Investigation of molecular mechanisms playing role in the progression of breast cancers - the complex role of syndecan-4 in MCF-7 cells

PhD thesis

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Budapest
2010

INTRODUCTION

Numerous markers are applied routinely to predict the agressivity of breast carcinomas. Syndecan-1 was reported as a prognostic factor in breast cancers, but the role of syndecan-4 is less known.

Syndecans are type-I transmembrane proteoglycans. Extracellularly they interact mostly with matrix proteins, growth- and other factors attached to their glycosaminoglycan chains. Among the 4 family members syndecan-4 was reported as the component of focal adhesions (Figure 1.), which are the cell-extracellular matrix contact points. The short cytoplasmic domain of syndecan-4 has been shown to interact with wide variety of compounds and proteins including protein kinase C-alpha (PKC-alpha), PIP2, PDZ domain containing proteins, FERM proteins, alpha-actinin or dynamin II.

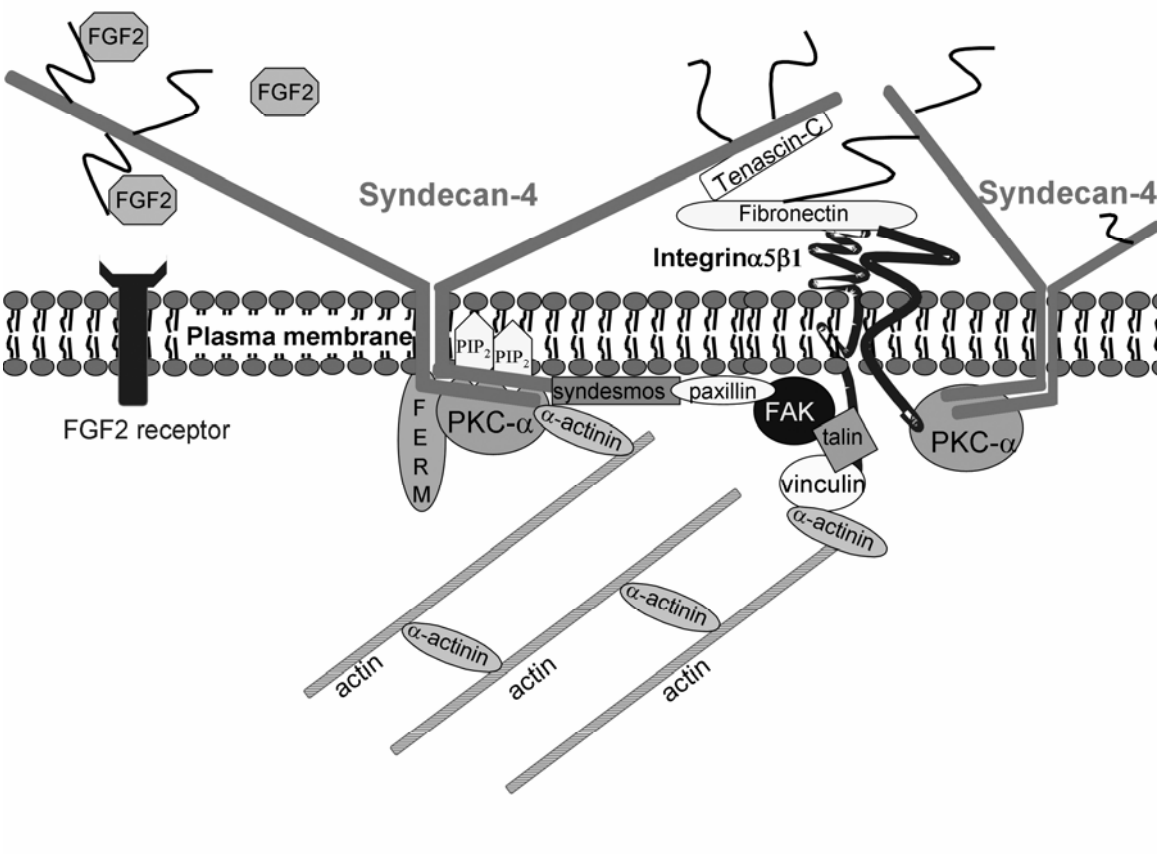

Figure 1. Interacting partners of syndecan-4.

Syndecan-4 involved directly in signal transduction super-activating PKC–alpha, and it can regulate negatively the activity of small GTP-ase Rac1. The cytoplasmic Ser179 of syndecan-4 has important regulatory role, its phosphorylation reduces the PIP2 binding affinity and consequently the activation of PKC-alpha. The small G protein Rho GTP-ases regulate many essential cellular processes, including actin dynamics, cell adhesion, migration and epithelial polarization. They have two conformational states: an inactive, GDP-bound, and an active, GTP-bound state. The guanine nucleotide exchange factors (GEFs) turn on their signalling by replacing the bound GDP from the inactive form with GTP.

The loss of cell-cell junctions can associate with the formation and progression of tumors. Polarity complexes play a role in the development of cell-cell junctions and epithelial polarization. Gene amplification and protein overexpression of the EGF family member HER-2 (ErbB2) associates with poor prognosis of breast cancers. It has been demonstrated, that the activation of HER-2 leads to loss of polarity, since it disrupts the Par polarity complex.

AIMS

During our experiments we intended to investigate the biological function of syndecan-4 and its role in signal transduction in MCF-7 breast adenocarcinoma cells. During our preliminary experiments, performed with different mutant and green fluorescent protein tagged syndecan-4 constructs, we found that the phosphorylation of syndecan-4 influences the phenotype of MCF-7 cells; furthermore, during the immunocytochemical characterization of the syndecan-4 constructs we observed the transmembrane syndecan-4 in the cytoplasm and nucleus.

Based on preliminary experiments our aims were as follows:

1. Characterisation of migrating and epithelial phenotypes induced by the mutations of Ser179 of syndecan-4, investigation of their effect for the actin cytoskeleton and focal adhesions, and unravelling of the biochemical changes due to the different phenotypes.
2. Characterization of the endocytosis and nuclear translocation of syndecan-4.
3. Investigation of the role of Ser179 phosphorylation of syndecan-4 during the M phase of the cell cycle, particularly during the cytokinesis.
4. Furthermore, we aimed to compare the methods used in determination of the prognostic and predicitive HER-2 status of breast carcinomas.

METHODS

Human syndecan-4 constructs

The human syndecan-4 cDNA was isolated by PCR technique and cloned into pCMV vector (Clontech). The green fluorescent protein (GFP) tag was inserted into the extracellular domain of syndecan-4 (wt-SDC4-GFP) to monitor the subcellular localization of syndecan-4 *in vivo*. The Ser179 syndecan-4 was mutated to phosphoresistant Ala (Ser179Ala) or phosphomimetic Glu (Ser179Glu) by PCR-based method in the pCBD construct. Next, truncated forms of SDC4 were generated with deletion of a part of the ectodomain representing the shed remnant.

Cell culture and transfection

The experiments were performed on MCF-7 breast adenocarcinoma cells, for certain control experiments we used HT1080 fibrosarcoma and C2/7 mouse myoblast cell lines. The plasmids were introduced to the cells by FuGene transfection reagent.

Investigation of cell migration

To investigate the cell migration wound-scratch assay was performed at 80% confluency on cells seeded on 6-well plate. The cultures were scratched, and phase contrast images were taken every 12 hours. The individual and collective migration of cells seeded on fibronectin was monitored by time-laps phase contrast microscopy.

The endocytosis of syndecan-4

The endocytosis of syndecan-4 was induced by phorbolester PMA (1 μM) in wild type syndecan-4-GFP expressing cells, and time-laps images were taken with confocal microscope at room temperature.

Immunocytochemistry and fluorescence microscopy

After fixation, permeabilization and blocking the cells were incubated with the appropriate primary and the fluorophore-conjugated secondary antibodies. After nuclear staining and mounting the cells were studied with Zeiss Axio Imager and Nikon Eclipse 600 fluorescent microscopes. The confocal images were taken with Bio-Rad MRC-1024 confocal microscope.

Western blot

The lysates of the cells were separated by SDS-PAGE and blotted to PVDF or nitrocellulose membrane. After blocking the membranes were incubated with the primary antibodies, followed by washing and incubation with the HRP-conjugated secondary antibodies. The peroxidase activity was visualized by the ECL procedure on X-ray films. The films were digitalized and analyzed.

Co-immunoprecipitation

The cell lysates were incubated with the antibody of interest, followed by the addition of protein A/G slurry. After incubation the eluted immunocomplex was analysed by Western blot.

Rac activity assay

Rac activity was estimated by applying Rac-activation kit (Upstate). The kit utilizes the p21-binding domain of PAK-1, bound to agarose beads, which specifically binds to and precipitates Rac-GTP from cell lysates. The cells were lysed, the lysates were incubated with PAK-PBD fused to agarose beads; the amount of bound active Rac1-GTP was detected by Western blotting.

Flow cytometry

The collected cells were washed, resuspended and fixed by addition of ethanol. The fixed cells were incubated with propidium iodide and flow cytometry was performed on Becton&Dickinson FACS Calibur and the data were analyzed by ModFit software.

Synchronization of cell cycle

The division of the cells was synchronized at the G1/S boundary by hidroxiurea (1,5 mM, 16h). At selected times after removing the hydroxyurea containing media the cells were prepared for flow cytometry or for Western blot analysis.

Cellular fractionation

The cells were lysed in a buffer without any detergent, after centrifugation of the cellular debris the supernatants representing the total cell lysates were ultracentrifuged (120000 *g*). After ultracentrifugation the supernatant corresponded to the cytoplasmic fraction, whilst the resuspendation of the membrane pellett resulted in membrane fraction. The distribution of the proteins between the different fractions was analysed by Western blot.

Immunohistochemistry

Sections were deparaffinized, followed by antigen retrieval. Following incubation with the primary antibody, the secondary antibody and the avidin-biotin peroxidase complex were applied. The chromogen substrate was VIP or DAB. Each slide was counterstained with haematoxilin, the reactions were scored on a 0 to +3 scale.

Determination of HER-2 gene amplification

Tissue sections were deparaffinized, digested in lysis buffer, followed by the isolation of DNA. Quantitative PCR (qPCR) was performed with the LightCycler-HER-2/neu DNA Quantification Kit according to the manufacturer's instructions. The calculation of the relative amounts of HER-2 DNA compared to the reference gene DNA was done by Relative Quantification Software. A ratio above 2.0 was regarded as being positive for HER-2 amplification.

RESULTS

To investigate the role and the subcellular localization of syndecan-4 *in vivo,* green fluorescent protein tagged constructs of syndecan-4 were generated. GFP was inserted into the extracellular domain to avoid the interference with the natural interacting partners of the cytoplasmic domain. The phosphorylation of the cytoplasmic Ser (Ser179) has regulatory role in the signal transduction by binding PIP2 and activating PKC-alpha. Therefore, we generated phosphoresistant (Ser179Ala) and phosphomimicking (Ser179Glu) mutants. The wild type and Ser179Ala syndecan-4 accumulated in the plasma membrane and intracellular vacuoles of interphase cells. In contrast, Ser179Glu mutant was hardly detected in the plasma membrane, it localized mainly in the cytoplasm.

1. Characterisation of migrating and epithelial phenotypes induced by the mutations of Ser179 of syndecan-4, unravelling of the biochemical changes due to the different phenotypes

Syndecan-4 influences the phenotype of MCF-7 cells in phosphorylation-dependent manner

The phosphorylation of Ser179 of syndecan-4 influences the migration of MCF-7 cells (Figure 2.). The non-transfected, wt-SDC4-GFP and Ser179Ala syndecan-4 expressing MCF-7 cells represented epithelial morphology. On the contrary, the Ser179Glu syndecan-4 expressing cells exhibited a less uniform organization, scattered evenly on the flask and showed migratory phenotype. The Ser179Glu mutation resulted individual migration of MCF-7 cells, which was confirmed by wound scratch assay and phase contrast time-laps images. Immunocytochemical results showed the presence of actin stress fibers and focal adhesions characteristic for migrating cells. At the same time, the epithelial polarization enhanced in the phosphoresistant Ser179Ala cells, and the junctional actin belt was developed.

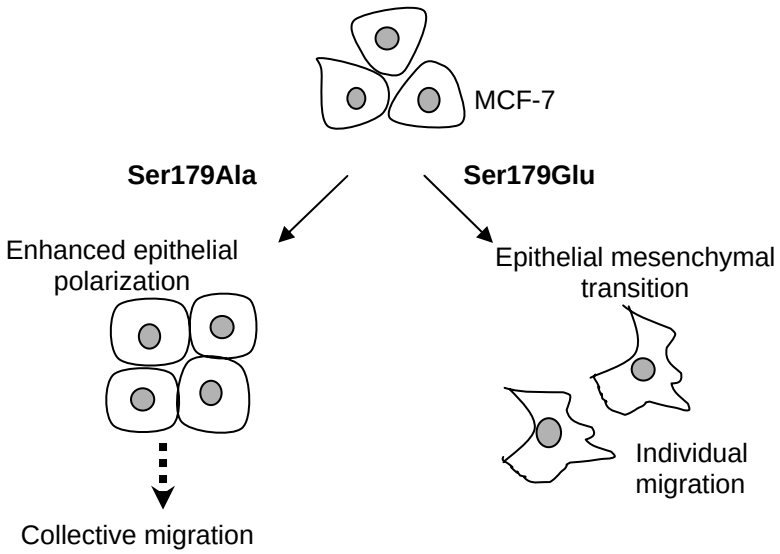

Figure 2. The mutations of Ser179 lead to phenotypical changes of MCF-7 cells. The phosphoresistant Ser179Ala mutation leads to enhanced epithelial polarization, whilst the phosphomimetic Ser179Glu mutation induces the individual migration of quiescent MCF-7 cells. The phorbolester PMA treatment of Ser179Ala cells resulted in collective migration, the cell-cell contacts remained intact.

Stimulation with PMA generated opposing responses in the different cell lines. The originally epithelial cells (wt-, and Ser179Ala) became migratory, single cells detached from the clusters; while tightly adhered Ser179Ala cells moved collectively in clusters upon PMA stimulation. On the contrary, the migrating Ser179Glu cells stopped migration and epithelial phenotype was observed.

Effect of phosphorylation of syndecan-4 for the composition of focal adhesions

In the Ser179Ala cells small focal complexes could be detected by anti-focal adhesion kinase (FAK) and anti-vinculin antisera, respectively. Immunostaining of the Ser179Glu line by anti-FAK antiserum visualized well-developed focal adhesions, but immunoassay with anti-vinculin failed to stain any of those. However, PMA treatment in Ser179Glu cells resulted in the formation of well-developed, vinculin containing focal adhesions. According to our results, the lack of vinculin does not concern alone for the existence of focal adhesions; however, vinculin is a widely used focal adhesion marker.

Effect of syndecan-4 for the activity of Rac1

The Rho GTP-ases (Rac, Rho, Cdc42) play central role in cell migration and polarization of epithelial cells. According to previous papers, syndecan-4 influences the activity of Rac1; but it is not known whether the phosphorylation of Ser179 can influence it.

In the Ser179Ala cells the level of Rac1-GTP increased to 1.9 ± 0.15-fold compared to that of the wt-syndecan-4 line, considered as basis. On the contrary, in the Ser179Glu line the Rac1-GTP decreased to 0.6 ± 0.1-fold compared to the basis. PMA influenced the migration of the cells; therefore, the level of Rac1-GTP was checked in PMA treated lines, too. Upon PMA stimulation (1μM, 30 min) the Rac1 activity decreased to 0.25 ± 0.05-fold and 0.4 ± 0.08-fold in the wt- and Ser179Ala syndecan-4 cells compared to that of the non-treated wt syndecan-4, respectively. However, the originally low level of Rac1-GTP of the Ser179Glu cells raised almost to the value of the non-treated one.

Syndecan-4 regulates cell-matrix adhesion and cell-cell contacts via determination of activity of Rac1. The phenotypical alterations were followed by changes in the actin cytoskeleton characteristic for migrating and polarized epithel cell. The reduced activity of Rac1 associated with migrating phenotype, whilst its increment enhanced the epithelial polarization.

Investigation of the mechanism of regulation of Rac1 activity by syndecan-4

Tiam1 is a type II PDZ domain containing, Rac-specific guanine nucleotide exchange factor (GEF), which promotes the formation of active GTP-bound Rac1. According to our results, Tiam1 interacts with syndecan-4. We investigated the interaction of Tiam1 and Rac1 in different cell lines. In the lysates of cells expressing Ser179Glu mutant the Tiam1-Rac1 interaction reduced markedly unlike in wt-SDC4-GFP and Ser179Ala lines. The phosphorylation of Ser179 syndecan-4 inhibited the interaction of Tiam1 and Rac1, hereby decreased the level of active Rac1.

Syndecan-4 influences the localization of Par6

Par6, is a member of the Par-polarity complex, contains PDZ domain, like Tiam1. Therefore, it raised the question whether it interacts with syndecan-4 bearing type-II PDZ binding site. All the three syndecan-4 variants (wt-SDC4-GFP, Ser179Ala and Ser179Glu) were co-immunoprecipitated with anti-Par6 antiserum. Since the Ser179 phosphorylation of syndecan-4 influences its intracellular distribution; therefore, syndecan-4 influenced the distribution of Par6 in phosphorylation-dependent manner.

2. Characterization of the endocytosis and nuclear translocation of syndecan-4

The **endocytosis** of the transmembrane heparan-sulfate proteoglycan syndecan-4 can be stimulated by the phorbolester PMA, FGF2 or by antibody recognizing the ectodomain of syndecan-4. We followed the endocytosis of syndecan-4 by confocal

microscopy *in vivo*. Five minutes after the induction syndecan-4 could not be observed in the plasmamembrane, and 2 hours later accumulated asymmetrically around the nucleus. According to the immunocytochemical studies, following endocytosis syndecan-4 can be observed in the early endosomes, and it can reach the perinuclear region through the *trans*-Golgi and *cis*-Golgi network by a retrograde transport.

During our experiments, we observed the **presence of syndecan-4 in the nucleus** as well, in the interchromatin region, in the nuclear speckles. The nuclear accumulation surmises the presence of a nuclear localization signal (NLS), which consists of basic amino acids usually. The RMKKK sequence in the cytoplasmic domain of syndecan-4 can be a potential nuclear localization signal. We investigated how the phosphorylation of syndecan-4 could influence the nuclear localization of syndecan-4. We certified by mutational analysis that only the acidic mutant (Ser179Glu) construct can enter the nucleus, the basic mutant (Ser179Ala) not. Therefore, the cytoplasmic domain of syndecan-4 contains a Ser179 phosphorylation-dependent, inducible nuclear localization signal. The factors, molecules bound to the core protein or heparan sulfate chains of syndecan-4 transported to the nucleus, as well.

3. *Investigation of the role of Ser179 phosphorylation of syndecan-4 during mitosis, particularly during the cytokinesis*

Syndecan-4 is present in the intercellular bridge

During mitosis following nuclear division an invagination of the plasma membrane (cleavage furrow) is created, which proceeds until the cytoplasm is constricted to a narrow intercellular bridge, with a dense structure in the middle defined as midbody, connecting the prospective daughter cells. In the final stage of cytokinesis, termed abscission, the ICBs are ultimately resolved.

In our experiments we demonstrated the accumulation of phospho-Ser179 syndecan-4 along the mitotic spindle, it was present in the intercellular bridges and at

the midbody. We proved its presence by mutational analysis, as well. The phosphomimetic Ser179Glu mutant accumulated in the intercellular bridges and at the midbodies, whilst the Ser179Ala mutant was not detected there.

The fluctuation of Ser179 phosphorylation and shedding of syndecan-4 during the cell cycle

The extracellular domains of syndecans can be released from the cell surface by a proteolytic cleavage in a process known as ectodomain shedding. Before and during mitosis normally adherent cells round up and reorganize their adhesive interactions with the extracellular matrix; executing cytokinesis the cells start reattaching and spreading again. Ectodomain shedding of syndecan-4 can help rounding-up with breaking the cell-matrix adhesion.

Based on the experiments performed on synchronized cells we established that both the phosphorylation and shedding of syndecan-4 change in cell cycle-dependent manner. The phosphorylation of syndecan-4 enhanced in G2/M phases, and simultaneously the ectodomain shedding elevated, by the completion of mitosis both decreased to a hardly detectable value.

In the case of phosphomimetic mutant the ectodomain shedding could not be observed, therefore we can state that the phosphorylation of Ser179 is the prerequisite of ectodomain shedding.

The effect of phosphorylation of Ser179 of syndecan-4 for the cytokinesis

The phosphoresistant Ser179Ala mutation enhanced the frequency of multinucleated cells, whilst the phosphomimetic Ser179Glu mutation delayed the abscission. The extended length of ICBs could be observed frequently in Ser179Glu mutant cells. The average length of ICBs of Ser179Glu cells (n=100) was extended 4.5-fold (45.4 ± 18.28 μm) compared to that of the wt-SDC4-GFP (9.8 ± 4.23 μm).

4. *Comparison of methods used in determination of HER-2 status of breast carcinomas*

The amplification of the HER-2 gene and overexpression of the protein were associated with poor prognosis in breast tumors. Two most commonly used methods for testing HER-2 status are immunohistochemistry, which measures semi-quantitatively the expression of the HER-2 protein, and fluorescent or chromogenic in situ hybridization (FISH or CISH), which detects amplification of the HER-2 gene. According to the algorithm in the routine pathology the 0 and 1+ cases by immunohistochemistry are considered to be negative, the 3+ cases are positive, in these cases the in situ hybridization is not necessary. On the contrary, in the cases of 2+ samples in situ hybridization must be performed. During the experiments our aim was to compare the results of the highly sensitive and quick qPCR method with immunohistochemistry and FISH. A total of 210 breast carcinomas were examined. In 45 cases immunohistochemistry as well as FISH and qPCR were performed. Analyzing the results of FISH and qPCR in 36/45 cases both methods showed the same results: 20 cases were negative and 16 positive. In 9 cases FISH and qPCR results were discordant. Based on our results using qPCR for the detection of clinically relevant HER-2/neu gene amplification, we suggest the cut-off level of the gene amplification ratio to define at least above 2.7.

CONCLUSIONS

- **We succeeded in generating functional green fluorescent protein tagged constructs of transmembrane proteoglycan syndecan-4** for investigating the biological behaviour and endocytosis of syndecan-4.
- **Syndecan-4 influences the level of Rac1-GTP in phosphorylation-dependent manner:** the phosphoresistant Ser179Ala mutation enhanced, whilst the phosphomimetic Ser179Glu mutant form decreased it.
- **The Ser179 phosphorylation of syndecan-4 inhibits the interaction of the Rac1 activator Tiam1 and Rac1,** hereby diminished the level of active Rac1.
- **The phosphorylation of Ser179 syndcan-4 regulates the transition between the epithelial and mesenchymal phenotypes.** The phosphomimetic Ser179Glu mutation induces individual cell migration, whilst the phosphoresistant Ser179Ala mutation enhances the epithelial polarization**.** The reduced activity of Rac1 associated with migrating phenotype, whilst its increment enhanced the epithelial polarization.
- **Phorbolester PMA treatment induces collective migration of Ser179Ala cells.** The cell-cell contact remain intact during migration, the cells migrate in a cluster.
- **The phosphorylation of Ser179 of syndcan-4 influences the presence of vinculin in the focal adhesions.** In the case of Ser179Glu mutation the focal adhesion marker vinculin was missing from the focal adhesions, but the presence of focal adhesion kinase was not influenced.
- **The cytoplasmic domain of syndecan-4 contains an inducible nuclear localization signal.** After endocytosis the transmembrane, heparan-sulfate proteoglycan syndecan-4 can reach the perinuclear area through the *trans*-Golgi and *cis*-Golgi network by **retrograde transport.** The phosphorylation of Ser179 of syndecan-4 can induce the development of a nuclear localization signal.

- **Syndecan-4 influences the cytokinesis in phosphorylation-dependent manner.** The phosphoresistant Ser179Ala mutation enhanced the frequency of multinucleated cells, whilst the phosphomimetic Ser179Glu mutation delayed the abscission. The phosphomimetic mutant form accumulated along the mitotic spindle, and enriched in the intercellular bridges and at the midbodies.
- **The phosphorylation and ectodomain shedding of syndecan-4 fluctuate in a cell cycle-dependent manner.** As the cell cycle progressed, the phosphorylation and shedding of syndecan-4 enhanced in G2/M phases, and at the end of the mitosis it decreased.
- **The shedding depends on the phosphorylation state of the Ser179, only the phosphorylated form can undergo shedding.** The shedding of phosphoresistant Ser179Ala mutant was abrogated.
- **Syndecan-4 can be a potential prognostic marker in breast carcinomas beside the routinely used markers (e.g.: HER-2). The quantitative PCR is an applicable technique for the determination of HER-2 status in breast carcinomas.** Important to determine the level of the clinically relevant gene amplification.

LIST OF PUBLICATIONS

Publications providing the basis of the dissertation:

Keller-Pinter A, Bottka S, Timar J, Kulka J, Katona R, Dux L, Deak F, Szilak L. (2010) Syndecan-4 promotes cytokinesis in a phosphorylation-dependent manner. Cell Mol Life Sci. (accepted, doi: 10.1007/s00018-010-0298-6)
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Letoha T, **Keller-Pintér A**, Kusz E, Kolozsi C, Bozsó Z, Tóth G, Vizler C, Oláh Z, Szilák L. (2010) Cell-penetrating peptide exploited syndecans. Biochim Biophys Acta Biomembranes. (accepted, doi: 10.1016/j.bbamem.2010.01.022)
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Kulka J, Tőkés AM, Kaposi-Novák P, Udvarhelyi N, **Keller A**, Lotz G, Schaff Z. (2006) Detection of HER-2/neu Gene Amplification in Breast Carcinomas Using Quantitative Real-time PCR – A Comparison with Immunohistochemical and FISH Results. Path Oncol Res. 12: 197-204.
IF: 1,241

International patents related to the subject of the dissertation:

„Intracellular targeting of molecules”
PCT WO 2008/010162
inventors: Szilak L, **Keller-Pinter A**, Timar J, Letoha T

„Syndecan-4 a modulator of Rac1-GTP”
Bejelentett nemzetközi szabadalom, ügyszám P0800464
inventors: Szilak L, **Keller-Pinter A**, Timar J, Letoha T

Abstracts:

Kulka J, Tőkés AM, Udvarhelyi N, **Keller A**, Pekár M, Schaff Z. (2004) Detection of Her2/neu gene amplification in breast carcinomas using quantitative real time PCR. Comparison with immunohistochemical and FISH results. Eur J Cancer, 2 Suppl: 101.

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Keller-Pinter A, Mendler L, Dux L. (2009) Heparan sulfate-dependent interaction of myostatin with syndecan-4. Neuromuscul Disord, 19: 550.

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