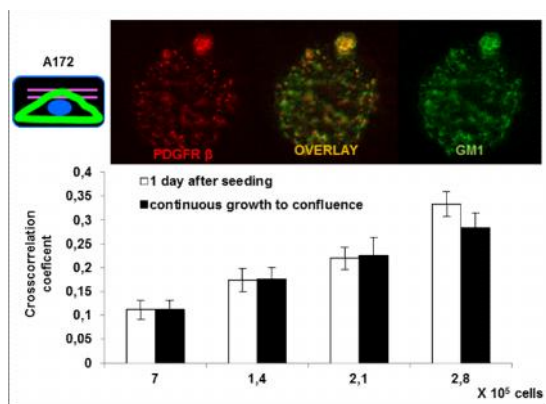
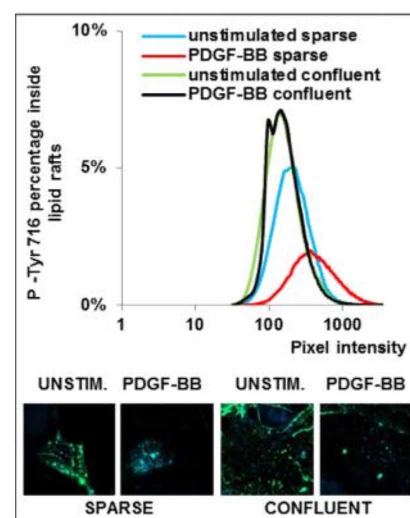
Final Report / OTKA 75752

Preamble

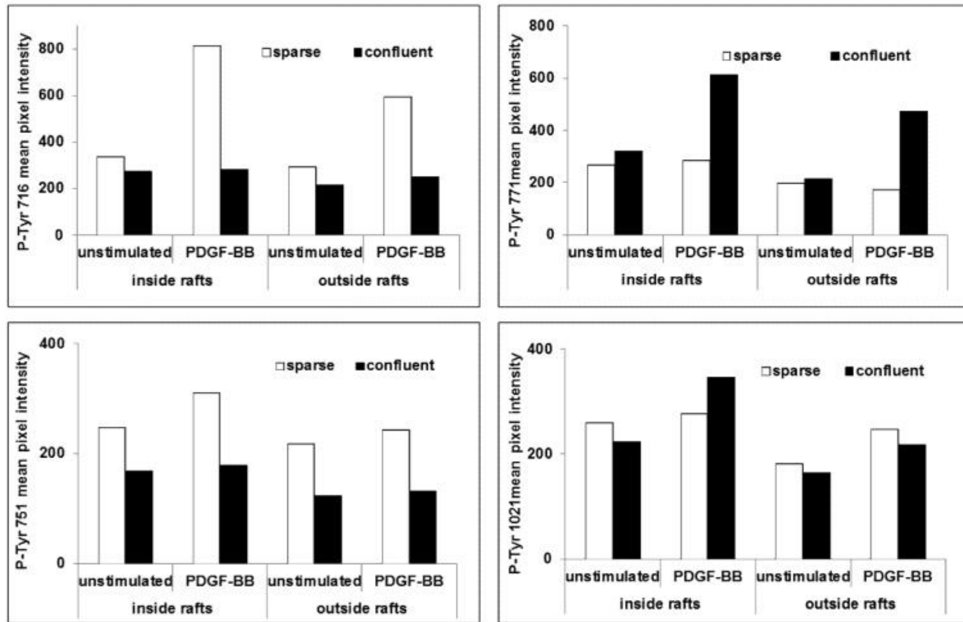
Platelet derived growth factor receptors (PDGFR) are receptor tyrosine kinases (RTK) that play an important physiological role in haematopoesis, embryogenesis, tissue homeostasis, physiological inflammatory reactions and wound healing. They also, along with other RTKs, have been implied in the proliferation and survival of tumor cells. Human glioblastoma cells express relatively high amounts of PDGF-BB as well as PDGFR that is constitutively activated by PDGF-BB in an autocrine loop. According to our earlier results, these cells, when confluent in cell culture, respond to PDGF-BB with a two-phase calcium transient. In contrast to confluent cells, cells that are sparse in culture exhibit mostly short spikes with lower peaks, hardly any influx, or no transients at all, which is not the result of altered cell cycle composition (e.g. G0/G1 block in confluence). Thus, signaling by the PDGFR appears to be regulated by cell confluence. Our own preliminary data also showed that the membrane expression of PDGFR increases with cell confluence, and that the majority of these receptors are localized to GM1-positive lipid rafts. The organizing role – both separating and collecting molecular species – of lipid rafts in signal transduction is now widely accepted. In this OTKA supported research we have undertaken to examine in some detail the as yet little-known confluence dependent regulation of receptor tyrosine kinases (PDGFR being one main target), with special respect to the role of lipid rafts. The planned work packages have been followed through, and the following main results have been achieved.

1. For characterizing the activation state and amenability to activation by PDGF-BB of the PDGFR as a function of localization and cell confluence, the system of A172 glioblastoma cells was explored in detail. Dependence of receptor expression on cell culture confluence (density), age, serum concentration was titrated. Most significant differences were found at day 2, or at 5x difference in cell density. No difference was found between same-age, different density cultures and cultures that were started with the same initial density and grown for different times. While one hour serum starvation maximized receptor expression, longer starvation periods variably upset cell metabolism. Under the same optimized conditions differential localization of PDGFR to GM1 rich domains was confirmed and quantitated. Labeling the various phosphorylated forms of the receptor in situ was optimized for the PI3K, Grb2/SOS, RasGAP, and PLC binding sites. A confocal microscopy/digital image processing method was developed for quantitating in-raft and outside raft relative phosphorylation of the different residues and also for determining the distribution of the total amount and of the phospho forms of the receptor.



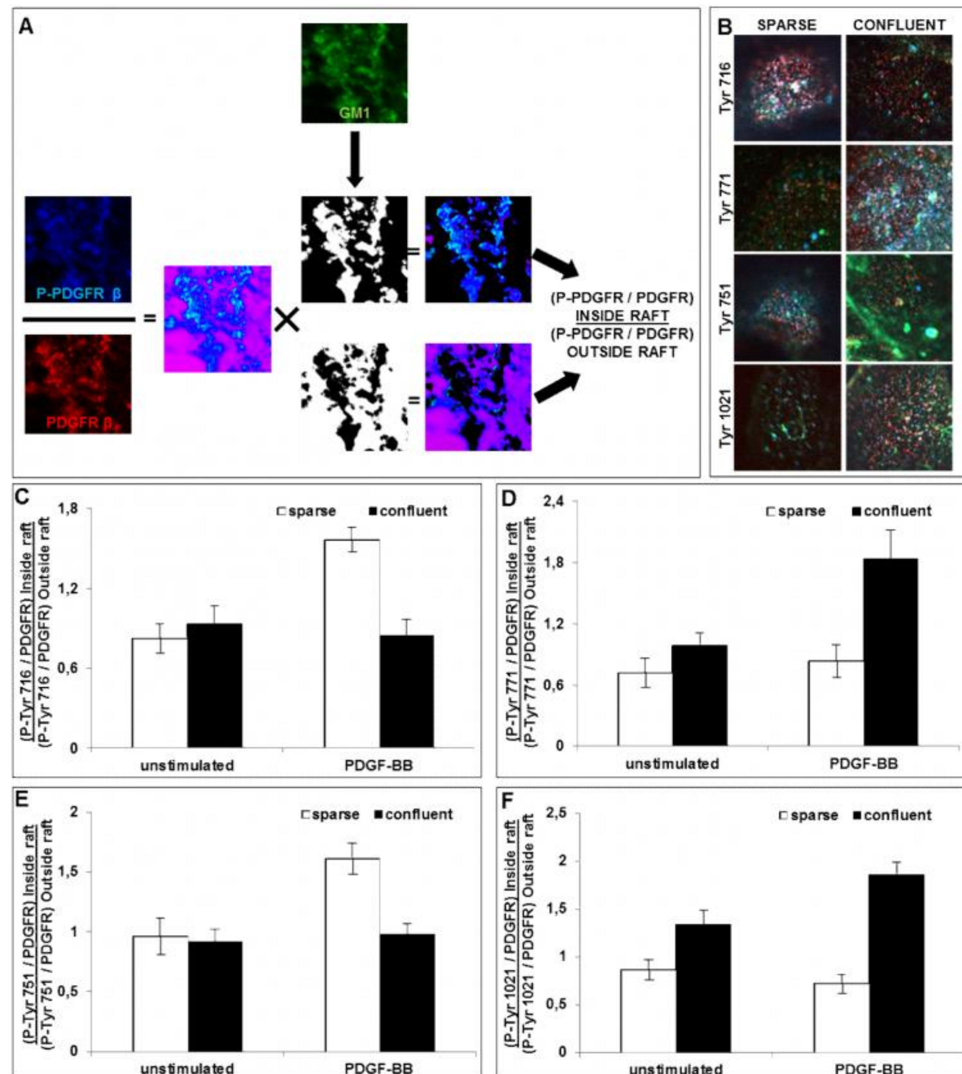


2. PDGF-BB stimulation caused a redistribution of PDGF receptors towards GM1 rich domains, which was more prominent in confluent monolayers. Global tyrosine phosphorylation increased both in confluent and non-confluent cultures. PDGF-BB



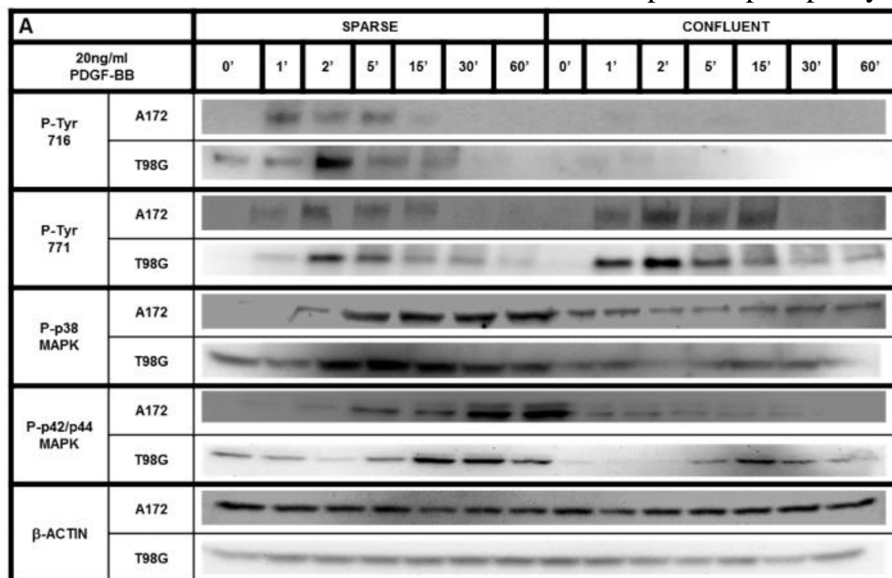
stimulation significantly increased relative receptor phosphorylation of the Ras / MAPK pathway specific tyrosine 716 residues and the phosphoinositide 3-kinase / Akt pathway specific tyrosine 751 residues in non-confluent (sparse) cultures. As cell monolayers reached confluence, tyrosine 771 residues that serve as adaptors for Ras-GAP which inactivates the MAPK pathway and tyrosine 1021 residues feeding into the phospholipase C-gamma / CaMK-PKC pathway were the docking sites significantly hyperphosphorylated following ligand stimulation.

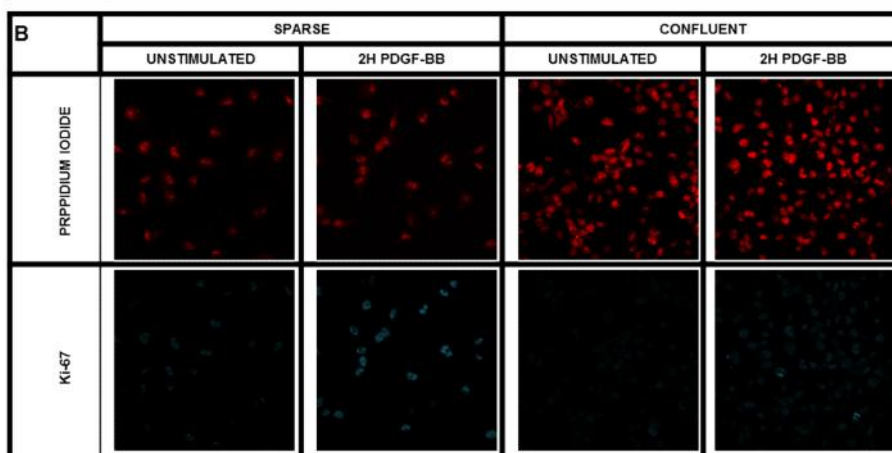
3. The role of lipid rafts has been indicated by the increased colocalization of the PDGFR in GM-1 rich membrane domains with increasing cell confluence. The observations on the distinctness of phosphorylation patterns in sparse and confluent cultures gave rise to the hypotheses that in confluent cultures more receptors are in rafts and specific phosphorylation of Tyr1021 happens there, while sparse cells keep more of their receptors outside



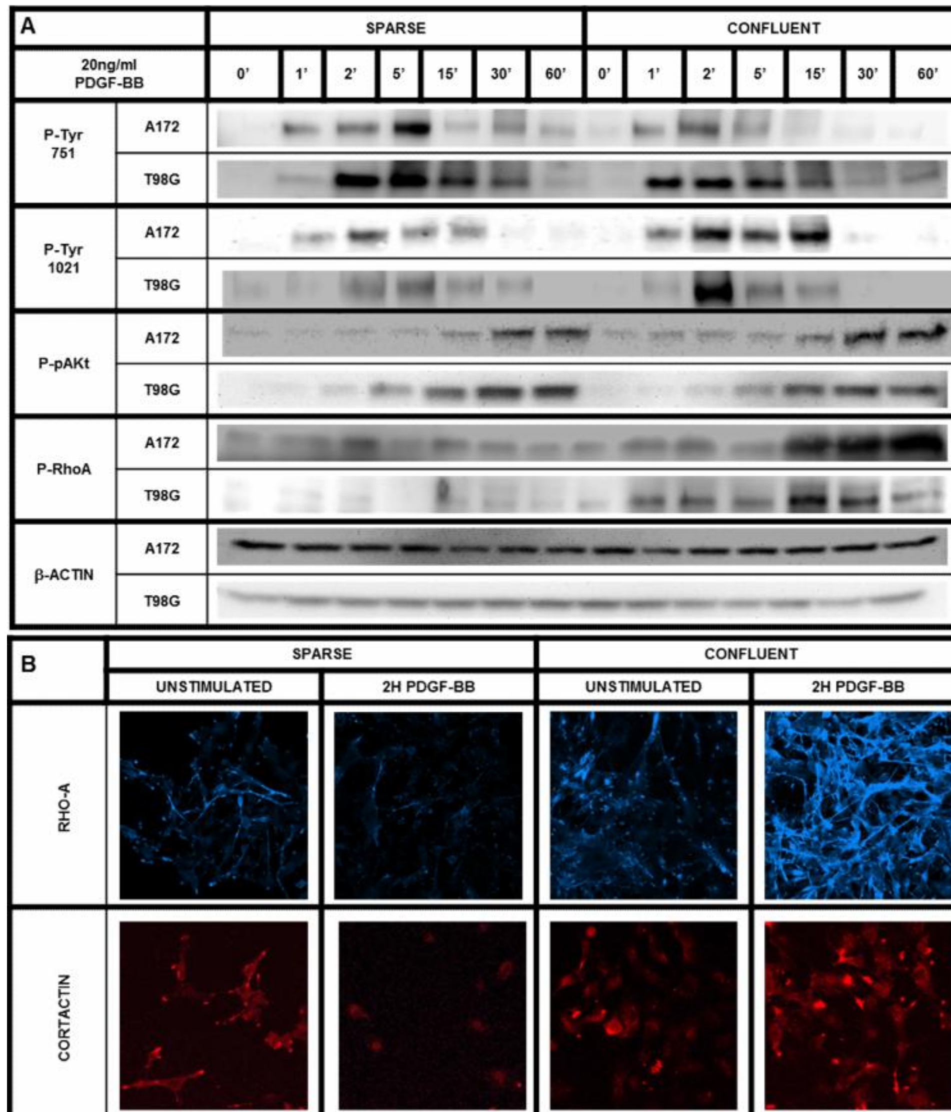
the rafts, where the other residues get preferentially phosphorylated. To substantiate this, we have developed microscopic methods to evaluate the relative degree of phosphorylation of specific tyrosine residues inside and outside lipid rafts. Surprisingly, depending on the status of cell confluence, each tyrosine residue was preferentially phosphorylated inside the lipid rafts. Mild perturbation of these domains using carefully titrated degree of cholesterol extraction with methyl-beta-cyclodextrin caused the decrease of phosphorylation process pertinent to the cell status. Thus, it remained to be seen what cellular factors within these lipid domains regulate the targeting of trans-phosphorylation as a function of cell confluence – and we have hypothesized that phosphatases can be on important set of tools in this differential regulation. (see under 5.). The differential recruitment of receptors to lipid rafts also raised several questions related to the targetability of such receptors in solid tumors. Reprogrammed T-cells can target various unique antigens the location of which could possibly modulate their collections into immune synapses. We've used the quantitative techniques developed for PDGFR to also characterize protein densities in immune synapses and reviewed the compartmentalizing role of lipid rafts from the point of view of immune cells. As a potential other lipid-raft modulated target on tumor cells we've identified DR4 and DR5 receptors whose relocalization to lipid rafts is inducible by platinum compounds (cisplatin and the novel, more potent LA-12) which in turn enhances TRAIL internalization and induces caspase-8 mediated apoptosis.

4. We have next set out to examine in how the specific phosphorylation of various tyrosine residues translates to cellular outputs. The most straightforward manifestations were found with regards to proliferation versus migration. In sparse cultures, MAPK phosphorylation was enhanced coherent with Tyr716 phosphorylation and Ki67 positivity upon PDGF stimulation of starved cells indicated the relevant proliferative response to MAPK activation. In confluent cultures, pMAPK did not increase, Tyr771 activating RasGAP was hyperphosphorylated, and the percentage of Ki67 positive cells was very low even after PDGF stimulus.





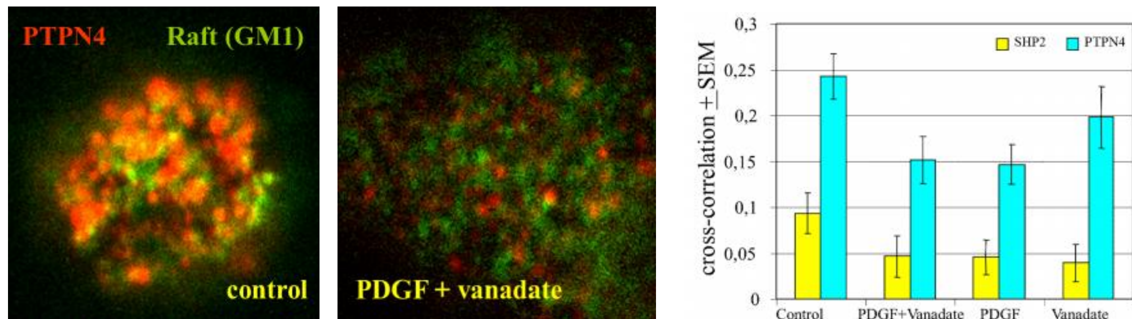
In line with the dominant phosphorylation of Tyr1021 in confluent cultures, phospholipase C-gamma mediated calcium release and PKC-dependent RhoA activation were the prominent output features that PDGF stimulus achieved here. RhoA phosphorylation was accompanied by a very outstanding increase of cellular cortactin immunofluorescence. Conversely, the sparse cells have displayed a decrease in cortactin signal, indicating negative regulation of this output by PDGF. Differential activation of the survival pathway in favor of sparse cells was less prominent.



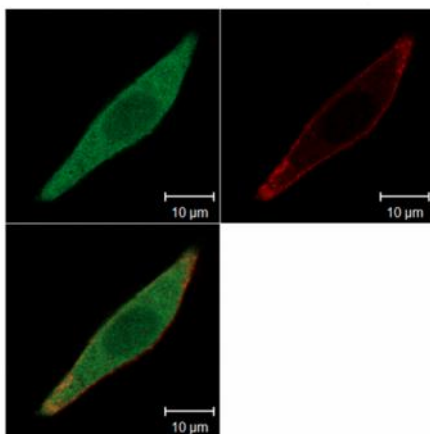
Nonetheless, phospho-Akt was also dominantly increased in sparse cultures, both when assessed by immunofluorescence and by western blot. We have investigated a panel of pro-apoptotic stimuli to ascertain that this increase in pAkt is relevant, and, coherent with literature, found that only activation of the intrinsic pathway is affected by the Akt kinase. Eventually, the best method of quantitatively producing apoptosis (assessable by Annexin V / PI) turned out to be heat-shocking, and sparse culture stimulated with PDGF were significantly less sensitive to this treatment. These observations suggest that the same stimulus, through a confluence dependent regulatory mechanism, is able to promote distinctly relevant signaling outputs, namely, cell division and survival in sparse cultures and inhibition of proliferation joined with promotion of migration in confluent monolayers that appear contact inhibited. These findings have stimulated us to extend the investigation to other glioblastoma cell lines so that a generalization can be provided, as well as to non tumorous primary cells, which, based on their receptor expression were chosen to be primary fibroblasts. On the one hand, the studies on more glioblastoma lines have proved that the principle can be generalized (albeit there are some minor differences in the outputs, e.g. the time course of phosphorylation of the effectors, or the isotype of certain enzymes involved, such as p38MAPK or p42MAPK). On the other hand, the observed regulation appears to be tumor specific. While primary cells also exhibit a higher expression of their PDGF receptors in lipid rafts with increasing cell

confluence, their growth/expansion is more strictly better regulated, and apparently do not switch to migratory signaling upon reaching confluence.

5. Phosphatases are one possible group of proteins to take part in such regulation. Of the

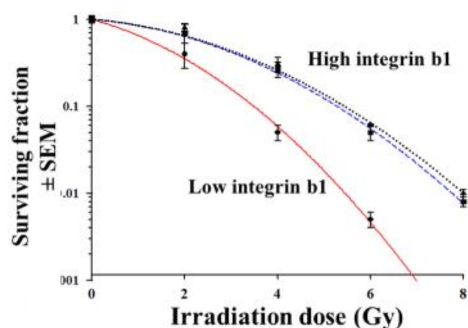


regulatory phosphatases, we have found PTPN4 especially abundant, but also SHP2 was well expressed. Investigating the spatiotemporal compartmentation of these enzymes we have noted that while a reasonable fraction of them is localized to GM1 positive domains, upon PDGF stimulus these phosphatases exit from the rafts, which can be substantiated by quantitative image processing. This raises the question whether specific phosphotyrosine residues might be more sensitive to certain phosphatases than others, thus providing for a differential regulation of the output signal under altered cell confluence conditions.



For further investigation of this phenomenon and also to determine the mode of interaction of PTPN4 with the PDGFR (which cannot happen via PY residues and SH2 domains), we have cloned PTPN4 into an eukaryotic expression vector and fuse it to cyan and yellow fluorescent proteins with various linkers. We have also cloned the PDGFR beta into expression vectors with fluorscent proteins. We have seen no Förster resonance energy transfer (FRET) between the eGFP-tagged PDGFR and TagRFP-PTPN4 in cells observed at various times after PDGF stimulus using conventional, intensity based fluorescence approaches. In the next steps, we have used frequency domain fluorescence lifetime imaging microscopy (FLIM) to observe changes in FRET at video rate, but found no interaction. Since the lack of FRET cannot be interpreted as the lack of molecular interactions, we also created a catching mutant of PTPN4 by site directed mutagenesis to stabilize the interaction between the receptor and the phosphatase (change Asparagine (GAC) toAlanine (GCC) at position 820 of the catalytic domain), but to no avail. Since positive approaches did not work, we decided on cloning a cell line in which PTPN4 can be inducibly knocked down with interfering RNA. As PTPN4 is not a well examined phosphatase (about 20 publications in the literature overall), there are no good validated RNAi against is. So, at the time of reporting, it still remains to be seen if PTPN4, and/or other phosphatases, would be responsible for selective dephosphorylation of PDGFR and producing cell contact dependent logical cellular responses to growth factor stimulus.

6. Since confluence gives rise to enhanced migratory responses, while the low density of cells promotes survival pathways, both of which may be related to signaling from integrins, we have started a screen of various integrins on a panel of 6 glioblastoma lines. We have found that integrins beta1, alpha5 and alphaV are the most prominent on most lines. We also found that integrin beta 1 tightly interacts with PDGFR and EGFR to generate greatly enhanced Akt phosphorylation. Interestingly, integrin beta 1 levels are not emphatically changed by cell



confluence, yet their interaction with the tyrosine kinases, possibly in a membrane-domain dependent manner, could prevail behind increased Akt phosphorylation. We have also observed that this interaction is correlated to radiation, and in general therapy resistance of glial tumors. Conversely, integrin knockdown lines show decreased integrin-receptor tyrosine kinase FRET and increased susceptibility to radiation induced cell death. As a therapeutic implication, we have shown that in clinical glioblastoma samples, those of higher grade exhibit increased RTK-ITGB1 interaction, and this parallels with therapy resistance.

7. In the quest for possible other regulatory factors driving the differential trans-phosphorylation as a function cell confluence, we have analyzed the transcriptome of sparse and confluent cultures, both in serum-starved and in serum-supplied steady state conditions. In spite of technical triplicates, the number of differentially expressed genes (over 1.5 x higher mRNA) at the not very low $p<0.05$ was in the range of a few tens for all comparisons, and all of them appeared to be more a consequence of the confluence state (keratins and associated proteins, as well as many growth arrest markers) than regulators of signaling (phosphorylation) processes or lipid metabolism. It therefore appears that the regulatory role of cell confluence is exerted via immediate metabolic/signaling pathways that are not dependent on expression of new proteins.

Based on our results, we have published, including methodological innovations, protocols and reviews, 14 papers (IF = 43.66) and 2 further manuscripts are currently under review.