Chondroitin Sulfate Proteoglycan-4 Does Not Protect Melanoma Cells During Inhibition of PI3K and mTOR Pathways

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Abstract. *Background: Chondroitin sulfate proteoglycan-4 (CSPG4) is commonly expressed in melanoma cells and induces melanoma cell proliferation and migration by enhancement of activation of the extracellular signal-regulated kinase 1, 2 (ERK1,2) pathway. The phosphoinositide 3-kinase (PI3K) -protein kinase B (AKT) and mammalian target of rapamycin (mTOR) pathways are also frequently de-regulated in melanoma. We hypothesized that CSPG4, by sustained activation of PI3K, may reduce the effect of the dual inhibition of PI3K-AKT and mTOR pathways. Materials and Methods: CSPG4-negative melanoma cell line WM1552C was transfected with CSPG4 and CSPG4 lacking cytoplasmic domain (melanoma-associated chondroitin sulfate proteoglycan (MCSP)ΔCD). To assess the effect of CSPG4 on the mTOR pathway, PF-5212384, a dual PI3K/mTOR inhibitor was used. Cell proliferation and downstream signaling from mTOR was assayed in the presence of CSPG4. Results: Forced CSPG4 expression did not provide any protection to melanoma cells from the pharmacological inhibition of mTOR pathway in vitro. In addition, we demonstrated that inhibition of signaling molecules downstream of AKT and mTOR was not diminished in the presence of CSPG4 when the cells were treated with the PI3K/mTOR inhibitor. Conclusion: CSPG4 expression does not have any impact on survival and signaling activity of melanoma cells during PI3K/mTOR inhibition.*

New targeted-drugs that inhibit components of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway (MAPK/ERK pathway) are being increasingly used to treat malignant melanoma (1). Vemurafenib is a serine/threonine-protein kinase B-Raf (BRAF) inhibitor that elicited encouraging treatment responses in patients with melanoma harboring a *BRAF* V600E mutation (2); however, resistance to this drug emerged after initial responses. Many different mechanisms of resistance were identified, including up-regulation in neuroblastoma Rat sarcoma NRAS signaling (3).

Chondroitin sulfate proteoglycan-4 (CSPG4) is a membrane-bound protein, covalently modified by the chondroitin sulfate glycosaminoglycan (4). CSPG4 is expressed in normal melanocytes and a variety of other normal and pathological cell types (5). In addition, CSPG4 is highly expressed by human melanoma cells, where it acts as a regulator of proliferation, cell adhesion, and motility (6). CSPG4 in radical growth-phase human melanomas caused persistent activation of critical survival and growth pathways, such as focal adhesion kinase (FAK) and ERK1/2, in addition to causing other tumor promoting consequences (7).

How CSPG4 affects the behavior of such tumor is not exactly known. It is not evident whether CSPG4 possesses any enzymatic activity of its own. It is reported that it participates in signal transduction with other tyrosine kinase receptors (8).

It was recently reported that inhibition of CSPG4 with a monoclonal antibody enhanced the effect of PLX4032, a selective BRAF inhibitor (9). The combination of the CSPG4 monoclonal antibody and PLX4032 inhibited *in vitro* melanoma cell growth to a greater degree than either agent alone. In addition, the CSPG4 monoclonal antibody delayed the development of PLX4032 resistance in treated cells. This study provided evidence that CSPG4 may have a direct or indirect role in enhancing resistance in melanoma cells to BRAF inhibition and a combined inhibition of both proteins could lead to better treatment outcome.

It has been reported that CSPG4 can directly interact with BRAF (8). Most efforts to investigate its role and potential mechanism of action have focused on the FAK and MAPK pathways.

One of the mechanisms of resistance to BRAF inhibition in melanoma cells with activating *BRAF* mutation is through activation of the protein kinase B (AKT)/ mammalian target

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of rapamycin (mTOR) pathway (10). The phosphatidylinositide 3-kinase (PI3K)/AKT/mTOR pathway is activated in melanoma and may play an important role in the pathogenesis of the disease (11). Recognizing the role of CSPG4 in the MAPK pathway, we hypothesized that CSPG4 may also interact with the PI3K/AKT/mTOR pathway and could instigate resistance to its inhibition. To address this, we examined the effects of inhibiting the PI3K/AKT/mTOR pathway in melanoma cell lines with PF-5212384 (PKI-587), a selective, adenosine triphosphate (ATP)-competitive, and reversible dual PI3K/mTOR inhibitor (12).

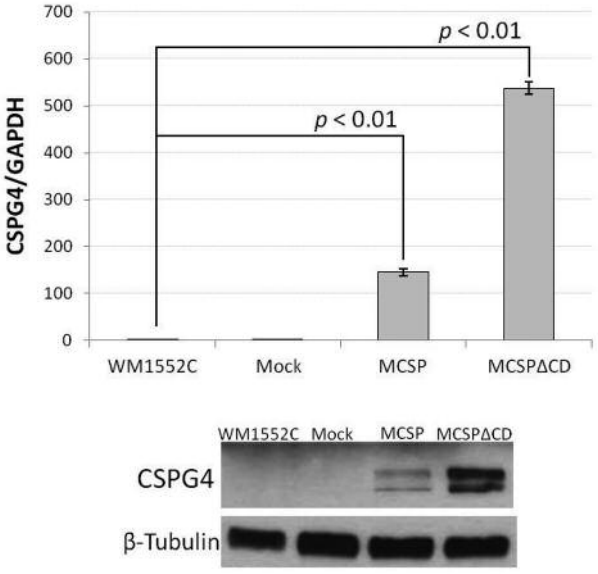

Figure 1. *Validation of chondroitin sulfate proteoglycan-4 (CSPG4) expression in WM1552C cells. The expression of CSPG4 gene was assessed with quantitative polymerase chain reaction and results were calculated by the average of it ratio to expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from duplicated samples. Significant (using Dunnett's test) CSPG4 expression was observed in CSPG4 transfected (WM1552C/(MCSP) and cytoplasmic domain-truncated CSPG4 (WM1552C/MCSPΔCD) cells compared to non-transfected WM1552C cells.*

Materials and Methods

Cell lines. The CSPG4-negative melanoma cell line WM1552C was used in this study (Department of Laboratory Medicine and Pathology [Dr. James B. McCarthy], University of Minnesota, Minneapolis, MN, USA). The WM1552C is a radical growth-phase primary cell line, derived from a stage 3 melanoma. WM1552C cells were transfected with *CSPG4* (WM1552C/MCSP), cytoplasmic domain-truncated *CSPG4* (WM1552C/MCSPΔCD) and empty vector (WM1552C/Mock) as previously described by Yang *et al*. from the University of Minnesota (7). Cells were cultured in 4:1 MCDB153:Leibovitz's L-15 medium (Sigma-Aldrich Corp, St. Louis, MO, USA) supplemented with 5 μg/ml insulin and 2% fetal bovine serum (FBS).

PF-5212384. PF-5212384 was obtained from Pfizer (New York, NY, USA) under a Material Transfer Agreement. Also known as PKI-587, this drug is a highly potent dual PI3K/mTOR inhibitor. The molecular weight of PF-5212384 is 615.73, and its half-maximal inhibitory concentration (IC_{50}) values for the various isoforms and common mutants of PI3K vary from 0.0004 to 0.008 μM. The IC_{50} for mammalian target of rapamycin complex 1 (mTORC1) is less than 3 nmol/l and for p-AKT at S473 less than 10 nmol/l (13). Stock solutions were made in dimethyl sulfoxide (DMSO) at a concentration of 4 mM and used for further studies.

Quantitative polymerase chain reaction (qPCR). Total RNA from WM1552C, WM1552C/Mock, WM1552C/MCSP and WM1552C/MCSPΔCD cells was extracted using RNeasy Mini Kit from Qiagen (Germantown, MD, USA), according to the manufacturer's protocol. Reverse transcription was performed to generate cDNA using High-capacity cDNA Reverse transcription kit from Life Technologies (Carlsbad, CA, USA). qPCR was performed with ViiA7 (Applied Biosystems, Grand Island, NY, USA) to detect the expression of *CSPG4* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) using probes against these genes. The ratio of *CSPG4* to *GAPDH* was calculated.

Cell proliferation assay. Cell proliferation assays were performed as described previously (14). Briefly, cells were seeded in triplicates at a density of 2.5×10^4/ml in 96-well plates. The medium was replaced the next day with medium containing dual PI3K/mTOR inhibitor, PF-5212384 an inhibitor, and cells were incubated for 24, 48 and 72 h. The WST-1 cell proliferation assay (Roche Applied Science, Mannheim, Germany) was then performed according to the manufacturer's instructions. Tetrazolium salt WST-1 was added to the medium at 1:10 final dilution and plates were incubated for 1 h at 37˚C. Absorbance was read at 450 nm and cell viability was calculated as normalized with untreated control.

Western blot analysis. Cells were plated at a density of 150,000 cells/ml in six-well plates. After 48 h, they were treated with inhibitor at 0.1, 1, 10 and 40 μM the indicated doses for 4 h. Cell lysates were collected as described previously (15). Cells were washed twice with ice-cold phosphate-buffered saline (PBS). Phosphosafe extraction reagent (Cell Signaling, Danvers, MA, USA) was used to lyse the cells for 5 min on ice according to the manufacturer's instruction. Lysates were cleared by centrifugation for 5 min at 14,400 ×*g* at 4˚C. Bicinchoninic acid protein assay was performed to quantify the amount of protein and 10 μg of protein was used to load the gels. The samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST) followed by probing with appropriate primary antibodies: anti-neural/glial antigen 2 (NG2) from Abcam (Cambridge, MA, USA); anti-phosphorylated mTOR at Ser2448, total mTOR, phosphorylated AKT at Ser473, total AKT, phosphorylated p70S6K at Thr389, phosphorylated 4E-binding protein 1 (4E-BP1) at Thr37/46, β-tubulin and β-actin were obtained from Cell Signaling (Danvers, MA, USA) overnight. After washing with TBST for 15 min three times, horseradish peroxidase-conjugated secondary antibody was added and the primary antigens were detected on X-ray film after incubation with chemiluminescent substrate.

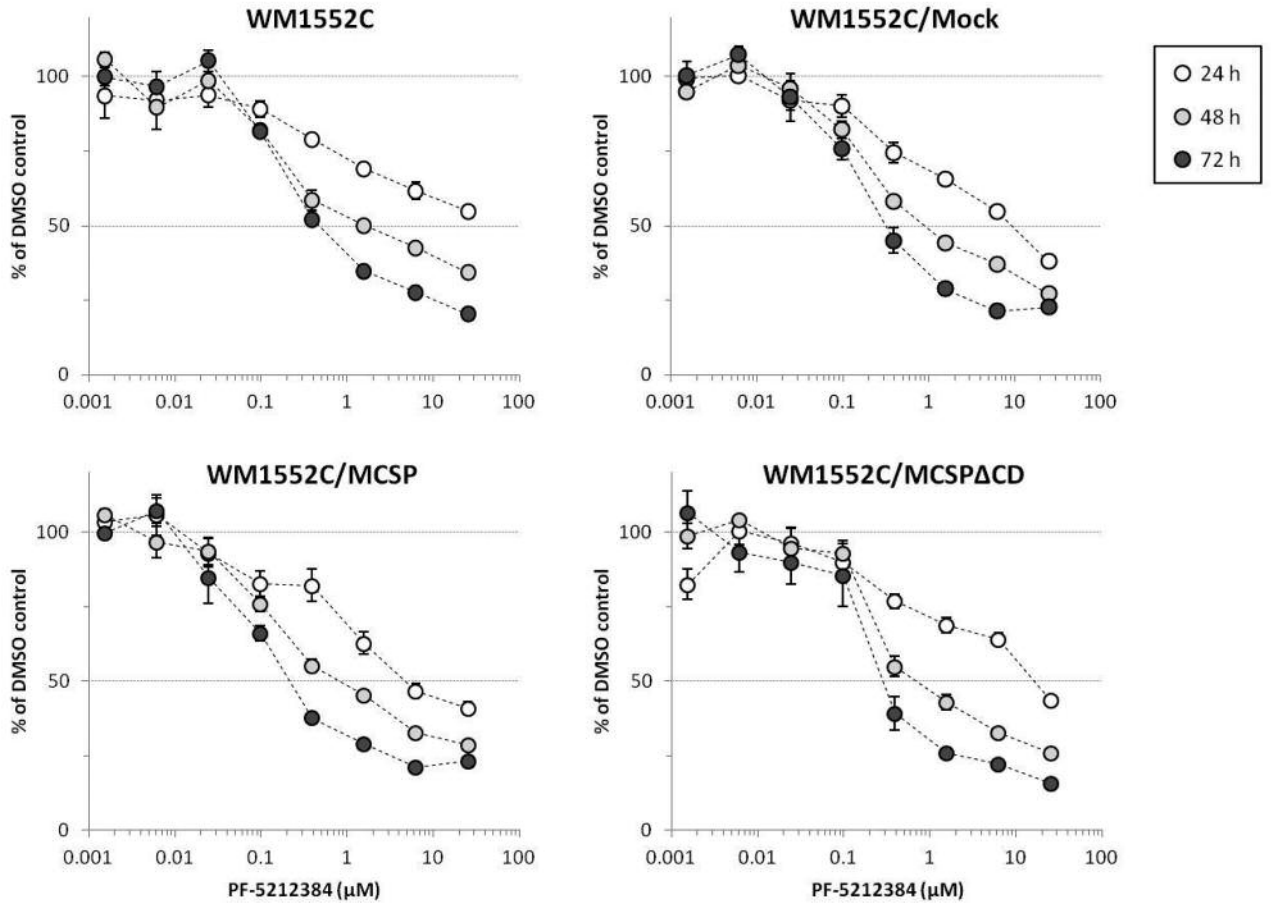

Figure 2. *Cell proliferation assay. Cells were treated with dual phosphatidylinositide 3-kinase/ mammalian target of rapamycin (PI3K/mTOR) inhibitor, PF-5212384, at different concentrations for 24, 48 and 72 h and WST-1 cell proliferation assays were performed to assess cell viability. The presence of chondroitin sulfate proteoglycan-4 (CSPG4) did not have any significant protective effect on cell viability.*

Statistical analysis. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) (16).

Results

CSPG4 is expressed in WM1552C cells transfected with CSPG4 vector. To establish the level of CSPG4 expression in WM1552C cell lines, RT-PCR was performed to detect its expression in WM1552C and transfected cells. Figure 1 shows the ratio of CSPG4 to GAPDH in different cell lines. WM1552C- and WM1552C-mock cells had very little expression of CSPG4 by TaqMan® gene expression assays (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA). In contrast, the WM1552C cells transfected with CSPG4 (WM1552C/MCSP), and WM1552C transfected with CSPG4 lacking cytoplasmic domain (MCSPΔCD) expressed a significant amount of CSPG4 compared to non-transfected WM1552C cells ($p<0.01$, by Dunnett's test).

These findings were confirmed using antibodies (Abcam Cambridge, MA, USA) against NG2, the rat homolog of CSPG4. The western blots confirmed that the highest level of CSPG4 was expressed in the WM1552C/MCSPΔCD, cells which were transfected with the cytoplasmic domain-truncated CSPG4 vector.

CSPG4 did not provide protection against proliferation inhibition in WM1552C cell lines. To determine whether CSPG4 provides protection against cell proliferation inhibition when the PI3K/mTOR pathway is inhibited, WST-1 cell proliferation assays were performed. Cells were treated for 24, 48 and 72 h with the inhibitor, as described in the Materials and Methods selection. As seen in Figure 2, there was no significant difference between the percentage of cells viable at the end of the treatment with and without *CSPG4* transfection. The maximum response to the inhibition of proliferation was achieved at the end of treatment of 72 h, with a drug concentration of 25 μM.

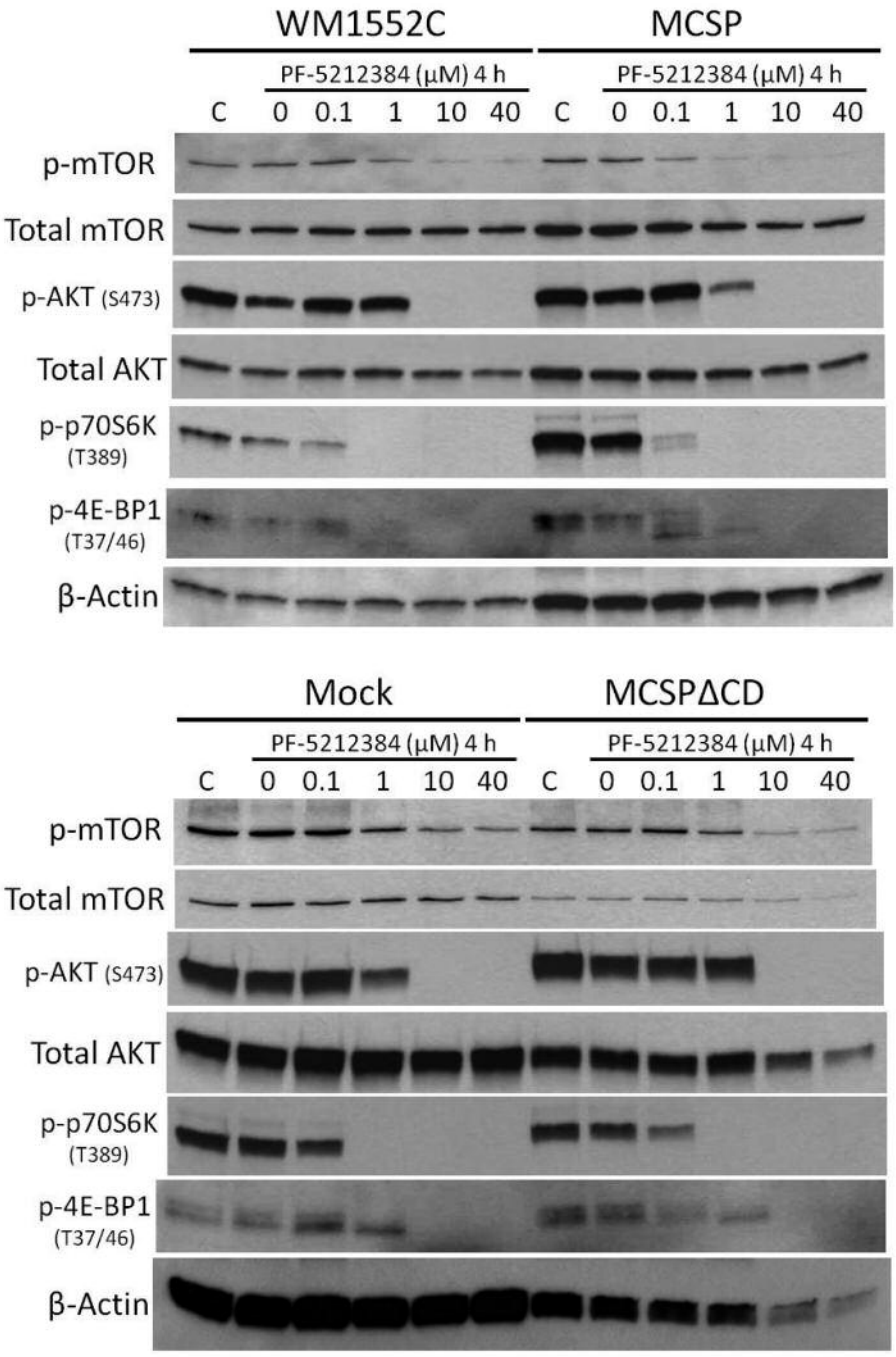

Figure 3. *Chondroitin sulfate proteoglycan-4 (CSPG4) expression does not preserve signaling downstream of protein kinase B (AKT) and mammalian target of rapamycin (mTOR) during dual phosphatidylinositide 3-kinase (PI3K)/mTOR inhibition. Cells were treated with dual PI3K/mTOR inhibitor, PF-5212384, for 4 h and cell lysates were blotted with antibodies against mTOR, AKT, p70S6k and 4E-binding protein 1 (4E-BP1). No protective effect was observed on the signaling inhibition in the presence of CSPG4.*

CSPG4 did not preserve the PI3K/mTOR signaling pathway in response to treatment with PF-5212384. To investigate whether CSPG4 provides protection against the inhibition of signaling downstream of mTOR when treated with PF-5212384, western blots were performed using antibodies against phosphorylated mTOR, AKT, p70S6K, and 4E-BP1.

The PI3K/mTOR pathway was inhibited in response to treatment with PF-5212384. There was no difference in the residual activity of the signaling molecules with and without *CSPG4* (Figure 3).

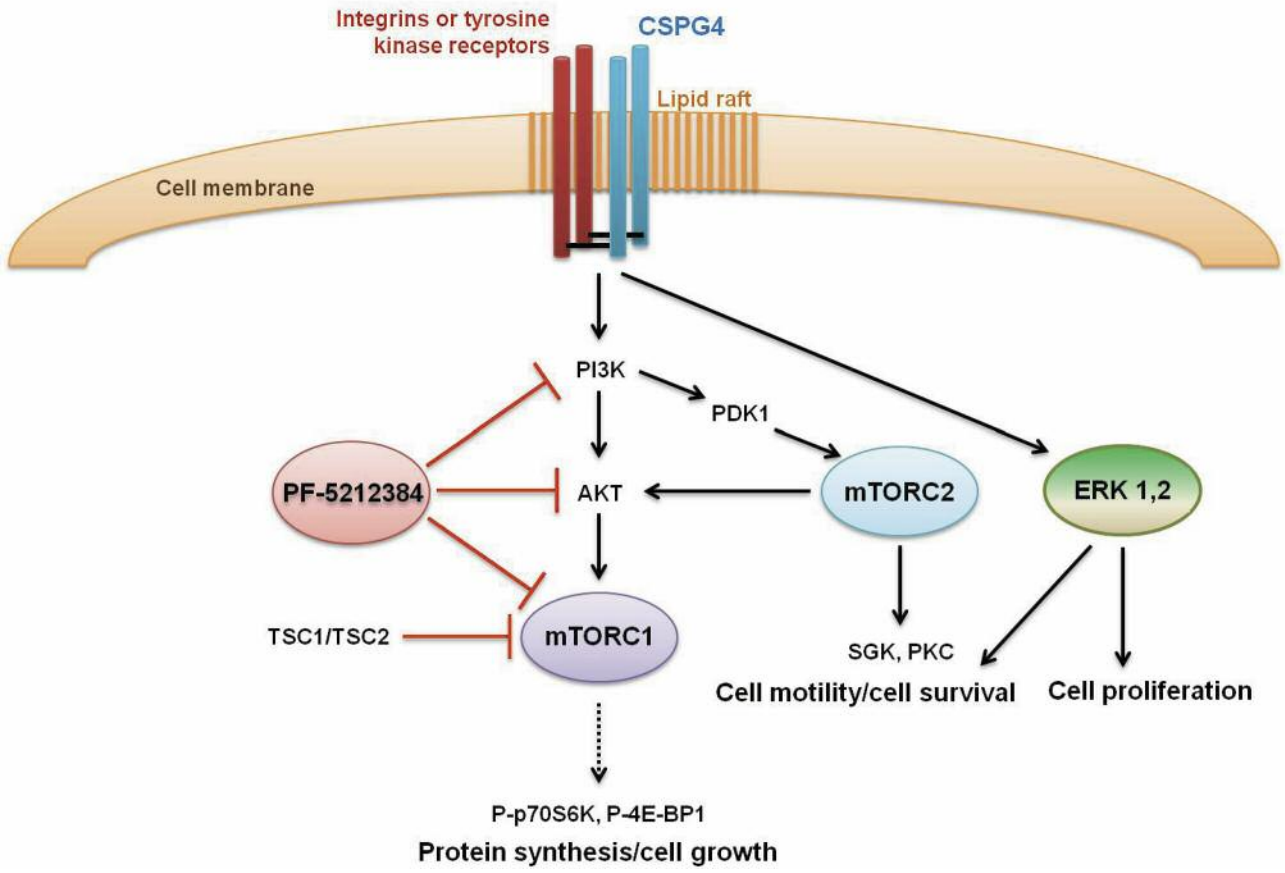

Figure 4. *Model of chondroitin sulfate proteoglycan-4 (CSPG4) effect on phosphatidylinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) pathways.*

Discussion

CSPG4 is robustly expressed in melanoma cells and may be associated with treatment resistance. Our study shows that CSPG4 does not cause melanoma cells to become resistant to growth inhibition in response to treatment with PF-05212384, a dual PI3K/mTOR inhibitor.

The PI3K/mTOR pathway consists of multiple signaling molecules which interact with each other. Signaling through the PI3K/AKT pathway is initiated by mitogenic stimuli such as growth factors. AKT is activated in a PI3K-dependent manner. AKT interacts with a number of molecules to regulate cell proliferation, differentiation and survival. The activity of AKT is negatively regulated by phosphatase and tensin homolog (PTEN). The proline-rich AKT substrate of 40 kDa (PRAS40) is known to have a critical role in the PI3K/mTOR pathway. PRAS40 is activated by PI3K/AKT and binds to mTOR to mediate the signals to it (17). Since CSPG4 may enhance activity of cell surface receptors and integrins (8), it could have provided a protective effect on cell proliferation (Figure 4) during the dual inhibition of PI3K and mTOR. However, we did not detect a protective effect of CSPG4 through ERK1/2 activation. This may be because simultaneous PI3K/AKT and mTOR pathway inhibition overrides any pro-proliferative effects of ERK1/2 activation. Another possible explanation for our findings could be the limited nutrient supply to the cells in culture. It is well-established that the PI3K/mTOR pathway is influenced by the availability of nutrients. The ability of the raptor–mTOR complex to phosphorylate downstream targets is enhanced in the presence of amino acids. Growth factors also increase the activity of this complex in a PI3K-dependent manner (18). Further experiments using selective inhibitors of mTOR, or PI3K, or AKT should be performed to dissect the role of CSPG4 in melanoma, particularly under the conditions of the tumor microenvironment.

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