High Podocalyxin levels promote cell viability partially through up-regulation of Annexin A2

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A B S T R A C T
Podocalyxin (PODXL) is a highly glycosylated and sialylated transmembrane protein that is up-regulated in various types of tumors and whose expression levels positively correlate with tumor grade. We previously found Podxl to be highly expressed in murine tumorigenic neural stem/progenitor cells (NSPs). Here we investigated the effects of elevated Podxl levels in these cells. NSPs overexpressing Podxl did not form brain tumors upon intracranial transplantations, indicating that high levels of this gene alone are not sufficient for tumor initiation. However, Podxl overexpression had a positive effect on cell number, sphere formation and cell viability, indicating that it might in this way contribute to the development and/or maintenance of tumors. Proteome analyses of Podxl-overexpressing and control NSPs revealed increased levels of Annexin A2 (ANXA2). We also found increased transcript levels, indicating that PODXL stimulates expression of the Anxa2 gene. Lack of Anxa2 in Podxl-overexpressing NSPs resulted in reduced viability of these cells, suggesting that PODXL-mediated pro-survival effects can at least in part be explained by increased ANXA2 levels. Finally, our data indicate that Podxl overexpression activates the MAP kinase (MAPK) pathway which in turn up-regulates Anxa2 expression. Our data indicate a novel molecular connection between PODXL and ANXA2; both exert pro-survival effects in NSPs, and PODXL positively regulates ANXA2 expression through the MAPK pathway.

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1. Introduction
Podocalyxin is a member of the CD34 family of sialomucins and was initially identified as the major protein on the apical domain of podocytes in the kidney [1]. High expression of the PODXL gene has been linked to tumors of several organs including breast, prostate, lung, liver, blood, colon, testicles, thyroid gland, pancreas, ovary, and brain [2–10] and is frequently associated with aggressive tumor variants [2,3,11]. With respect to brain tumors, a correlation between high PODXL expression and increasing glioma grade and decreasing patient survival rate has been reported [2]. Consistent with this, we found Podxl to be highly expressed in murine tumorigenic neural stem/progenitor cells [12] and thus sought to investigate the consequences of Podxl up-regulation in these cells.

The exact role of PODXL in tumors is not yet established. Several in vitro studies using tumor cell lines support a facilitation of cell migration and invasion by PODXL [6,8,10,13,14], which might in part be related to the anti-adhesion property conferred by sialic acid residues located on its extracellular mucin domain [14].

To investigate the effects of PODXL, most studies have either knocked-down or overexpressed the gene in tumor cell lines. The drawback of such lines is their phenotypic and genotypic drift occurring under the selection pressure of long term cell culture conditions. We followed a gain-of-function approach using NSPs which represent cells of origin for different brain tumor types [12] and as primary cells are not associated with the disadvantages of tumor cell lines.

We show that overexpression of Podxl in NSPs has a positive effect on cell numbers and sphere formation in vitro, the latter reflecting in vitro self-renewal capacity. Our experiments furthermore reveal that high PODXL levels increase cell viability in vitro and in vivo. We identified ANXA2 as a protein that is up-regulated in PODXL-overexpressing cells and our results indicate that this occurs through the MAPK pathway. Finally, we show that lack of
Anxa2 in Podxl-overexpressing NSPs diminishes the PODXL-mediated pro-survival effect. In summary, we propose a novel molecular link between PODXL and ANXA2, two proteins which positively affect the survival of cancer cells and are present at high levels in several tumor types.

2. Materials and methods

2.1. Animals

Animals were kept according to the Lund University animal facility regulations. All animal procedures were approved by the Ethics Committee at Lund University. C57Bl/6j mice were from Charles River and Anxa2^{−/−} mice on a C57Bl/6 background have been described previously [15].

2.2. Plasmid construction

The CMMP retroviral vector construct has been described previously [16]. To generate CMMP-Podxl-ires-GFP, full length mouse Podxl cDNA was PCR-amplified from mouse kidney cDNA using primers 5'-TTAGATCTGCCACCATGCCTCCCACTACGGCG-3' and 5'-TTTCGAGTCATCAGAGGTGTGTCTTC-3'.

2.3. Isolation of NSPs from postnatal mouse brains

NSPs were isolated and cultured as described previously [12].

2.4. Culture of cell lines

Platinum-E cells (Cell Biolabs) were maintained in DMEM (Life Technologies), 10% FCS (Biochrom), 10 μg/ml of puromycin (Sigma) and 10 μg/ml Blasticidin (Life Technologies). MDA-MB231 cells (provided by Sebastian Braun, Lund University) were cultured in DMEM/F12 with 10% FCS.

2.5. Retrovirus production

Platinum-E cells, plated one day before in CellBIND flasks (Corning Inc.) at a density of 5 × 10^5 cells/75 cm^2 were transfected with 40 μg of plasmid/flask by the calcium phosphate precipitation method. Medium was changed 16 h after transfection. 24 h post-medium change, supernatant was collected and centrifuged as viral harvest. Titration of the viral supernatant was done by flow cytometry on wild type NSPs.

2.6. Overexpression of Podxl in NSPs

Viral supernatant was added to NSPs at an MOI of one. GFP-positive cells were sorted using a FACSariaII instrument (BD Biosciences).

2.7. MAPK pathway inhibition

1 × 10^5 NSPs/well were seeded in 6-well plates. Cells were treated with 10 μM U0126 (Sigma) for 6 h. Control cells were incubated in medium containing 0.1% DMSO.

2.8. Intracranial transplantation

1.75–5.0 × 10^5 CMMP-Podxl-GFP- or CMMP-Empty-GFP-transduced NSPs were injected into the right frontal brain lobe of C57Bl/6j mice in a 3 μl cell suspension [12]. All procedures were carried out in an aseptic manner.

2.9. Immunostaining

Cryosections of brains (N = 3 per condition) were processed for immunostaining as described previously [12]. Primary antibodies used were α-Podxl (1:500, Abcam); α-cleaved caspase-3 (1:100, Cell Signaling Technology). Fluorescently labeled secondary antibodies (1:300) were from Dianova. Images were taken with an Olympus BX51 microscope. To quantify apoptotic cells within the transplanted GFP-positive cell population, images from 3 sections/brain/condition were analyzed using ImageJ. The Student’s t-test was used to calculate statistical significance.

2.10. Neurosphere assay

Sorted CMMP-Podxl-GFP or CMMP-Empty-GFP NSPs were plated at clonal density (1 cell/μl) in triplicates. Spheres were counted one week later. Subsequently, the cells were harvested by centrifugation and total viable cell numbers were counted. Cells were then newly plated for consecutive rounds. The Student’s t-test was used to calculate statistical significance.

2.11. BrdU assay

1 × 10^5 cells/well were plated in 96-well plates. Cell proliferation was assessed using BrdU (10 μM final concentration, Roche) according to the manufacturer’s protocol. Measurements were done with a microplate reader (Anthos 2010 reader).

2.12. SDS-PAGE and immunoblotting

Protein isolation and Western blotting were performed as previously described [12]. Antibodies used were α-Podxl (1:300, Abgent); α-Annexin A2 (1:1000, Abcam); α-c-Jun, α-phospho-c-Jun (1:500, both from Cell Signaling Technology); α-Erk1/2 (1:1000, Cell Signaling Technology); α-beta Actin-HRP (1:10000, Abcam); α-rabbit HRP (1:3000, Abcam).

2.13. Analysis of cell viability by flow cytometry

NSPs were digested with accutase (PAA Laboratories) for 10–15 min at 37˚C. Cells were then stained using an APC-conjugated Annexin V kit (BD Pharmingen) according to the manufacturer’s instructions. Analyses of the GFP-positive Podxl-NSP and Empty-NSP cell fractions were performed using a BD FACSariaII instrument. Control samples were unlabeled GFP-positive NSPs, while NSPs labeled individually with Annexin V or PI were used to set gates of each cell population. Viable cells were identified as being negative for both Annexin V and PI, early apoptotic cells were positive for only Annexin V, and late apoptotic cells were positive for both dyes. The Student’s t-test was used to calculate statistical significance.

2.14. Label-free liquid chromatography-mass spectrometry (LC-MS)

Protein was processed and analyzed according to the Protein Technology Laboratory Center (Lund University) protocol [17]. One-way ANOVA was used to calculate statistical significance between the two groups of samples consisting of three biological replicates each.

2.15. PODXL knock-down

Knockdown of PODXL in MDA-MB231 cells was achieved by viral transduction using different shRNA constructs and puromycin selection of transduced cells (10 μg/ml of puromycin (Sigma) was
added two days after transduction). To generate the shPODXL-1 and shControl-1 lines, a retroviral GFP vector from OriGene (Rockville, MD) containing a small-hairpin RNA construct targeting human PODXL (catalog number TG310316A; 5’-CCTAACACCA-CAGCCGACAACTGAGC-3’) and a non-effective shRNA cassette (catalog number TR30013) respectively were used to produce viral particles. The shPODXL-2 and shControl-2 lines were generated using shRNA lentiviral particles (catalog numbers sc-44029-V and sc-108080) from Santa Cruz Biotechnology (Dallas, Texas). Knockdown of PODXL was assessed by Western blotting.

2.16. Quantitative PCR

Total mRNA was extracted (Allprep DNA/RNA kit, Qiagen) and cDNA was synthesized from 2 μg of RNA. Real-time PCR was performed (Power SYBR Green PCR master mix, Applied Biosystems) with a StepOnePlus™ Real-Time PCR system (Applied Biosystems). The following primers were used: mouse Anxa2 Fw 5’-AAGGGAGGCTCTCTCGGATACC-3’, mouse Anxa2 Rv 5’-ACCTCTCAGGCAATGCT-3’, human ANXA2 Fw 5’-GGTCTCCCGCAGTGAAGTGGACAT-3’, human ANXA2 Rv 5’-GGCCAGGCAATGCTTAGGCAACTA-3’, mouse Gapdh Fw 5’-GAGCTGGCTCTGAGCAGATGACTA-3’, mouse Gapdh Rv 5’-GGTCTCCCGCAGTGAAGTGGACAT-3’, human GAPDH Fw 5’-GAAGGTCGGTGTGAACGGATTTGGC-3’, human GAPDH Rv 5’-GAGCTGGCTCTGAGCAGATGACTA-3’. GAPDH expression was normalized to Gapdh expression. The Student’s t-test was used to calculate statistical significance.

3. Results

3.1. Podxl overexpression leads to increased cell and sphere numbers without influencing proliferation

Previously, we generated three different brain tumor types (gliomas, primitive neuroectodermal tumors, atypical teratoid/rhabdoid-like tumors) in mice by overexpressing combinations of mutant RAS and MYC in NSPs [12]. Gene expression analyses of these tumor cells in comparison to non-tumorigenic NSPs revealed an up-regulation of Podxl in the former. To address the role of high Podxl levels in brain tumor cells, we overexpressed Podxl in NSPs (Podxl-NSPs) isolated from the lateral ventricles of wild type murine brains since these cells represent cells of origin for different brain tumors [12]. Mouse Podxl CDNA was inserted upstream of the IRES sequence of a CMMP-IRES-eGFP retroviral vector. Negative control particles were generated from a plasmid without insert, henceforth referred to as Empty control.

To investigate the in vitro self-renewal capacity of Podxl-overexpressing cells, we carried out neurosphere assays. One week after plating single cells, spheres were counted and then disaggregated to determine single cell numbers. This was designated passage one of the assay. Few passages after sorting, we observed morphological differences between Empty control (Empty-NSPs) and Podxl spheres (Podxl-NSPs), Podxl spheres lost their round appearance and sharp borders and became more irregular and loose (Fig. 1a). At passage one of the assay, more than three times as many Podxl-spheres as Empty-spheres were counted, and more than four times as many cells (Fig. 1b). This is consistent with a previous study on POXDl in glioma oncospheres [2]. However, with subsequent disaggregation and re-plating of cells, this difference was not maintained: no increased cell and sphere numbers were found in Podxl-NSP cultures in comparison to Empty-NSP cultures over the following three passages of the assay. This result could be explained by the fact that Podxl-overexpressing spheres become less compact with increasing passage number (Fig. 1a), thus single cells might not be harvested during the centrifugation step of the assay and might therefore be excluded during counting, as opposed to fewer but more compact Empty-spheres.

To assess cell proliferation, we performed a BrdU assay using cells at the same passage number as that used for the neurosphere assay. No difference between Podxl- and Empty-NSPs was noted in cultures of floating spheres or attached cells (data not shown). These results indicate that Podxl overexpression leads to an increase in in vitro self-renewal capacity, cell numbers, and loss of neurosphere compactness, but does not seem to increase cell proliferation in our cell system.

3.2. Overexpression of Podxl does not induce tumor formation

Since PODXL is up-regulated in several malignant tumors and is an indicator of poor prognosis, we sought to assess the ability of Podxl to contribute to tumor development in vivo. Podxl-NSPs and Empty-NSPs were inoculated into the frontal lobe of mice and animals were monitored for tumor formation. Nine months post-transplantation, no tumors had developed. We therefore conclude that overexpression of Podxl in NSPs is in itself not sufficient for brain tumor initiation.
3.3. Podxl-NSPs have a higher viability in vitro and in vivo

Since we did not detect any proliferative advantage of Podxl-NSPs compared to Empty-NSPs based on the BrdU assay, despite consistently higher numbers of cells and spheres at the first passage of the neurosphere assay, we reasoned that increased cell survival of Podxl-overexpressing cells might explain our observations.

To this end, we measured cell surface Annexin V as a marker for apoptotic cells and uptake of PI as a marker for dead cells by flow cytometry. As shown in Fig. 2a, the Podxl-NSP population contained approximately 32% more living cells compared to the Empty population (mean percentage of 84.5 for Podxl versus 52.6% for Empty, p = 0.01). More importantly, there were nearly three times fewer Podxl-than Empty control cells in early apoptosis (mean of 6.4% for Podxl versus 19.1% for Empty, p = 0.03). The same trend was observed for late apoptosis (p = 0.01). Our data suggest that NSP cultures overexpressing Podxl possess a higher cell viability and lower apoptotic fraction.

Two weeks post-transplantation, brains were collected and stained with the apoptosis marker cleaved caspase-3. Podxl-NSPs (GFP-positive) transplanted into brains contained 5.6-fold (p = 0.04) fewer cleaved caspase-3 positive cells in comparison to Empty-NSPs (GFP-positive) (Fig. 2b–c). We therefore conclude that Podxl overexpression leads to a reduction of apoptosis in vitro and also in vivo.

3.4. Podxl-overexpressing NSPs up-regulate Anxa2

To gain a better understanding of how high PODXL levels lead to increased cell viability at the molecular level, we investigated the proteome of Podxl-NSPs and Empty-NSPs. A liquid chromatography-mass spectrometry analysis revealed ANXA2 as one of eight proteins (supplementary data) that were significantly changed (2.3-fold increase, p = 0.02). Increased ANXA2 protein levels in Podxl-NSPs were confirmed by Western blotting (Fig. 3a). Moreover, we detected increased Anxa2 transcript levels in Podxl-NSPs (Fig. 3b, p = 0.03), indicating that Podxl overexpression has an impact on both the transcript and protein level of this gene.

3.5. ANXA2 transcript and protein levels are reduced upon down-regulation of PODXL

To further investigate the correlation between PODXL and ANXA2 levels, we next asked if a reduction of PODXL transcripts also affects ANXA2 expression. Since endogenous levels of PODXL protein are very low in NSPs (data not shown), we chose the breast cancer cell line MDA-MB231 that expresses high levels of PODXL [18]. Moreover, MDA-MB231 cells are invasive, a characteristic of ANXA2-overexpressing tumors [19–21].

To down-regulate PODXL in these cells, we made use of shRNA delivered by retroviral or lentiviral transductions, generating two knockdown (shPODXL-1 and shPODXL-2) and two control lines (shControl-1 and shControl-2). PODXL protein levels were reduced by at least 80% in these two lines (Fig. 3c). We detected lower ANXA2 protein (Fig. 3c) and transcript (Fig. 3d) levels compared with respective scrambled control lines.

3.6. Absence of Anxa2 lowers the viability of Podxl-overexpressing NSPs

Based on our LC-MS data and following our observations that...
Podxl-overexpressing NSPs were more viable and less apoptotic (Fig. 2a) and that up- or down-regulation of PODXL led to changes in ANXA2 levels, we wondered if PODXL might contribute, via ANXA2, to the increased cell viability. Different studies support a pro-survival function of ANXA2. Knock-down of Anxa2 in mouse gliomas results in increased apoptosis of transplanted cells [19]. Upon down-regulation of ANXA2, decreased activation of survival proteins has also been observed [22]. Waters et al. [23] reported that knock-down of Anxa2 in the mouse skin cell line JB6 leads to reduced cell viability upon TNFα treatment and reduced expression of pro-survival factors such as NFκB. In addition, over-expression of ANXA2 in Hela cells was found to increase the transcriptional activity of NFκB and to increase the expression of anti-apoptotic genes [24].

We overexpressed Podxl in NSPs derived from Anxa2 knockout mice (Anxa2−/−Podxl) and compared the viability of these cells with wild type NSPs overexpressing Podxl (Anxa2+/−Podxl). Indeed, Anxa2−/−Podxl NSPs showed a lower fraction of viable cells than Anxa2+/−Podxl NSPs (66.8 versus 84.5%, p = 0.004) (Fig. 4a). The absence of Anxa2 also increased the population of cells in early apoptosis (13.9 versus 6.4%, p = 0.007) and a similar trend (16.2 versus 7.9%, n.s.) was observed for the late apoptotic population. We therefore conclude that the positive effect of PODXL on cell viability might at least in part occur via regulation of Anxa2 expression.

3.7. PODXL regulates ANXA2 through the MAPK pathway

PODXL has previously been shown to activate the MAPK pathway [8]. Upon induction, the MAPK pathway activates the transcription factor activator protein 1 (AP-1) [25,26]. Since Annexin A2 is downstream of AP-1 [27], we hypothesized that up-regulation of Podxl leads to AP-1 activation and subsequent Anxa2 up-regulation.

Consistent with this assumption, we detected increased levels of phosphorylated c-Jun, c-Jun being one of the main components of AP-1, and of ERK1/2 in Podxl-NSPs (Fig. 4b). Upon application of the dual MEK1 & MEK2 inhibitor U0126, a reduction of ANXA2 protein levels was detected in Podxl-NSPs (Fig. 4c). These results indicate that PODXL might regulate Anxa2 expression through the MAPK pathway.

4. Discussion

Our study establishes an anti-apoptotic effect of high PODXL levels which is in part mediated by up-regulation of Anxa2. We found that overexpression of Podxl in NSPs led to an increased number of formed spheres and increased total cell number in comparison to control cells. These results are in accordance with a report by Binder et al. [2] on PODXL-positive and -negative cell comparison to control lines. These findings were not revealed, they found that knock-down of Podxl knock-down leads to lower ANXA2 transcript levels (p = 0.03). *P ≤ 0.05.
overexpression experiments. Moreover, decreased apoptosis of PODXL-overexpressing cells was also found in vivo upon intracranial transplantation.

That overexpression or knock-down of PODXL in tumor cell lines does not have any impact on apoptosis is in contrast to our findings in primary neural stem/progenitor cultures and might be explained by different factors. Compensatory effects of other PODXL-related genes such as CD34 or PODXL2 might occur upon knock-down of PODXL, or dysregulation of other confounding genes that are aberrantly expressed in such cancerous cell lines might take place.

To identify the mechanisms by which high Podocalyxin levels promote cell survival, we sought to identify genes differently expressed between Podxl- and Empty-NSPs and found that ANXA2 protein and transcript levels are elevated in the former. Decreased ANXA2 protein levels in Podxl-NSPs upon inhibition of the MAPK pathway indicate that PODXL might regulate ANXA2 expression via this pathway. While the tetrameric cytoplasmic form of ANXA2 is stably associated with the cytoskeleton and extracellular matrix, the monomeric form may translocate to the nucleus as a response to DNA damage \[28\]. Similar to PODXL, ANXA2 is up-regulated in various tumor types and its expression in human gliomas correlates with pathological grade \[2,29\]. ANXA2 overexpression leads to degradation of the extracellular matrix, increases cell invasion and angiogenesis, and decreases apoptosis \[18,29,30\]. We found that lack of Anxa2 reduces cell viability and increases the apoptotic fraction of Podxl-overexpressing cells, suggesting that the pro-survival function of high PODXL levels is in part due to ANXA2 up-regulation. The exact cellular anti-apoptotic mechanisms of PODXL and ANXA2 remain to be established. ANXA2 is located in different cellular compartments and implicated in various cellular processes (for review, see Ref. \[28\]). Both PODXL and ANXA2 are found at plasma membranes and PODXL is the major protein on glomerular podocytes that are constantly exposed to high fluid pressure generated by ultrafiltration within the Bowman’s capsule. Interestingly, ANXA2 is involved in efficient plasma membrane repair and tumor cell membranes have been reported as particularly fragile \[31\]. Since culturing of NSPs and their preparation for cell viability analyses by flow cytometry involves mechanical dissociation, such treatments likely lead to plasma membrane damage. In case of our intracranial transplants, one could envisage hypoxia-induced membrane damage in view of the high number of transplanted cells. Finally, although not the focus of our study, high levels of both PODXL and ANXA2 might also cooperate to enhance the migratory and invasive behavior of cells as exemplified by individual overexpression and knockdown studies of the two genes \[8,10,18,20,32\].

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Transparency document

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Appendix A. Supplementary data

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