

The biological material represented by human skin fragments surgically removed from the areas affected by premalignant lesions, basal cell carcinoma and squamous cell carcinoma, were performed with safety margins. Dr. Juravle Florin-Daniel, primary plastic surgeon, reconstructive and aesthetic microsurgery, performed the surgical operations, and providing the evidence involved patient informed consent. Patient data was retrieved from the appropriate observation sheets.

Of the 62 biological samples taken from 58 patients, 46 are from non-malignant lesions, 14 are basal cell carcinomas and 2 are squamous cell carcinomas. Techniques and protocols used in biological material analysis were histology, transmission electron microscopy and immunoelectron microscopy.

The protocol for specimen processing for Electron Transmission Microscope investigations was carried out according to the standard (Ploaie and Petre, 1979), and the immunoelectron microscopy was the double-immun labeling.

RESULTS AND DISCUSSIONS

A. ULTRASTRUCTURAL INVESTIGATIONS IN PREMALIGNANT SKIN LESIONS

1. Seborrheic keratosis (SK)

Electron microscopic analysis of surgical removal specimens from a patient suffering from seborrhoeic keratosis showed that a continuous basement membrane usually separates epidermis from the fibrotic dermis (**Fig. 1**) at the level of dermal-epidermal junction zone (DEJZ) (Mirancea, **Moroşanu** *et al*., 2011).

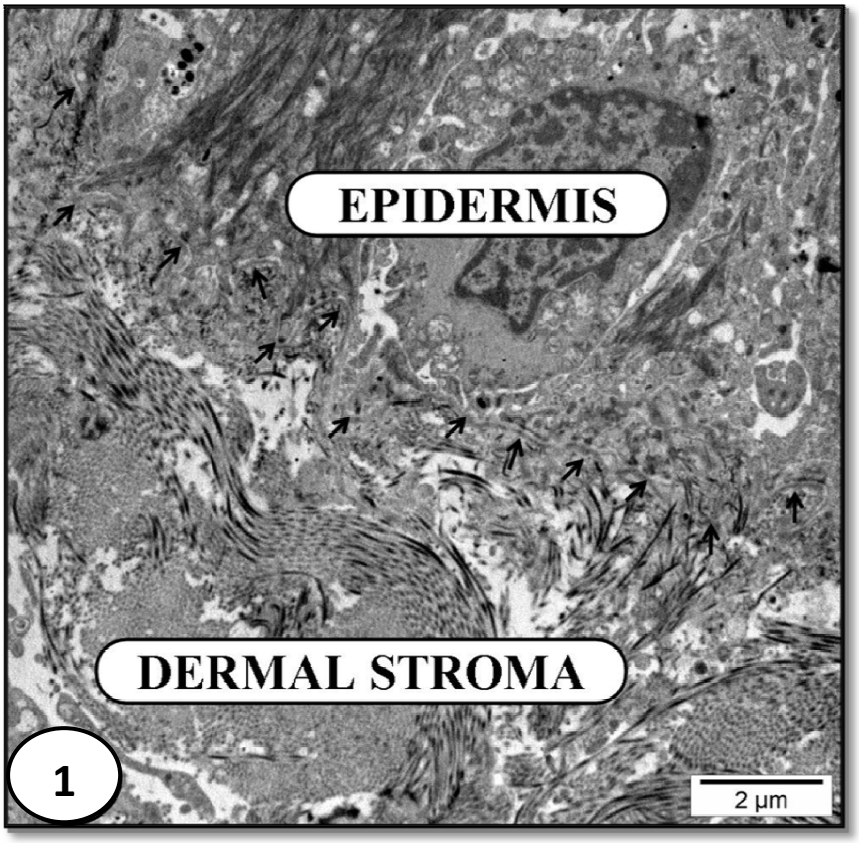

Fig. 1 - A continuous basement membrane (arrows) separates the epidermis from the fibrotic dermis. Seborrheic keratosis (according to Mirancea, **Moroşanu** *et al.,* 2011).

The basement membrane features distinct and continuous *lamina lucida*, but also the *lamina densa*, and the intermediate filaments connect normal hemidesmosomal junctions. Dermal-epidermal junction zone also presents infrastructure anomalies in some places with redundant portions of the basement membrane, no hemidesmosomes along the dermal-epidermal junction zone profiles, or infrastructure components resulting from the destruction of hemidesmosomes (**Fig. 2, 3**). Trabecular vesicles derived from plasma membranes (endocytosis cavities, clathrin-coated vesicles) can also be observed (**Fig. 4, 5**) (Mirancea, **Moroşanu** *et al.,* 2011).

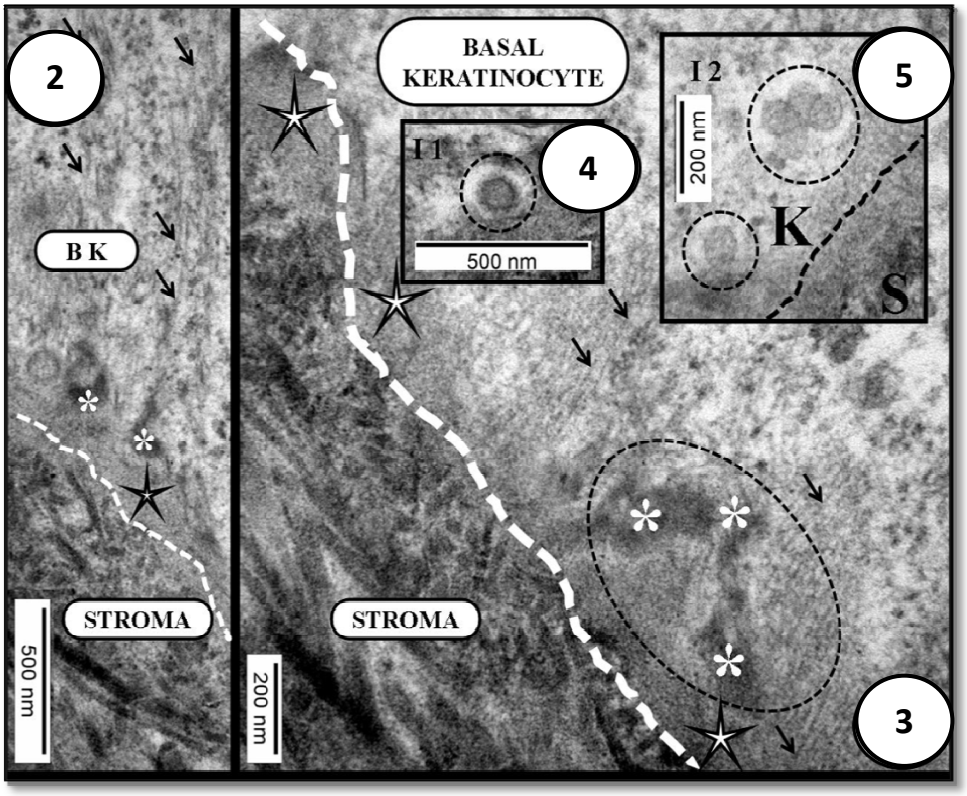

Fig. 2 - The basal keratinocyte (BK) profile can be seen as the remnant of the hemidesmosomal junctions, visible here as a dense amorphous material (asterisks) to which some keratin filaments (arrows) arrive. At the level of the dermal-epidermal junction (discontinued line), an amorphous material is visible, probably a basement membrane deposition (black star). Seborrheic keratosis (according to Mirancea, **Moroşanu** *et al*., 2011).
Fig. 3 - All ultrastructural abnormalities described above in **Fig. 2** are better visible on another profile of the dermal-epidermal junction area. Seborrheic keratosis (according to Mirancea, **Moroşanu** *et al.*, 2011).
Fig. 4 - A clathrin-coated vesicle inside the basal keratinocytes. Seborrheic keratosis (according to Mirancea, **Moroşanu** *et al*., 2011).
Fig. 5 - The endocytosis vesicles near the plasma membrane of a basal keratinocyte are directed to the stroma. Seborrheic keratosis (according to Mirancea, **Moroşanu** *et al*., 2011).

SK is benign malignant tumor (Hafner *et al.,* 2010), but some medical cases have been reported when SK led to the development of basal cell carcinoma (Akasaka and Kon, 1997) or malignant melanoma (Zabel *et al.,* 2000), without knowing if it is just coincidence or malignant transformation. The ultrastructural modifications at the level of the dermal-epidermal junction zone of SK mimic those described in basocellular and squamous cell carcinoma (Mirancea, **Şerban** *et al.,* 2009; Mirancea, **Moroşanu** *et al.,* 2010).

Following the electron microscopic examination, we were able to observe in the SK the existence of telocytes, a special stroma cell phenotype, telocytes that we identified and described for the first time in SK (manuscript in preparation). As a rule, in SK the telocytes exhibit one or two telopodial extensions (**Fig. 6**). The telocytes have a euchromatic nucleus, in which a nucleolus is sometimes distinguished (**Fig. 6**). In the telocyte cytoplasm, the main cytometric organelles are concentrated in the vicinity of the nucleus.

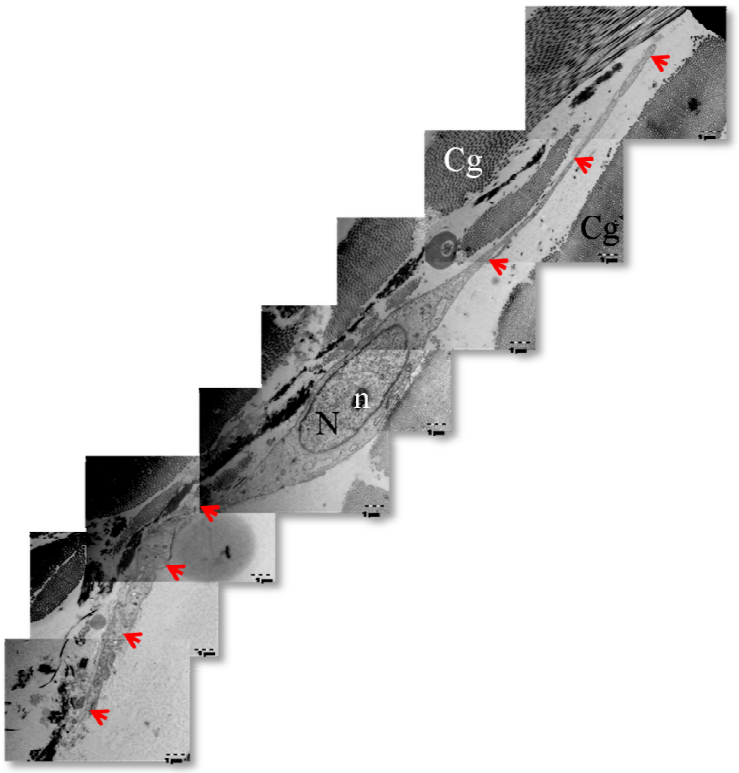

Fig. 6 - Bipolar telocyte, showing two telopodes (arrows) and a nucleolated (n) nucleus (N). Cg - collagen. Seborrheic keratosis. Sample T3.

2. CAVERNOUS HEMANGIOMA

Semithin sections from patients with cavernous hemangiomas analyzed under the optical microscope showed a lot of large and irregular capillaries as a structure with abnormally thin walls that are prone to loss (Mirancea, **Moroşanu** *et al*., 2011).

On the ultrathin sections examined at the transmission electron microscope, the presence of the red blood cells extravasated inside the dermal stroma, under the dysplastic to anaplastic epidermis, was observed. Almost all keratinocytes have lost the epithelial phenotype: there are no desmosomal junctions or are poor, the intermediate filaments are absent, the basement membrane is missing at the dermal-epidermal interface and the hemidesmosomal junction cannot be detected.

Eucromatine is predominant within most of the epithelial cell nuclei, and giant nucleoli can be seen (**Fig. 7**). At a greater enlargement, the red blood cells are extruded in direct contact with epidermal dysplastic cells up to anaplastic.

No basement membrane is inserted. Residues of a hemidesmosomal junction represented by the external plaque can be detected. No intermediate filament has reached the base pole (**Fig. 8**). In **Fig. 9** shows a short profile at the dermal-epidermal junction zone where the fibrous stroma is in direct contact with the basal keratinocytes. Desmosomal junctions are also affected, and cytokeratin filaments rarely reach or do not reach to these intercellular junctions.

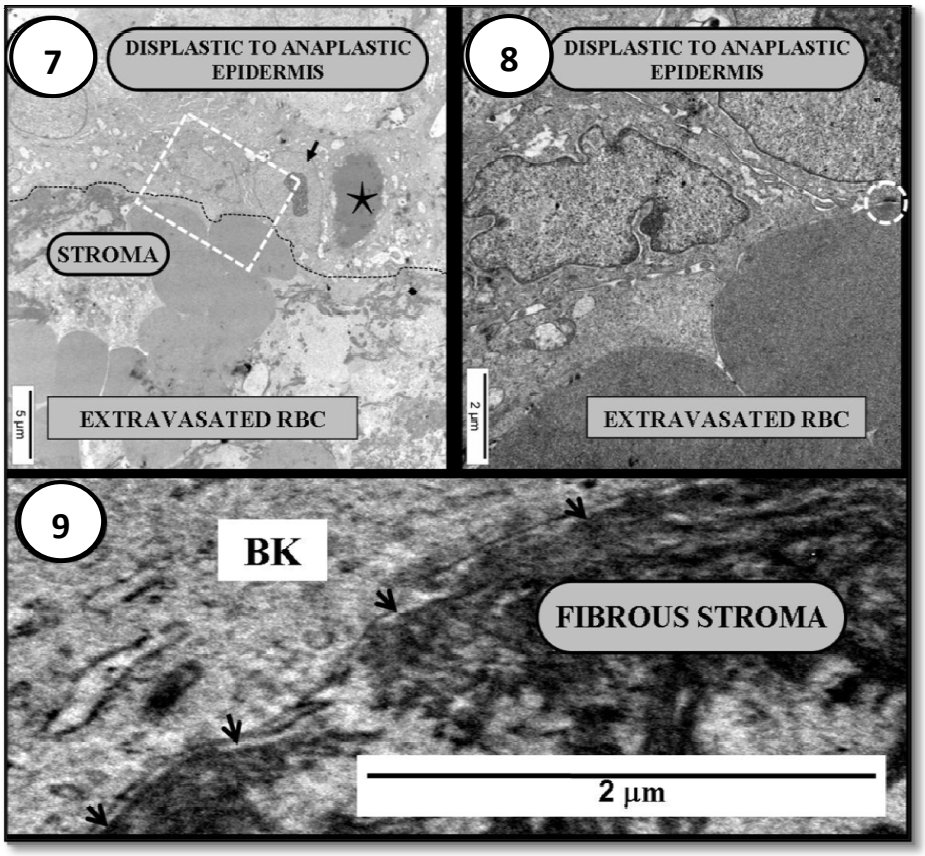

Fig. 7 - Extravasated red blood cells (RBCs) inside the dermal stroma below the dysplastic to anaplastic epidermis. Keratinocytes have almost lost their epithelial phenotype: there are no desmosomal junctions or, when present, they are precarious. Intermediate filaments are absent. The arrow marks an anaplastic cell with a huge nucleus and the star marks an apoptotic cell. Moreover, the basement membrane is missing from the dermal-epidermal interface (the discontinued black line) and no hemidesmosomal junction can be detected. Cavernous hemangioma (according to Mirancea, **Moroşanu** *et al*., 2011).

Fig. 8 - Detail in the area enclosed in **Fig. 7**. Extravasated red blood cells (RBCs) are in direct contact with epidermal dysplastic cells up to anaplastic. No basement membrane is inserted. The encircled area delimits a remnant of a hemidesmosome junction represented by the external plaque. No intermediate filaments reach the basal pole. Cavernous hemangioma (according to Mirancea, **Moroşanu** *et al*., 2011).

Fig. 9 - A short profile at the dermal-epidermal junction. The fibrotic stroma comes in direct contact (arrows) with a basal keratinocyte (BK). Cavernous hemangioma (according to Mirancea, **Moroşanu** *et al*., 2011).

Some epithelial cells were engaged in an apoptotic process. Along the intercellular contacts, desmosomal junctions cannot be detected. All of these infrastructure abnormalities provide a real support for diagnosis: the epidermis appears as a dysplastic to anaplastic epithelium. Anomalies of blood microvasculature have led to the formation of extensive haemorrhagic areas affronted with epidermal epithelium (Mirancea, **Moroşanu** *et al*., 2011). Without the normal barrier associated with the endothelial wall and the associated basement membrane and the absence of the pericytes, paracrine factors will induce a very intense and unlimited effect on the epidermis. Thus, the basement membrane and the hemidesmosomal junctions are the first targets for lytic enzymes. The aggressive action of this type of paracrine factors easily released by extravasated blood cells (Mueller and Fusenig, 2004) to epidermal cells leads to the change of the epithelial phenotype: cell-cell and cell-extracellular matrix junctions are destroyed, the cytokeratin cytoskeleton has disappeared which has led to cellular depolarization.

B. ULTRASTRUCTURAL AND MOLECULAR INVESTIGATIONS IN BASAL CELL CARCINOMA AND SQUAMOUS CELL CARCINOMA

1. BAZAL CELL CARCINOMA

a) Ultrastructural alterations from the tumor-peritumoral stroma interface

In this doctoral thesis I studied the dynamic changes of carcinoma phenotypes (basal cell carcinoma and squamous cell carcinoma) following the tumor-peritumoral stromal interface changes, from the peritumoral stroma, but also from the desmosomal and hemidesmosomal junctions. Thus, we noticed that tumor cell phenotypes in both types of carcinoma were severely altered, yet retaining some infrastructures that recall their epithelial origin.

In the event of disturbances in the distribution of BM molecules and hemidesmosomes, depolarization of the epithelium occurs with its involvement in malignant proliferation. Tumors developed by the epithelium on the stromal tumor interface showed an irregular growth of the cell, revealing very severe damage to cellular stratification, and consequently the loss of cell polarity (Mirancea, **Moroşanu** *et al.,* 2010).

In BCC, keratin filaments are severely impaired (does not connect the basement plasma membrane of tumor cells affronted to tumor stroma), because hemidesmosomes are defective or missing (**Moroşanu** *et al*., 2013) (**Figs. 10, 11, 12**). Following electron microscopic examination, we sometimes observed debris of hemidesmosomal junctions, and intermediate filaments can be seen as perinuclear bundles or rare cytokeratin filaments (Mirancea, **Moroşanu** *et al*., 2011). Since desmosomes are also severely affected or lacking, the tonofilaments have formed conglomerates of keratin filaments that have captured and internalized desmosomes within the cytoplasm. Due to alteration of intercellular junctions, including the internalization of desmosomes, intercellular spaces become abnormally enlarged.

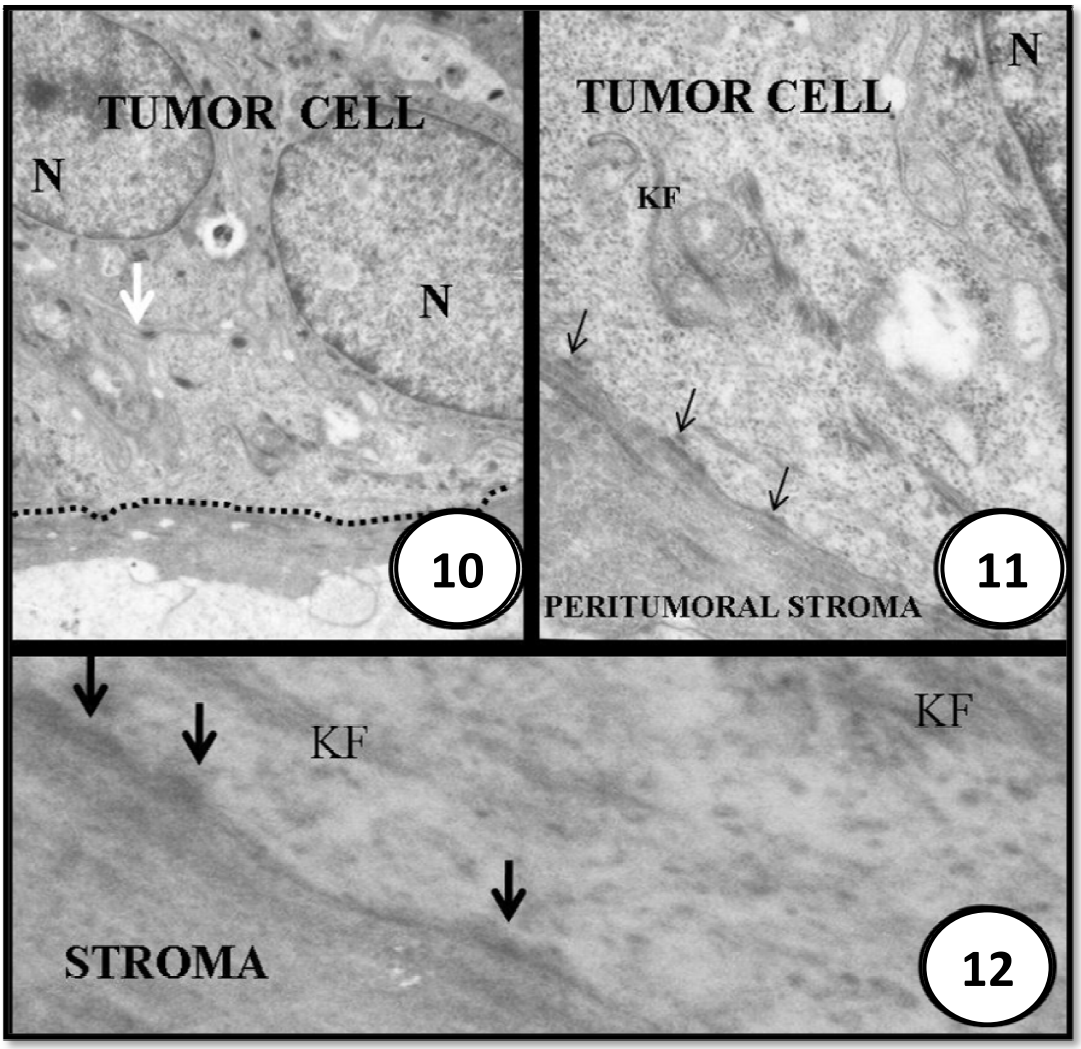

Fig. 10 - Basal pole from two tumor cells with euchromatic nuclei (N). The white arrow marks a deficient desmosomal junction. The boundary between tumor cells and the peritumoral stroma is delimited by black dots. (× 13.200). BCC (according to Mirancea, **Moroşanu** *et al*., 2010).
Fig. 11 - The basal pole of a tumor cell. Keratin filaments (KF) do not reach defective hemidesmosomes (arrows). N = nucleus. (× 24.300). BCC (according to Mirancea, **Moroşanu** *et al*., 2010).
Fig. 12 - Details of defective hemidesmosomes (arrows) that do not have internal plaques and therefore keratin filaments (KF) fail to connect hemidesmosomes (× 65.000). BCC (according to Mirancea, **Moroşanu** *et al*., 2010).

The "*conduit system*" phenotype identified for the first time in basal cell carcinoma is a particular one since, different from other "*conduit systems*" reported so far in the literature in other tissue types as having a basement membrane separating the amorphous fibrilar matrix the remainder of the tissue we describe does not have a visible basement membrane, the isolation of its matrix being carried out by tumor cells that are still solidarized by desmosomal junctions and forming its walls (**Fig. 13**). These unique structures seem to play an important role in the transport of fluid and low molecular weight molecules such as chemokines (Sixt *et al*., 2005; Roozendaal *et al*., 2008; Roozendaal *et al*., 2009).

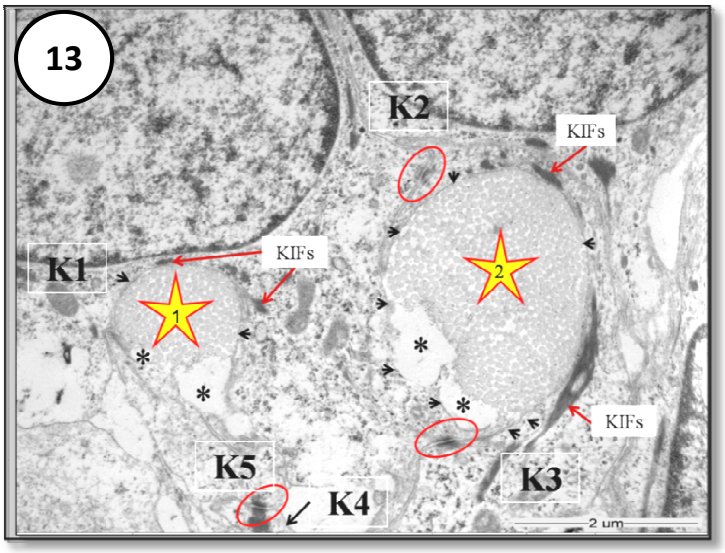

Fig. 13 - Within the tumor mass, there are particular "*conduit systems*" (large stars 1 and 2) delimited by tumor cells (K1-K5), which still retain intermediate keratin filaments (KIF) and desmosomal junctions (elliptical areas). "*Conduit systems*" with a core represented by cross-sectional collagen fibrils are delineated by the tumor cells of the tumor cells (arrows). Empty spaces can be seen inside each "*conduit system*" (asterisks) (manuscript in preparation). BCC.

At tumor tumor-peritumoral stroma interface, **plasma membrane dissolution** of tumor cells were observed, which has contributed to the formation of membrane microvesicles.

The **membrane recombination** process is a special type of intercellular communication, through which dissolution occurs at the site of the two cells affronted plasmalemma membranes, allowing for open cell communication (Mirancea *et al*., 2013). Cancer cells can fuse with normal cells (stromal, epithelial, macrophages) but also with other cancer cells, resulting in **hybrids** with new properties (Mohr *et al*., 2015) and increased heterogeneity (Coward and Harding, 2014).

Some tumor cells exhibited **membrane microvesicles** or numerous lysosomal-like structures (**Fig. 14**, detail in **Fig. 15**) (Mirancea, Şerban *et al*., 2009).

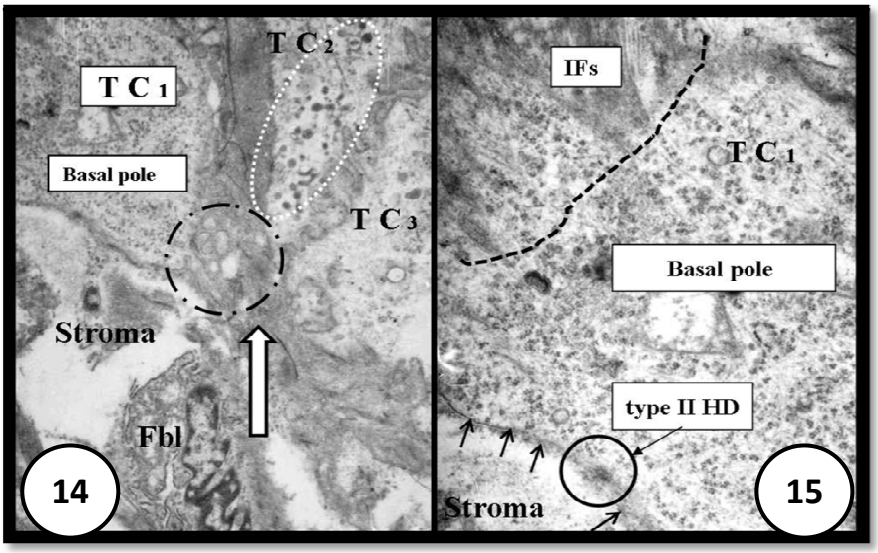

Fig. 14 - The basal poles of several basal cell epithelial cells (TC1-TC3) at the associated stromal interface. The circular area surrounded by black circular lines and dots describes a modified segment of the basal pole of the TC1 tumor cell by removing vesicles. Inside another tumor cell (TC2), many lysosome-like infrastructures (white ellipsoid area) can be detected; Fbl = fibroblast; (× 10.500). BCC (according to Mirancea, **Şerban et al.,** 2009).
Fig. 15 - The enlarged area of the TC1 tumor cell of **Fig. 14.** The keratin (KF) filaments do not reach the basal pole due to lack of internal hemidesmosis plaque (circular area) (× 10.500). BCC (according to Mirancea, **Şerban et al.,** 2009).

In almost all our investigated cases of BCC, as well as SCC (Mirancea, **Moroşanu** *et al*., 2013; Mirancea, **Moroşanu** *et al*., 2010), we detected microvesicles released by tumor cells. Cancer cells release large amounts of membrane microvesicles that can be transferred to non-transformed cells (stromal cells such as fibroblasts, endothelial cells and infiltrating inflammatory cells). Such events can contribute to tumor angiogenesis as well as tumor migration, invasiveness and ectopic dissemination to develop a secondary tumor (metastasis), drug resistance, and cancer stem cell hierarchy (Lee *et al*., 2011).

Some areas of the neoplastic epithelial epithelium have shown extensive prolongations of cells that penetrate deep into the peritumoral stroma, and the basement membrane and hemidesmosomes accompany these extensions (but in some areas are missing) called **invadopodia**. Because of the fragility of the plasma membrane, the cellular content tends to herniate in the peritumoral stroma. Sometimes the basal cell of the tumor cell is filled with a multitude of lysosome-like vesicles and apparently empty.

b) Ultrastructural aspects of the tumor stroma in the immediate vicinity of tumor cells

The dermal-epidermal junction zone is severely altered during epidermal carcinogenesis, so in line with BM tumor progression it becomes illusory or even totally disappearing, which is a distinctive sign of an unfavorable evolution of BCC.

In the ultrastructural investigations performed by TEM, we have noticed that at the associated peritumoral stromal interface, the basement membrane is often missing or observable amorphous material attached to the plasma membrane from the peritumoral stroma interface. Sometimes, the boundary between the tumor cells and the adjacent stroma appears to be linear, but no basement membrane can be detected, and the hemidesmosomal junctions are totally missing, and hence the tonofilaments do not reach the basal base of the plasma membrane (**Fig. 16**). Since BM is lacking, instead, the tumor cell emits invadopodia that penetrate deep inside the peritumoral stroma. Along the peripheral area of the invadopodium with the micromedium, including its tip, neither hemidesmosomes nor BM can be seen (**Fig. 17**).

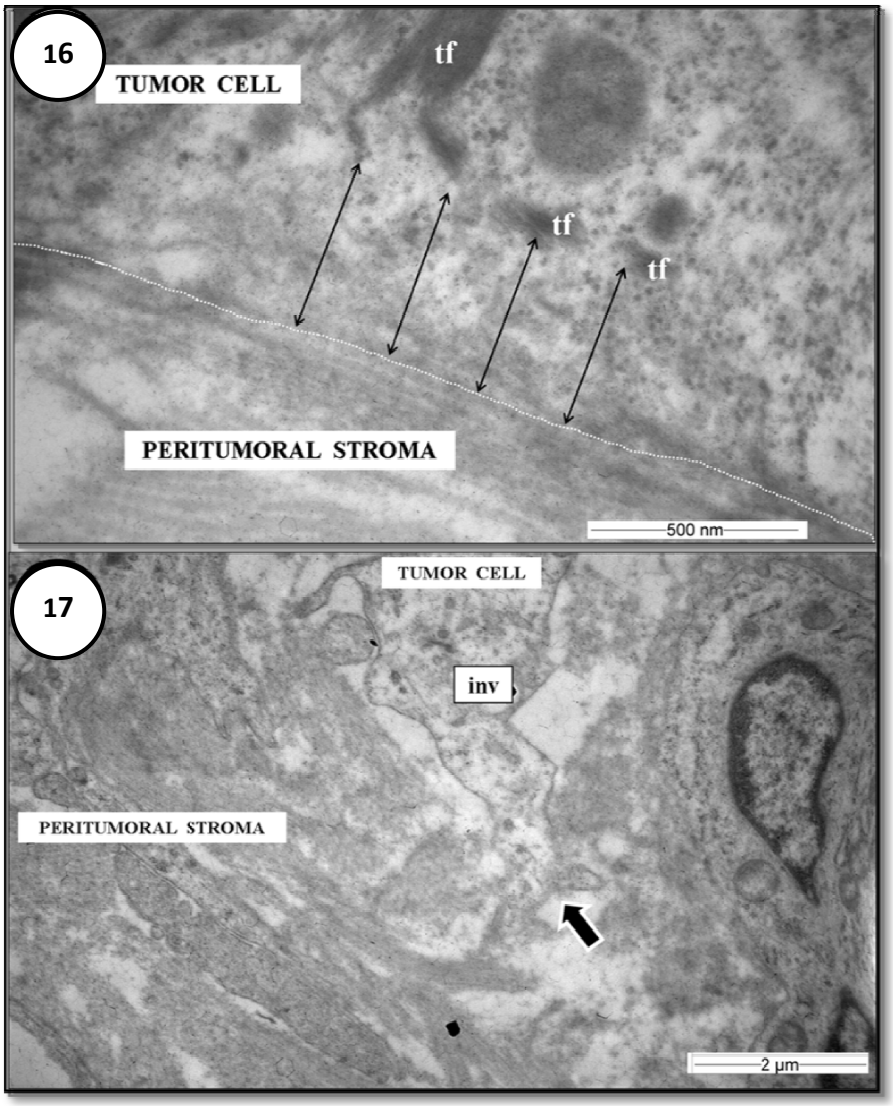

Fig. 16 - Small sector of a malignant cell affronted with the peritumoral stroma. The dotted line marks the interface between a tumor cell and the peritumoral stroma, where no basement membrane can be detected. All hemidesmosomal junctions are missing. The tonofilaments (tf) do not reach the basal pole of the plasma membrane, so a remarkable space (two-headed arrows) separates the basal profile cytoskeleton. BCC (according to **Moroşanu** *et al.,* 2013).
Fig. 17 - A tumor invadopodium (inv) passes deep inside the peritumoral stroma. Along the peripheral area of the invadopodium with the micromedium, including its tip (arrow), hemidesmosomes and basement membrane cannot be seen. BCC (according to **Moroşanu** *et al.,* 2013).

In the case of BCC, the anchoring plaques are destroyed, Cg VII fibers being ectopically localized or even totally missing.

The tumor stroma is massively infiltrated with inflammatory cells that have released the lysosomal content (involved in the destruction of the adjacent extracellular matrix) (Mirancea, **Moroşanu** *et al*., 2013). In some areas, extravasated blood cells mix with invasive tumor cells. The remarkable vascular permeability that causes red and white cells to disseminate inside or in peritumoral spaces (Mirancea and Mirancea, 2010; Mirancea *et al.,* 2014; Constantin *et al.,* 2015; Stratman and Davis, 2012), so that inflammatory blood cells become rich sources of pro-inflammatory agents, a precondition for well-known tumor status as a disease that does not heal (Dvorak, 1986).

2. SQUAMOUS CELL CARCINOMA

At the tumor-peritumoral stroma interface in the case of SCC, it was observed that while tumor mass portions penetrate inside the stroma, stromal strips penetrate inside the tumor mass (Mirancea, **Moroşanu** *et al*., 2009).

Desmosomal junctions between the adjacent tumor cells on the stroma interface are missing or precarious, and the intercellular spaces are filled with numerous fine cellular extensions (**Fig. 18**, frame **C**). The connection between desmosomes and tonofilaments of keratin is missing. Sometimes, affronted plasma membranes from two adjacent tumor cells show **plasma membrane recombination** associated of clathrin-coated vesicles (**Fig. 18**, frame **A**) (Mirancea, **Şerban** *et al.,* 2009).

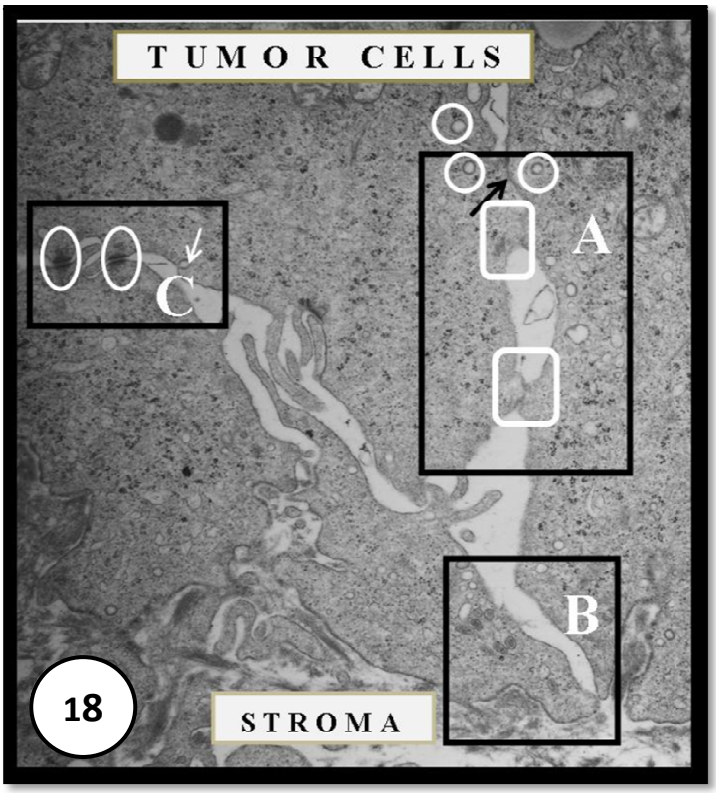

Fig. 18 – Overview of a basal pole of tumor cells affronted with each other or with peritumoral stroma. Intercellular spaces are large. Desmosomal junctions are almost lacking, but where they are present (elliptical areas in **C**), are affected, and their connection to tonofilaments is lacking. Sometimes, to some extent, plasma membranes affronted by two adjacent tumor cells exhibit membrane recombination (white fragments in **A**). Within the edge of some invadopodia there are lysosomes; hemidesmosomes are missing (frame **B**) (× 19.000). SCC (according to Mirancea, **Şerban** *et al*., 2009).

At the basal pole interface of tumor cells with peritumoral stroma, the basement membrane (BM) is missing; very rare, only small basal amorphous material peaks may be detected. Inside the invadopodia there are lysosomes. The hemidesmosomal junctions are also missing, and when present they have defects in the internal plaques so that their connection to the intermediate keratin filaments is abolished. Occasionally, precarious hemidesmosomes can be detected and ectopically located (in the basal-lateral position).

Both epithelial tumor cells and the adjacent stroma undergo obvious changes in the stromal tumor interface. The tumor cells presented the process of releasing the vesicles to their contact with stromal tissue. Within the peritumoral stroma, blood microvessels are very fragile, so that red blood cells and white blood cells can be detected in large amounts in the immediate vicinity of the tumor, even among tumor cells.

C. TELOCYTE - a particular phenotype of interstitial cell component of the tumor stroma

Given the particular interest shown by various laboratories throughout the scientific world for the recently discovered cell phenotype known as telocyte, in this thesis we have given special importance to the knowledge of ultrastructure of telocyte and its homo- and heterocellular relationships.

In the skin, telocytes are found in the immediate vicinity of collagen fibers and elastic fibers, as well as mast cells, fibroblasts, adipocytes, blood vessels, nerves and around the stem cell cluster at the base of the hair follicle („*bulge*") (Rusu *et al*., 2012, Ceafalan *et al*., 2012). Insides of both BCC and SCC telocytes are found mainly within the fibrotic peritumoral stroma. Most of the telocytes that we observed appear as bipolar cells having an ovoid nucleus with predominant eucrhomatin; heterochromatin appears predominantly attached to the inner membrane of the nuclear envelope (**Fig. 19**). The cell body together with the two cellular extensions called telopodes has about 50 µm. Often, the rough endoplasmic reticulum (RER) is located in the immediate vicinity of the nucleus, and the Golgi apparatus appears in the perinuclear region. The telopods show the alternation of dilated segments (podoms) and thin segments (podomeres) (Mirancea, **Moroşanu** *et al*., 2013).

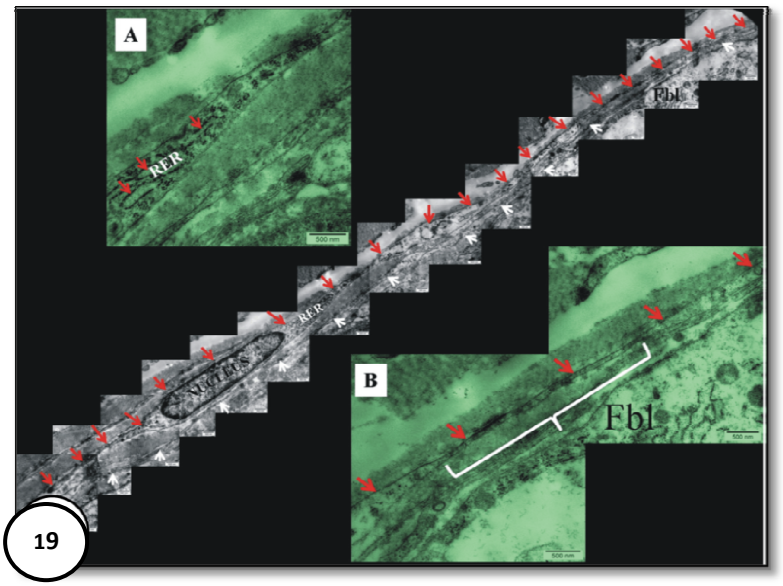

Fig. 19 - A bipolar telocyte has an ovoidal nucleus with predominant euchromatin; heterochromatin appears prevalently attached to the inner membrane of nuclear envelope. Body cell together with the two cell extensions (telopodes) (red arrows) measured approximately 50 µm; one telopode is very long. An extremely long slender telopode (white head arrows) belonging to another telocyte whose cell body (including nucleus) is not visible, runs parallel with the nucleated telocyte. Rough endoplasmic reticulum (RER) is located in close vicinity of the nucleus (A). In (B), a detail for the long telopode (red arrows) shows the alternation of dilated segments (podoms) and thin segments (podomeres) (accolade). Fbl = Fibroblast. (**A**) and (**B**) digitally colored in green. BCC (according to Mirancea, **Moroşanu** *et al*., 2013).

Sometimes, the plasma membranes of the two linked telocytes (a homocellular junction) become fused and carry out the recombination phenomenon of the plasma membranes. In the current study, we did not identify telocytes in direct contact with tumor cells or putative cancer stem cells, and telocytes appear localized at different distances from tumor cells (Mirancea, **Moroşanu** *et al*., 2013).

BCC and SCC tumor cells are capable of carrying out (1) cell-cell direct communication (a) homocellular communication (somatic / stromal synapses) or (b) heterocellular (somatic / stromal synapses between two or more adjacent telocytes), and (2) cell-cell indirect communication by microvesicle release (Mirancea, **Moroşanu** *et al*., 2013), and sometimes a telocyte can simultaneously perform homocellular junctions with another telocytes and heterocellular junctions with other cell types.

1. Cell-cell direct communications / junctions (homocellular and heterocellular)

In normal tissues, telocytes produce stromal synapses between them - homocellular junctions and other types of cells – heterotypic junctions (Popescu and Faussone-Pellegrini, 2010; Gherghiceanu and Popescu, 2012; Rusu *et al*., 2012, a, b). Intercellular junctions on the one hand, and those between cells and the extracellular matrix, play a key role in cell growth, tissue morphogenesis, cell polarity maintenance, and tissue pathophysiology, including renewal and repair (Mirancea, **Moroşanu** *et al*., 2013).

❖ Homocellular junctions

The intercellular space, which separates two telocytes involved in a somatic homocellular junction, is within the macromolecular interaction (about 10-30 nm) (Mirancea, **Moroşanu** *et al*., 2013). Some telocytes establish stromal synapses at the ends of their telepods (**Fig. 20**).

Detailed ultrastructural aspects of somatic synapses performed by end-to-end telomeres have shown that a dense electron microscopic material is attached to the internal surface of the plasma membranes of both telocytes (the molecular composition of such internal plaques) (Mirancea, **Moroşanu** *et al*., 2013). A telocyte can simultaneously develop homo- and heterocellular junctions.

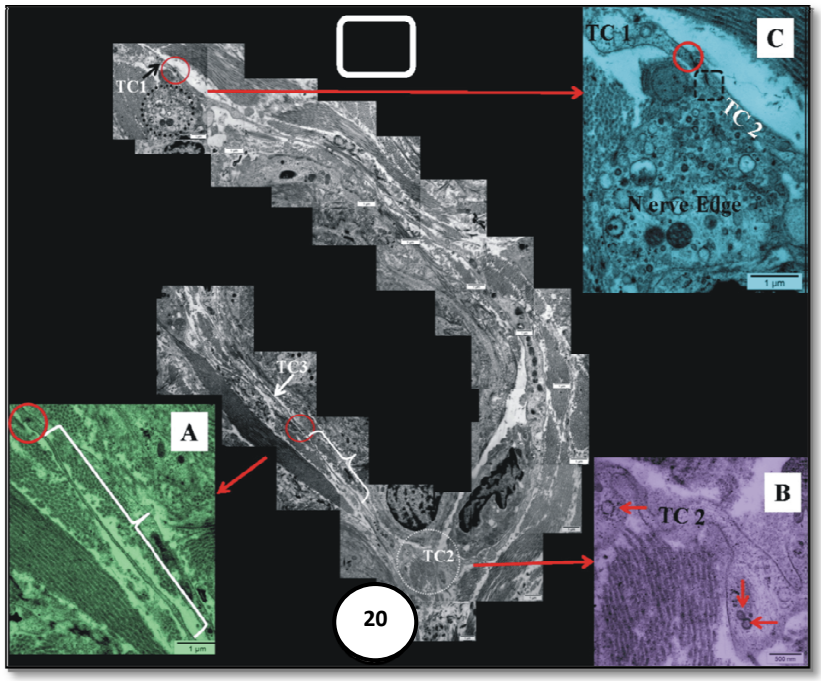

Fig. 20 - Two-dimensional (2-D) sequenced concatenation of 18 serial electron micrographs. Inside of the peritumoral dermal tissue from BCC of the skin, three telocytes are connected each other by homocellular junctions/stromal synapses (red circles areas). The nuclear body of telocytes is not visible. The longest telopode measured approx. 55 µm. In (**A**), detail for a thin telopode (accolade) of the telocyte TC2 connected by a somatic synapse (encircled area) with telocyte TC3 (digitally colored in green). In (**B**), detail for the white dotted encircled area in **Fig. 20**, showing clathrin coated vesicles (red arrows) (digitally colored in pink). In (**C**), telocytes TC2 makes synapses with TC1 and a naked nerve edge (digitally colored in blue). BCC (Mirancea, **Moroşanu** *et al*., 2013).

❖ Heterocellular junctions

Recently published papers (Gherghiceanu and Popescu, 2012; Rusu *et al.,* 2012, Popescu *et al.,* 2005) reported that telocytes form a structural tandem with mast cells. Mast cells are considered to be constituents of the conjunctive tissues and are very important players in pathophysiology due to the large amount of inflammatory mediators deposited inside the cytoplasmic granules. It seems that the lack of heterologous junctions of telocyte-mast cells in the tumor stroma may be involved in overexpression of inflammatory mediators. Limiting the heterologous cell junctions suggests a possible involvement in inducing alteration of cell-cell communications in the peritumoral stroma and subsequently throughout the tumor, which favors invasive behavior.

Ultrastructural investigations in the investigated BCC cases indicated the presence of heterotypic contacts of a telocyte with mast cells, striated muscle cells, macrophages (**Fig. 22**) and peripheral nerves or free nerve endings (**Fig. 21**). Unlike normal skin (Rusu *et al.*, 2012, Ceafalan *et al.,* 2012), we have noticed that homotypic and heterotypic cell junctions of telocytes are very limited.

The numerical deficiency of the telocytes themselves, but especially the numerical decrease of the heterotypic junctions can be assimilated to the loss of function at the level of stromal homeostasis. In cases of BCC investigated by us there is a reduction of heterotypic junctions of telocytes with fibroblasts, mast cells, endothelial cells, nerves and consequently abnormalities of skin regeneration and / or repair (**Moroşanu** *et al.,* 2013).

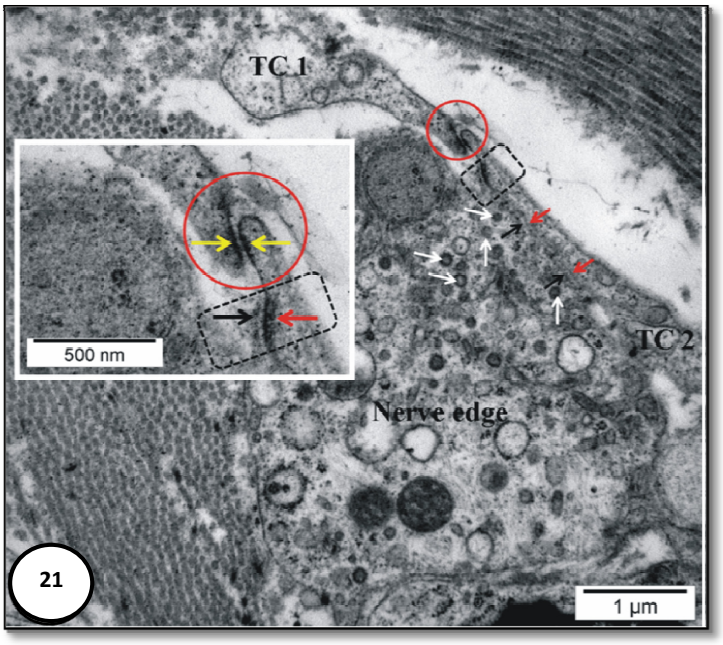

Fig. 21 - Two telocytes (TC1 and TC2) are connected by homocellular junction (encircled area). Heterocellular junctions (dotted square), including synaptic junctions between TC2 telocyte and a nerve end, are visible. Inside the nerve end, polymorphic presynaptic vesicles (white arrows) are visible. A relatively long profile of the synaptic cleft is visible as a narrow hole of approx. 30-50 nm delimited by presinaptic axolema (black arrows) and telocyte 2 plasma membrale cell (red arrows) in post-synaptic position. In the box: electronodense plaques (yellow arrows) are visibly attached to the inner face of the plasma membrane at the level of the homocellular junctions. The red arrow indicates an ectodomain plaque belonging to the telocyte, while the black arrow indicates a dense plaque attached to the plasma membrane of the nerve end. BCC (according to Mirancea, **Moroşanu** *et al.,* 2013).

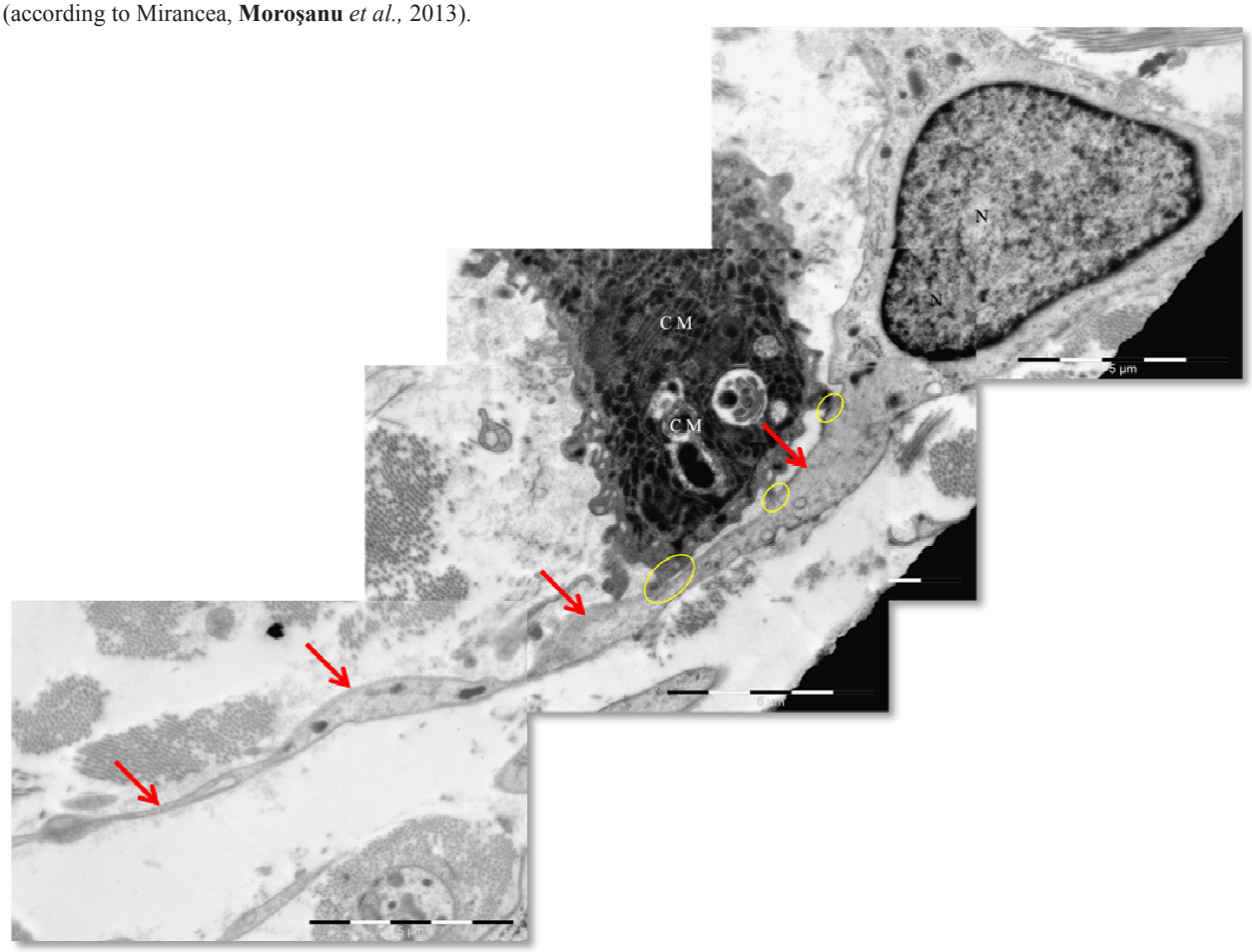

Fig. 22 - Heterotypic relationship (yellow circles) between a nucleated telocyte (N), established between its telopode and a macrophage. Sample T52. BCC.

2. Indirect cell-cell communication mediated by "*shedding membrane vesicles*"

By their telopods, telocytes release small microvesicles *("shedding membrane vesicles",* median diameter 180 nm) in the form of single or reserve membrane microvesicles (**Fig. 23**). Micro- and macromolecules released from microvesicles play a paracrine role in signaling over long distances by transmitting signals to neighboring cells (Popescu, 2011; Gherghiceanu and Popescu, 2012). Membrane microvesicles are involved in some pathophysiological processes through membrane surface trafficking and by horizontal transfer of proteins and RNAs between adjacent or neighboring cells - paracrine effects in order to make rapid changes in the phenotype required for a variety of conditions (Coccuci *et al*., 2009).

The extracellular vesicles released by telocytes can play a role in intercellular communication, cell signaling, and maintenance of tissue homeostasis (Mirancea, **Moroşanu** *et al.,* 2013; Creţoiu and Popescu, 2014).

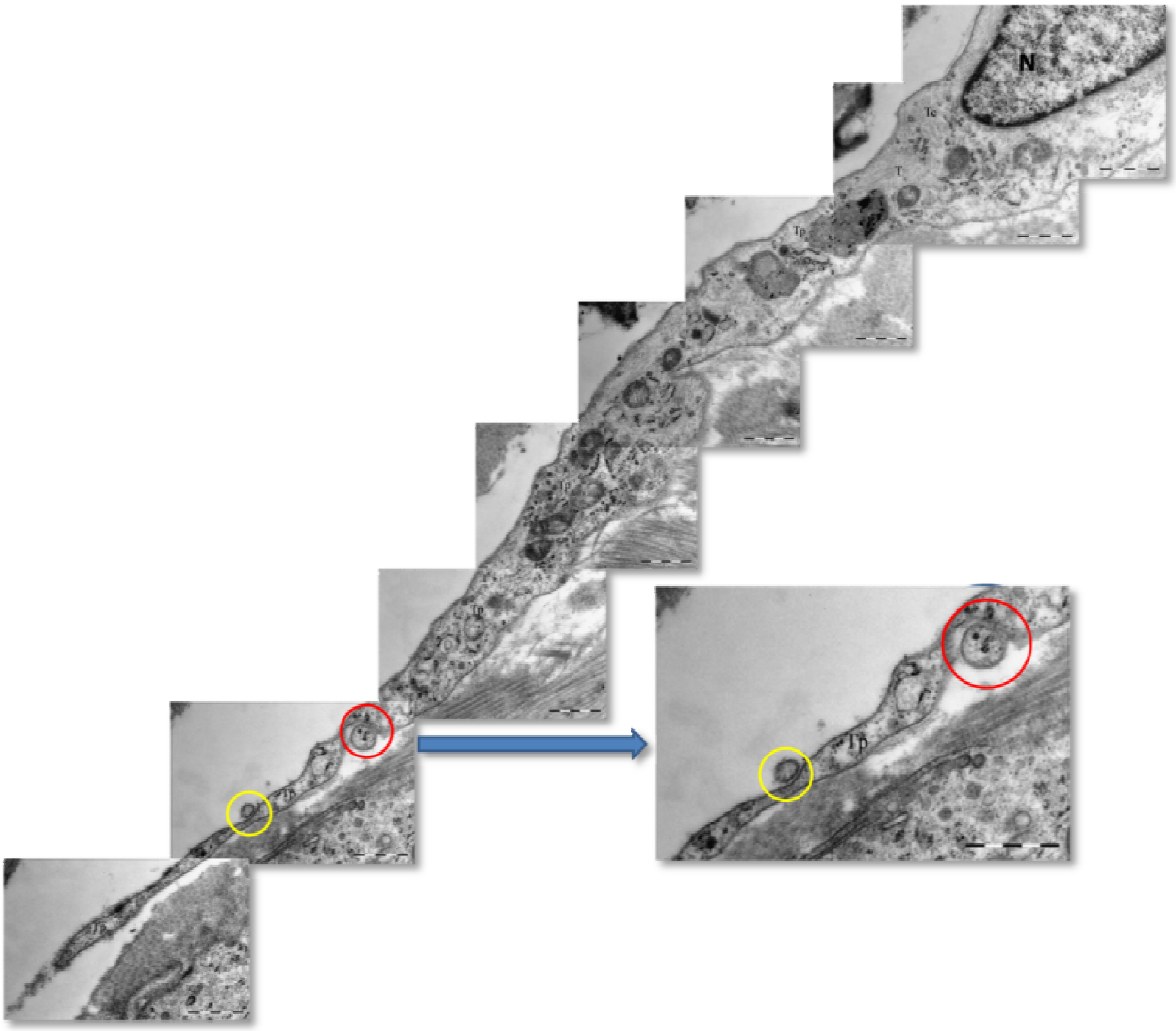

Fig. 23 - Overview of a nucleated telocyte (N) where microvesicles are seen at different stages of release in extracellular space. In detail, a vesicle is seen to be detached from the telocyte plasma membrane (red circle) and another already detached (yellow circle). Sample T52. CBC.

D. Investigation of alterations in the distribution of molecular components of the tumor-peritumoral stroma interface through IEM in BCC and SCC

In the dermal-epidermal junction zone, in normal skin, hemidesmosomes and basement membrane contribute fundamentally to the permanent maintenance of firm epidermis adhesion to the dermis.

In the present study, we paid special attention to investigating the ultrastructural alterations of hemidesmosomes and the distribution of their various molecular components in both basal cell carcinoma and squamous cell carcinoma.

For the correct evaluation of the distribution disturbances of the essential molecules comprised of hemidesmosomes, we used normal human skin specimens.

In order to evaluate the distribution of the molecular components of the hemidesmosomes, preferably the hemidesmosomal junction remnants that can still be identified in the adjacent stromal affronted area of the tumors, we have systematically followed them by evaluating the presence, ectopic distribution or total absence of the various molecules in the intracytoplasmic compartment, the transmembrane region, and the extracellular compartment (from peritumoral stroma adjacent to tumor cells).

• **Pankeratin and Keratin 14**

Immunoelectron microscopy applied to healthy human skin showed positive reactions for pankeratin and K14 of basal keratinocytes both in the upper cytoplasm and in their basal cytoplasm, which demonstrates good solidarity of the cytoskeleton in basal cell hemidesmosomes, a prerequisite for maintaining the polarity of the keratinocyte basal and normal epidermal homeostasis.

When immunoelectron microscopy was applied to malignant tumors of BCC and SCC, immune recruitment for pankeratin and K14 is absent in the basal cell cytoplasm of some cells adjacent to tumor stroma. The absence of the IEM signal for pankeratin and K14 at this level correlates with the presence of invadopodia emitted by tumor cells visible in the TEM at the adjacent stromal interface that leads to the alteration of cellular polarity, a precondition for detachment from tissue constraints and the migration of these cells.

• **Plectin / HD1**

TEM analysis has shown that hemidesmosomes are defective for the inner plaque and, knowing that plectin (also called HD1) is a keratin binding link molecule, IEM found that while in the case of healthy epidermis *in situ*, plectin (HD1) is detectable in the inner plaque of the hemidesmosomal junction, it is poorly represented (**Fig. 24**), ectopically localized (**Fig. 25**) or even absent in the case of tumor cell homologues (BCC or SCC) from the peritumoral stromal interface. Lack of plectin explains why cytoskeletic keratin filaments do not enter invadopodia, leading to depolarization of malignant cells and facilitating invasive behavior. Individual migration or cell mass is a very important process during morphogenesis, healing of wounds, as well as during aberrant

invasion and malignant metastasis (Mirancea and Mirancea, 1998; Wang *et al*., 2005; Margulis *et al*., 2006; Breitkreutz *et al*., 2009; Mirancea *et al*., 2009).

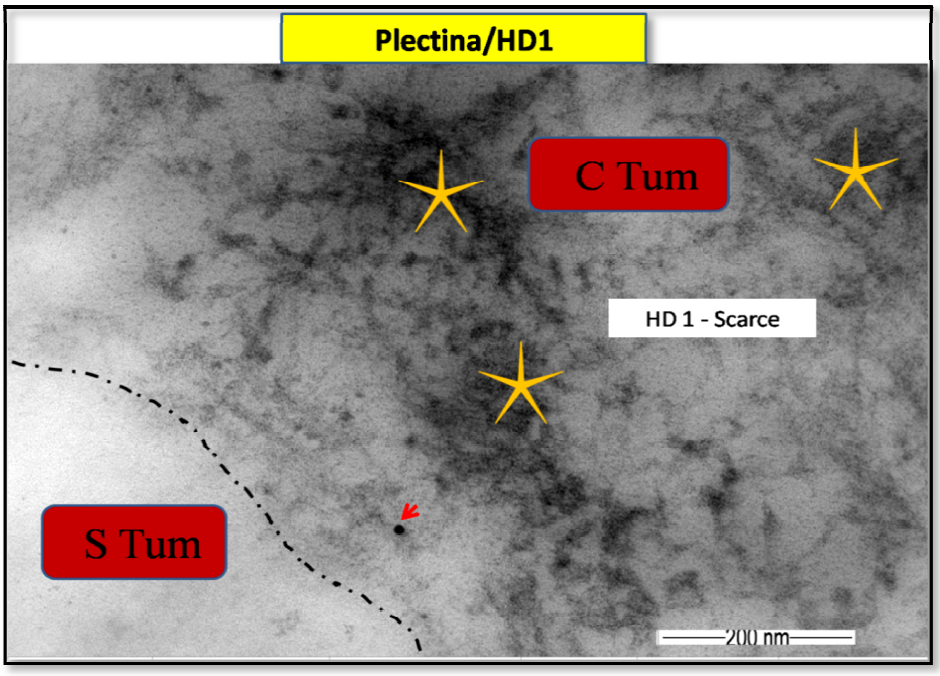

Fig. 24 - HD1 / Plectin detected by immunoelectronmicroscopy appears poorly represented, located close to the normal position (arrow). Secondary antibody conjugated to colloidal gold of 10 nm. C Tum = tumor cell. S Tum = tumor stroma. The yellow arrows mark the cytoscheletic keratin fibers. CBC (Manuscript in preparation).

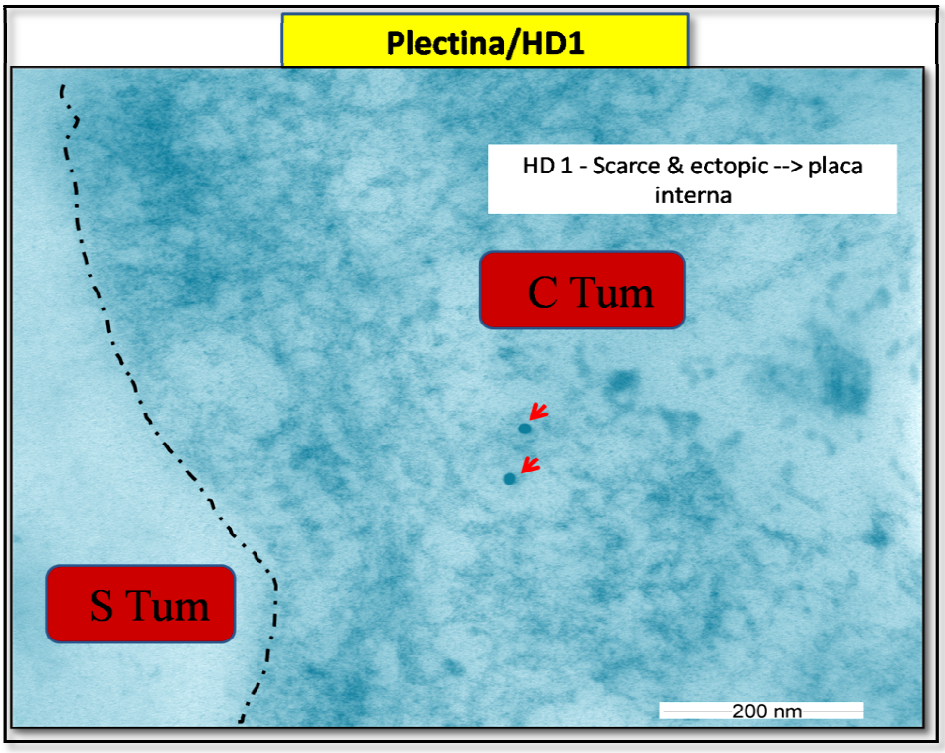

Fig. 25 - HD1 / Plectin detected by immunoelectronmicroscopy is poorly represented and ectopically localized, far away from the normal, much intracytoplasmatic position (arrowheads). Secondary antibody conjugated to colloidal gold of 10 nm. C Tum = tumor cell. S Tum = tumor stroma. The cytoscheletic keratin filaments are diffuse oriented (digitally colored with blue). CBC (Manuscript in preparation).

• **α6β4 integrin**

The ultrastructural immunodetection response for integrin concomitant for the α6 and the β4 chain at the invadopodia of SCC was negative, although, as shown in **Fig. 26**, at least on certain portions of the invadopodium, hemidesmosomes with subbasal dense plate are still distinguished. At the level of invadopodia which at the TEM level show plasma membranes dissolutions on extended portions, no remnants of hemidesmosome infrastructures can be detected, the immune reaction is totally absent.

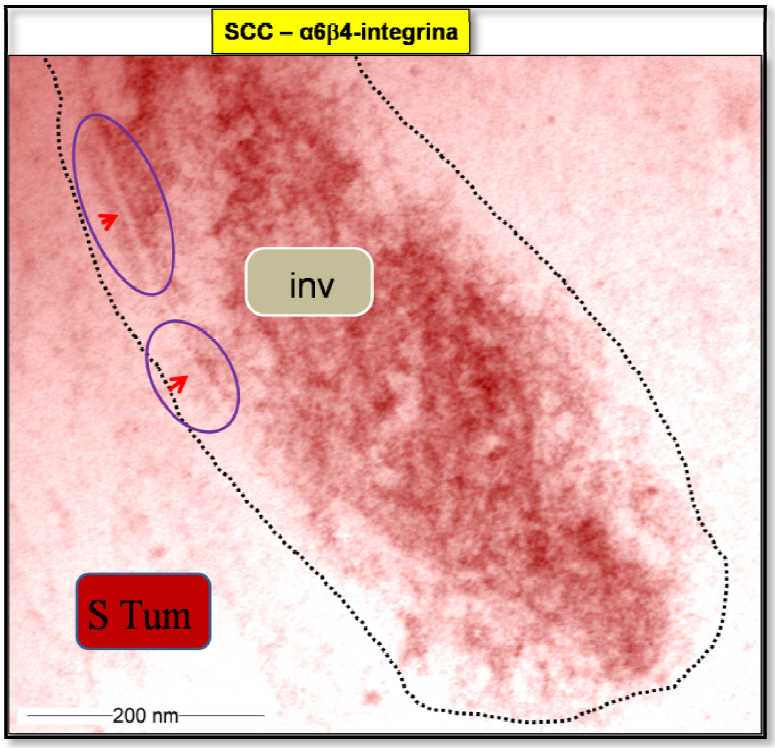

Fig. 26 - Detail of an invadopodium (inv) projected by a tumor cell of squamous cell carcinoma in the peritumoral stroma (S Tum). Plasmalem of the invadopodium is destroyed so that the boundary between this and the peritumoral stroma is illusive (the dotted curve). The immunostaining reaction for α6β4 integrin is negative because at the level of hemidesmosome residues (elliptical areas), which further distinguish the sub-basal dense plate marked by the arrowhead, no colloidal gold particle (digitally colored pink image) can be detected. SCC (Manuscript in preparation).

• **BPAG 2 (180 kDa)**

In normal human skin, BPAG 2 (180 kDa) has three segments: (1) intracytoplasmic, (2) transmembranary, and (3) extracellular that is involved in the hemidesmosomal filament composition. At the invasive growth site of BCC and SCC tumor cells, the IEM immunodetection reaction is very weak, most often totally negative.

• **Laminin V (LN 5)**

The immunodectection of laminin 5 by immunoelectron microscopy appears to be well represented as intensity and as localization in the basement membrane in normal skin but was very poorly represented, most often totally missing from the area of affrontation of invasive tumor cells with the peritumoral stroma, aspect which correlates with the absence of BM in the TEM examination.

• **Type IV collagen (Cg IV)**

The immunodetection of type IV collagen by immunoelectron microscopy appears to be well represented as intensity and as localization at the level of basement membrane in normal skin, predominantly distributed in the *lamina densa* of BM, sometimes being more deeply detected under BM. The latter aspect corresponds to the existence of small anchoring plaques for type VII collagen fibrils, as reported in the literature. The evaluation of immune response for type IV collagen in the case of BCC and SCC by IEM is similar to that obtained for laminin (it was very poor, most often missing totally from the area of affrontation of invasive tumor cells with the peritumoral stroma). Of course, this aspect correlated with the absence of BM previously described by TEM investigation.

• **Type VII collagen (Cg. VII)**

The immunostaining reaction for type VII collagen applied to normal human skin fragments gave an intense positive signal (**Fig. 27**), which shows that dermal epidermis anchoring fibers are numerous and provide strong solidity to dermal-epidermal junction zone. Different from the normal skin tissue situation, IEM immunostaining reaction of type VII collagen in tissue fragments taken from BCC or SCC tumors at the level of contact between tumor cells and tumor stroma is negative, which suggests that fiber synthesis and consolidation of anchoring represented by type VII collagen is not performed at the invasive growth front of the tumor protrusions in the adjacent stroma.

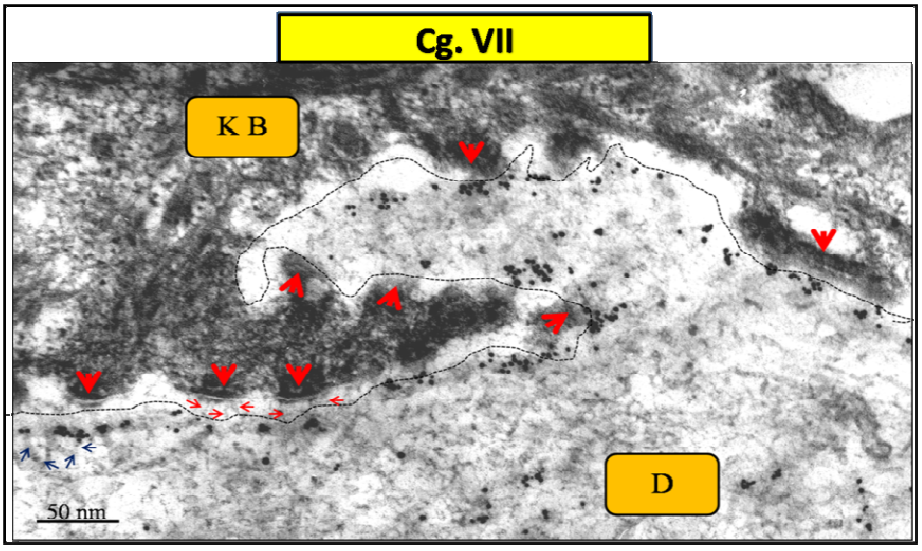

Fig. 27 – Immunostaining reation identifies type VII collagen (electronodense particles under the basement membrane that can be also seen in the deep dermis), which is the basis of basement membrane anchor fibers, basal keratinocytes (KB) in the underlying dermis (D). To amplify the signal, the indirect immune reaction method followed by the silver enhancement reaction was applied. The dotted line shows the dermal-epidermal junction. The arrowheads indicate the hemidesmosomal junctions, the red arrows mark the anchor filaments, and the blue arrows indicate the arched anchor fibers. Normal human skin (Manuscript in preparation).

From the comparative analysis of the pattern of distribution of various species of molecules at the level of dermal-epidermal junction zone in the normal human skin with that of the tumor invasive growth area, especially in the affrontation of some tumor cells with adjacent stroma, it is found that it is dramatically changed. The poor immunodetection signal that identifies quantitative

diminished synthesis or ectopic redistribution of certain proteins and glycoproteins from the hemidesmosomal junctions and BMs, or their total absence clearly demonstrates that severe invasive growth disturbances occur in the synthesis of specific molecules involved in maintaining the integrity of the hemidesmosome junctions and basement membrane.

CONCLUSIONS

The study carried out within this doctoral thesis made it possible to obtain original results on the relevant ultrastructural and molecular alterations at the tumor-peritumoral stroma interface in the case of (1) **premalignant skin lesions**, (2) **basal cell carcinoma** (BCC) and (3) **squamous cell carcinoma** (SCC).

We selected and **exhaustive analyzed electronomicroscopically specimens from patients diagnosed with invasive BCC and SCC** with the most significant ultrastructural alterations that document how tumor cells "build" and expose cell-molecular mechanisms of initiation and support of invasiveness and of migration to occupy ectopic territories (colonization of tissues distant from the primary tumor) and to generate secondary tumors (metastasis).

A. Premalignant skin lesions:

1) Ultrastructural abnormalities reported in two cases of benign lesions (seborrheic keratosis and cavernous hemangioma) in the transition to the premalignant phenotype provide an *in situ* model that allows deciphering subtle infrastructure changes accompanying malignant transformation;

2) The analysis of severe alterations of dermal-epidermal junction zone in the case of seborrheic keratosis (KS) and of epidermal epithelium dysplastic to anaplastic as an unusual phenotype in cavernous hemangioma contributes to a better understanding of the malignant transformation;

3) Nearly all keratinocytes in dermal-epidermal junction zone have lost epithelial phenotype: desmosomal junctions are damaged or cannot be detected, few cytokeratin filaments are connected to these intercellular junctions;

4) At the stroma interface no hemidesmosomal junctions can be detected, and the basement membrane is missing;

5) The capillaries are large and irregular in structure, with abnormally thin walls prone to the extravasation of the blood cells within the dermal stroma below the dysplastic to anaplastic epidermis;

6) We have identified for the first time the existence of telocytes and described the homo- and hetercellular relationships they have in the case of seborrheic keratosis and cavernous hemangiona.

B. Nonmelanomic cutaneous carcinomas (BCC and SCC)

Electron microscopic examinations of skin samples from patients diagnosed with basal cell carcinoma (BCC) showed that malignant transformed keratinocytes at the peritumoral stroma interface lost the characteristic phenotype of epidermal keratinocytes.

1) At the interface between fibrotic stroma and tumor epithelial cells, **basement membrane (BM) and hemidesmosomal junctions** are severely altered (can be detected as rudimentary type II hemidesmosomes) or are completely absent on large areas;

2) **Desmosomal junctions** are largely profoundly altered and sometimes desmosomes are internalized;

3) The coupling of intermediate keratin filaments with desmosomal plaques is abolished so that **cytoskeletic filaments** can be seen in the form of perinuclear redistributed bundles;

4) Tumor cells from the stroma interface emit **invadopodia**;

5) Electronomicroscopic investigations show that some tumor cells have **high cell membrane fragility.** This abnormal aspect is documented by numerous images that have surprised more disparities / destructions less or more extensive of malignant cells plasma membrane, especially those affronted with peritumoral stroma;

6) Our studies show that the fragility of the tumor cell membrane *in situ* can culminate in the realization of **recombinations** of tumor cell plasmalemmas with the adjacent plasmalemmas of normal stromal cells resulting in malignant-normal hybrid cells (chimeric cells);

7) We consider that the formation of **malignant-normal hybrid** cells *in situ* as a result of membrane recombinations increases the degree of heterogeneity of tumors and difficulty in skin carcinoma therapy;

8) Vesicular structures called extracellular **microvesicles** are released by tumor cells at the tumor-stroma interface;

9) Tumor-associated **capillaries** are sometimes devoid of basement membrane and pericytes, exhibit fenestrations such that extravasated inflammatory cells mix with tumor cells;

10) We have identified and characterized ultrastructural for the first time the so-called "***conduit system***" within tumors of basal cell carcinoma delimited by tumor cells;

11) The "***conduit system***" phenotype identified for the first time in basal cell carcinoma is a particular one since, different from other "*conduit systems*" reported so far in the literature in other tissue types as having a basement membrane separating the amorphous fibrilar matrix by the rest of the tissue, the one described by us does not have a visible basement membrane, the isolation of its matrix being carried out by the tumor cells that are still solidarized between them by desmosomal junctions and forming the walls thereof;

12) The presence of such a system of labyrinthic channels that appear as an extension of the peritumoral matrix (the matrix content is mainly formed from fibril collagen) within a tumor remains a

mystery. We consider that by the extension of the peritumoral matrix inside the tumor, it is provided **the supply of small-sized matrix molecules with a messaging role, supporting the growth and development of tumor cells**;

13) The **comparative examination of the ultrastructure** of tumor epithelial tumors *in situ* (basal cell carcinoma - **BCC** and squamous cell carcinoma - **SCC**) highlighted that ultrastructural alterations at the tumor-peritumor stromal interface in cases of basal cell carcinoma with invasive behavior (increased plasticity and plasmalemmal high fragility) have been found in the cases of squamous cell carcinoma investigated by us;

14) For the first time, **telocyte** - a particular stromal cell phenotype - has been identified and extensively investigated in some benign tumors with infrastructure alterations with aspect of premalignant lesions and especially in basal cell carcinoma and squamous cell carcinoma;

15) **Telocytes** are detectable as a distinct phenotype of interstitial cells present within the tumor stroma in **BCC** and **SCC**, respectively;

16) Telocytes, through their **homo- and heterocellular connections**, form a 3-D network in the tumor stroma, which may be involved in the coordination of long-distance intercellular signaling;

17) Through stromal cell synapses and the transfer of microvesicles (paracrine cell signaling) **telocytes play an important role in intercellular signaling**;

18) Compared to the normal skin condition, telocytes in BCC and SCC are capable of establishing homocellular junctions but **a very limited number of heterocellular junctions**, suggesting a possible involvement in **inducing altered cell-cell communication** in the peritumoral stroma and subsequently throughout tumor mass;

19) It is remarkable that both in BCC and SCC **telocytes are prevalently sequestred in dense fibrous tissue,** which seems to restrict the heterotypic relationships they might have with other cell types. These aspects suggest disturbances of malignant malignant epithelial tissue homeostasis, as they appear in the cases investigated in this study;

20) We prioritize our observation of r**ecombination of membranes between telocytes and other stromal cell types;**

21) **The heterotypic relationship that telocytes manifest with cutaneous mast cells** appears to be interesting since mast cells are involved in a wide range of diseases, particularly those related to fibroproliferation and neovascularization;

22) There are morphological evidence suggesting that telocytes play a role in their intercellular cell communication through mast cell contacts by **degranulation of mast cells and the release of active fibroblast growth factor into the extracellular space** of the peritumoral microenvironment.

C. Alterations in the distribution of molecular components of the peritumoral tumor tissue interface in BCC and SCC

Immunoelectron microscopy (IEM) has made it possible **to identify alterations in the pattern of distribution of different molecular species from the BCC and SCC tumors interface with the adjacent stroma.**

a. Alteration of the distribution of various molecules of the tumor-peritumoral stroma junction region: Plectin / HD1, BPAG 1 (230 kD), BPAG 2 (180 kD), α6β4 integrin, laminin, Cg IV, Cg VII) which is manifested by 1) their paucity, (2) ectopic localization or (3) total absence correlates with alterations of various cellular infrastructures, mainly hemidesmosomal, which leads to a dramatic change of cellular polarity, tendency to detach, migration and invasiveness of tumor cells in the peritumoral stroma;

b. Both transmission electron microscopy and immunoelectron microscopy investigations show that in epithelial cells affronted to the adjacent stroma, gradual alteration to the loss of cell-cell and cell-extracellular matrix junctions, ectopic localization or absence of cytoskeleton-binding molecules, and with the basement membrane lead to severe depolarization of epithelial cells, a precondition for the detachment and migration of malignant transformed cells.

In both invasive BCC and SCC cases, increased plasticity and high plasma tumor plaque that allow plasmalemal recombinations, invadopodia formation, *shedding membrane vesicles* and even focal membrane plasma dissolution correlate with ectopic location or absence of hemidesmosomal (e.g., HD1 / Plectin and BPAG1) linker molecules with the cytoskeleton or basement membrane (e.g., BPAG2, α6β4 integrin, collagen type VII). All of these changes, as well as the absence of basement membrane, contribute to cellular depolarization and increase the invasive capacity of tumor cells within the peritumoral stroma.

Identification and description of extracellular vesicle-generated infrastructures from tumor cells and peritumoral stroma telocytes contribute to the strengthening of the hypothesis that by their molecular epigenetic load content they can contribute to the horizontal transfer of information for the generation and maintenance of malignancy of tumors BCC and SCC.

The original results obtained in this fundamental scientific research study contained in the PhD thesis contribute to the intimate knowledge of cellular and molecular alterations occurring at the tumor-peritumoral stroma interface with involvement in the manifestation of the invasive behavior of malignant transformed cells.

Some of the original results obtained have been published in 5 scientific papers and are visible through quotations from the international scientific literature, and another part is included in 2 manuscripts for publication.

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