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Expression of the apelinergic system and its influence on functional properties of tumor cells

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DOI: 10.31383/ga.vol5iss1pp26-39

Abstract

Small peptide Apelin and its cognate receptor APJ, are known to play a role in tumor angiogenesis and overall cancer progression. Certain authors suggest that the Apelin receptor is also a factor in cancer immunotherapy. In this article, our goal was to study the effects of *in vitro* targeting of the Apelin/APJ system on the tumor cells functional properties. Protein surface and mRNA expression of Apelin and APJ had been largely examined in various tumor-derived cell lines. In contrast to the tumor tissue, the results of this study demonstrated that most tumor cell lines exhibited somewhat moderate expression of Apelin/APJ. Similar effects of APJ stimulation and inhibition had been observed in *in vitro* functional assays, which was due to their unusually low expression levels. Low APJ expression in cell lines has been overcome by stable APJ overexpression. In such conditions, stimulation of apelinergic system APJ-overexpressed cells affected cell functional properties in comparison to the wildtype cell lines, where overexpression of APJ receptor resulted in increased migration. On the other hand, no effect on cell proliferation was observed. Consequently, Apelin/APJ signaling in tumor-derived cell lines is not expected to play a direct and crucial role in *in vitro* cancer survival. Further investigation should focus on *in vivo* role of the apelinergic system, as demonstrated in the recently published studies, where apelinergic system is claimed to be a promising target for anti-cancer therapy.

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Received

May, 2021

Accepted

June, 2021

Published

June, 2021

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Keywords

Apelin, Apelin receptor, cancer, cell migration, gene expression

Introduction

Apelin receptor (APJ; gene symbol APLNR) and Apelin (**APJ** endogenous binding **ligand**), constitute the apelinergic system and occur in a variety of peripheral tissues (O'Carroll et al. 2013). The existence of Apelin receptor was confirmed in 1993 through homology cloning (O'Dowd et al. 1993),

and five years later, Apelin was isolated from bovine stomach homogenate (Tatemoto et al. 1998). This system is known to play an important role in various physiological functions, some of which are related to cardiovascular system (Szokodi et al. 2002; Ashley et al. 2005), energy metabolism (Boucher et al. 2005; Heinonen et al. 2005), fluid homeostasis (De

Mota et al. 2004), as well as angiogenesis (Cox et al. 2006; Kidoya and Takakura 2012). Interestingly, some authors associate the apelinergic system with several pathologies such as diabetes, obesity, chronic heart failure, cancer and other conditions (Shin, Kenward, and Rainey 2017). Therefore, Apelin/APJ is a system of high complexity, characterized by the presence of multiple ligand isoforms and a range of intracellular signaling cascades that can be triggered. Over the last two decades, it has been discussed that Apelin may have a significant role in tumor growth, neoangiogenesis, as well as in induction of metastasis (Table 1). In addition, some authors have confirmed the involvement of the apelinergic system in the progression of cancers such as cholangiocarcinomas

(Hall et al. 2017). Another research demonstrated the role of Apelin and APJ in cell migration by activating APJ-overexpressing human embryonic kidney cells with Apelin (Hashimoto et al. 2005). Various cancer cells, such as human lung adenocarcinoma, stomach cancer, oral cell carcinoma, and ovarian cancer cells, become more migratory when apelinergic activation occurs (Lv et al. 2016; Feng et al. 2016; Heo et al. 2012; Neelakantan et al. 2019). To spread information on the role of this system in pathophysiological processes, this study focused on the examination of the apelinergic system's expression profile in certain cancer cells, as well as on changes in functional properties in cells in which this system was stimulated or inhibited.

Table 1. Role of apelinergic system in cancer. Adapted from Wysocka et al. (Wysocka, Pietraszek-Gremplewicz, and Nowak 2018).

Entity of disease	Patient/Tissue/Cell line (Reference)	Synopsis
Brain	Glioblastoma patient/tissue (Kalin et al. 2007; Harford-Wright et al. 2017)	Involvement in the microvascular proliferation in glioblastoma.
Lung	Non-small lung cell carcinoma tissue/cell line (Berta et al. 2010) Lung adenocarcinoma cell line (Yang et al. 2014)	Elevated microvessel density and poor overall survival.
Multiple myeloma	Plasma (Maden, Pamuk, and Pamuk 2016)	Patients whose disease was advanced had a significantly poor prognosis compared with patients in good health.
Ovary	Ovarian cancer (Hoffmann, Fiedor, and Ptak 2017; Neelakantan et al. 2019)	After upregulation, Apelin induces the proliferation of ovarian cancerous cells, acting as a mitogen factor.
Breast	Tissue of breast cancer patient (Wang, Greeley, and Qiu 2008) Breast cancer cell line (Wang et al. 2006)	Paracrine or autocrine mechanisms of apelin signaling.
Blood	Chronic lymphocytic leukemia (Acik et al. 2019)	Elevated Apela (endogenous APJ agonist) may potentiate progress of chronic lymphocytic leukemia.

Material and methods

Substances

Pyroglutamated Apelin-13 isoform was used for all cell-based assays and it was purchased from Abcam (Cambridge, UK). On the other hand, a small molecule, non-peptide APJ antagonist (code name SMANT01) was used to oppose the effects of

Apelin (for the details of the substance identity, refer to author of the article). All substances were diluted in water at a stock concentration of 10 mM.

Eukaryotic cells and cell culture

Immortalized cell lines used in this study as well as cell media are listed in Table 2. Eukaryotic cells were cultured in a CO₂-incubator at 37°C, 95%

relative humidity and 5% CO₂. Cell lines were regularly tested for mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Lonza). Culture of suspension cell lines was done in sterile polystyrene cell culture flasks (T25, T75) with the appropriate cell culture medium as shown in Table 2. Cells were usually passaged three times per week, maintaining the minimal and maximal cell density, as recommended by ATCC and DSMZ. Depending on their confluence, adherent cells, growing in monolayers, have been passaged 2-3 times a week after achieving confluency of 80-90%. For cell detachment, medium was removed following the washing of cell layer with sterile PBS or 0.2% (m/v) ethylenediaminetetraacetic acid (EDTA) in PBS.

Cells were then incubated with 1x trypsin for 2-5 minutes at room temperature or in the incubator at 37°C, stopping the reaction by adding culture

medium (1:2 v/v). After centrifugation of desired cell suspension for 5 minutes, supernatant was removed, following cell resuspension in the appropriate medium. Cells were then transferred back in a cell culture flask.

For the purpose of this study, pro-angiogenic macrophages (M2) were used as a control when examining Apelin and APJ gene expression, since their stable expression has been described in a previously published study (Harbaum et al. 2014). To generate the M2-macrophage phenotype, healthy donors' peripheral blood mononuclear cells (PBMCs) were isolated and subsequently used for stimulation. The Monocyte Isolation Kit (Miltenyi Biotec) was used to separate human monocytes from PBMCs using negative selection parameters. Monocytes were further polarized to M2 macrophages using the existing protocol (Mia et al. 2014).

Table 2. Eukaryotic cells

Denotation	Cultivation medium	Reference / source
HL-60	RPMI + 10% FBS	ACC 3
Kasumi 1	RPMI + 20% FBS	ACC 220
Molm13	RPMI + 10% FBS	ACC 554
MONO-MAC-1	RPMI + 10 % FBS + 1% sodiumpyruvate + 1% non-essential amino acids	ACC 252
MV4-11	RPMI + 10% FBS	ATCC® CRL-9591
OCI-AML3	α-MEM + 20% FBS	ACC 582
OCI-AML5	α-MEM + 20% FBS + 2.5 ng/mL GM-CSF	ACC 247
OCI-M1	RPMI + 10 % FBS	ACC 529
TF-1	RPMI + 10% FBS + 2.5 ng/mL GM-CSF	ACC 334
THP-1	RPMI + 10% FBS	ACC 16
UKE-1	IMDM + 10% FBS + 10% HS + 1 μM Hydrocortison	Established in our research group (Fiedler et al. 2000)
A549	DMEM + 10 % FBS	ATCC® CCL-185
HT29	DMEM + 10 % FBS	ATCC® HTB-38
MDA MB231	DMEM + 10 % FBS	ACC 732
MDA MB468	DMEM + 10 % FBS	ACC 738
OVCAR8	RPMI + 10 % FBS	RRID:CVCL 1629
U118	DMEM + 10 % FBS	ATCC® HTB-15

Flow cytometric analysis

Flow cytometric analyses were carried out on the FACS Calibur using the software CellQuest Pro. The raw data was analyzed using the Java-based FlowJo program. Cells were stained with the appropriate quantity of antibody and incubated for up to 30 minutes at 4°C in the dark. Cells were then washed with and resuspended in PBS.

Synthetic oligonucleotides

Eurofins Genomics (Ebersberg, Germany) produced all of the oligonucleotides utilized in this investigation. Primers, designed by Primer-BLAST designing tool (Ye et al. 2012), were diluted in double distilled H₂O, following the manufacturer's recommendation. The used primers are listed in Table 3.

Table 3. Synthetic oligonucleotides

Denotation	Sequence 5' → 3'	GC%	No. Bases
Primers used for cloning			
hAPLN_pOTB7_fw	GTAAAACGACGGCCAGTAACTATAACGG	46	28
NotI_hAPLN_pOTB7_rev	ATCAGCGGCCGCTCAGGAAACAGCTATGACCATGTGCC	58	38
LeGO_APJ_fw	AAAGAATTCAAGCTTGGATCCGATATCG	39	28
LeGO_APJ_rev	TTTAGGCCTTCTAGAGTATACTGGCGCG	50	28
Primers used for Real-time RT-qPCR			
hAPLN 2002	GGAAGTGCAGCAGGAATAGC	55	20
hAPLN 2162	ACACACAAAGTTGGGCATCA	45	20
hAPJ 479	CTATGGGGCAGACAACCAGT	55	20
hAPJ 647	GATATCAGCTGAGCGCCTCT	55	20
hGAPDH for 822	GTCAGTGGTGGACCTGACCT	60	20
hGAPDH rev 1066	TGCTGTAGCCAAATTCGTTG	45	20

*Functional assays**Proliferation assays (cell count analysis)*

The count of viable cells following stimulation/inhibition was used to assess the effects of Apelin and SMANT01 on the proliferation rate of cell lines. In a 24-well plate, cells were seeded in triplicate at a concentration of 0.3×10^6 cells/mL. After three to four days of incubation, the cell number was determined using the trypan blue exclusion method on the Vi-Cell™ XR semiautomatic cell counter (Beckman Coulter).

WST-1 assay

Solid tumor cell lines had been planted at an optimal seeding density of 10 000 cells/100 L/well and cultured with Apelin and SMANT01 at various concentrations during a 24-48 hr timeframe. According to the specified directions, WST-1 reagent was administered to each well and absorbance was read against a background control. The substances' effect on proliferation was assessed by reading the absorbance of treated cells and comparing it to that of control cells (Cook and Mitchell 1989).

Colony formation assays

The effects of APJ stimulation and inhibition on cell colony formation were investigated using MethoCult™ H4230 Methylcellulose-Based Medium (Stemcell Technologies). Following a seven-day incubation at 37°C, 21% O₂, 5% CO₂, and 95% relative humidity, cells were sown in triplicates at a density of 250 cells/mL/dish. Manual counting under an inverted microscope was used to determine the colony count.

Migration assay

The Incucyte™ microscope equipment (Essen BioScience) was used to perform an automated cell migration test. Wells containing cells were rinsed once with basal media after the wound was placed. The cell media was then replaced with new media that had been treated with Apelin or SMANT01. Every 1 hr, the instrument recorded cell migration status, reflecting the score of moved cells as the surface of the gap between cell fractions.

Hypoxia assay

Tumor cell lines were grown at 37°C in a hypoxic incubator with 1% O₂ and 5% CO₂. Prior to the hypoxia assays, cells were sown in 6-well plates and grown under normoxic conditions until they reached 80% confluency. After being transferred to the hypoxic incubator, the cells were held for 2, 4, 8, and 24 hr before being subjected to RT-qPCR.

Gene expression analysis

The LightCycler® 96 equipment was used for real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) gene expression analysis. Pfaffl (Pfaffl 2001), ΔC_t ($2^{-\Delta C_t}$) or $\Delta\Delta C_t$ (ΔC_t with normalization to control sample) methods were employed to quantify gene expression. Refer to the Synthetic oligonucleotides section for primers used in real-time RT-qPCR (Table 3).

Cloning APJ and APLN in lentiviral vectors

Using restriction cloning, the vectors carrying the gene of interest (GOI) were generated.

Transduction of solid tumor and leukemic cell lines was accomplished using a third-generation lentiviral vector system (LeGO). The vector maps and

sequencing data for the vectors indicated above are available online (Riecken 2012). Cloning was accomplished using PCR amplification of the gene of interest in order to construct overhangs with precise restriction sites. Refer to the Synthetic oligonucleotides section for primers used in real-time restriction cloning (Table 3).

Results and Discussion

Expression and regulation of Apelin and APJ expression

Finding the gene whose expression is changed in cancer tissue has been one of the most important breakthroughs thus far in understanding tumor biology and patient prognosis (Liang and Pardee 2003). A broad search for the presence of Apelin/APJ in tumor cell lines and tumor tissue was carried out in this study.

Apelin/APJ mRNA levels, as well as APJ surface expression, were investigated in diverse tumor tissues and cell lines. The surface expression of APJ in glioblastoma specimens from four individuals was determined to be moderate, based on the results of flow cytometric analysis (Figure 1). No statistical analysis was carried out due to a paucity of normal brain tissue.

In context of acute myeloid leukemia (AML), flow cytometric analysis revealed the similar, low to moderate APJ surface expression in bone marrow cells and peripheral blood cells. Surprisingly, no variations in APJ surface expression were identified in either compartment. (Figure 2, A).

By analyzing the expression profile in leukemic blasts and comparing to the gene expression in healthy myeloid cells (M2 macrophages, as a special pro-angiogenic population of the cell lineage), APLN and APJ mRNA expression levels were found to be low (Figure 2, B). The overall expression profile of Apelin/APJ in tumor tissue was, however, contrary to certain studies (Harford-Wright et al. 2017), where high expression was found. Moreover, based on the recently published findings (Zhao et al. 2018), the question arises whether the tumor endothelium is the primary location of APJ. For that reason, future isolation and gene analysis of tumor endothelium is more likely to show gene upregulation of apelinergic system.

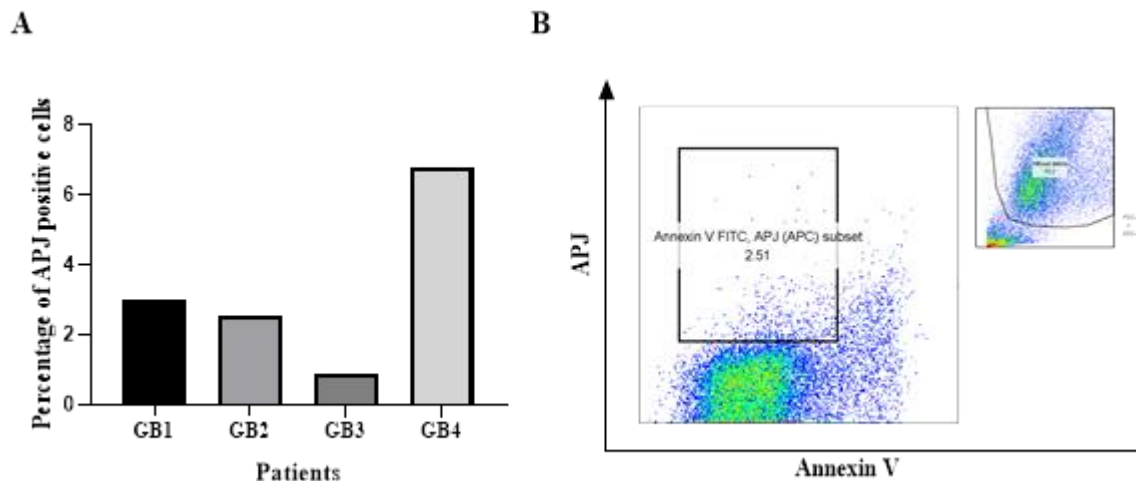


Figure 1. APJ surface analysis in human glioblastoma samples. (A) APJ surface expression in tumor samples isolated from glioblastoma patients. (B) APJ flow cytometric analysis from one glioblastoma specimen.

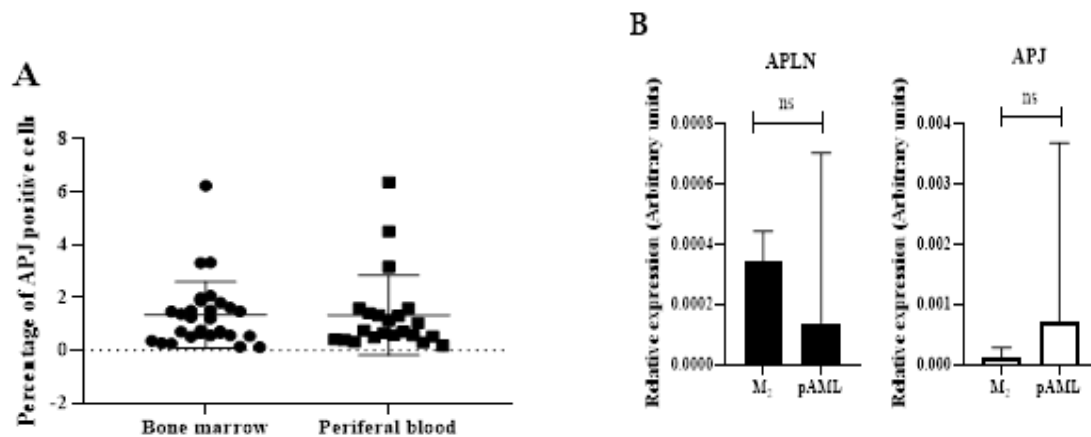


Figure 2. APJ surface expression on primary AML cells. (A) APJ expression from AML patients whose cells were isolated from bone marrow and peripheral blood (n=29). (B) APLN and APJ gene expression profile in AML cells (n=24) and M₂ macrophages as a control (n=3). The graph is based on mean + SD and all samples were evaluated in triplicates employing Δ Ct method. Expressions were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Statistical significance levels: ns (non-significant); Unpaired t-test.

It was recently demonstrated that blood levels of Apela (together with Apelin, endogenous agonist for the APJ receptor), were elevated in individuals with chronic lymphocytic leukemia (CLL) (Acik et al. 2019). Accordingly, it was anticipated that a similar situation may occur in acute myeloid leukemia (after APJ overexpression), and a thorough screening of AML cell lines was carried out (Figure 3). Although APJ gene expression was found to be low in the majority of examined cell lines, in comparison to the M2 macrophages serving as a control, the APJ expression was higher.

Furthermore, APJ mRNA levels were found to be low in the majority of solid tumor cell lines, followed by high Ct values (Figure 4, A). Apelin-stimulated gene expression regulation has also been examined in AML and solid cell lines (Figure 4, B). Apelin stimulation over 24 hr resulted in a decrease in APJ mRNA expression in the HepG2 cell line, but an increase in APJ gene expression in the A549 cell line. APLN/APJ expression profiles were also examined in solid tumor cells subjected to hypoxic settings (Figure 4, C).

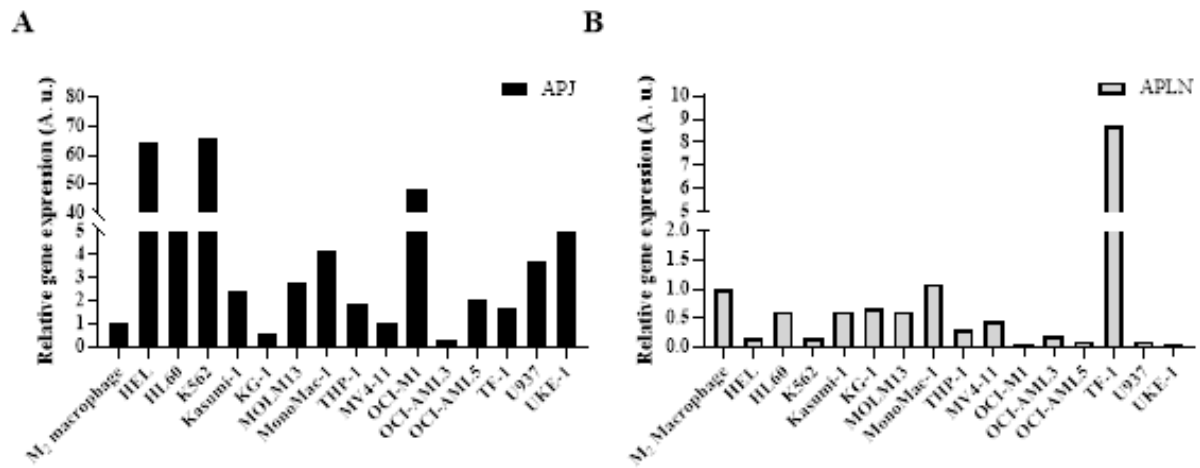


Figure 3. APJ/Apelin expression profile in AML cell lines. (A and B) APLN and APJ gene expression, with M2 macrophage serving as a control. All specimens were tested in triplicate, and the findings of the expression were standardized to glyceraldehyde-3-phosphate dehydrogenase expression (GAPDH).

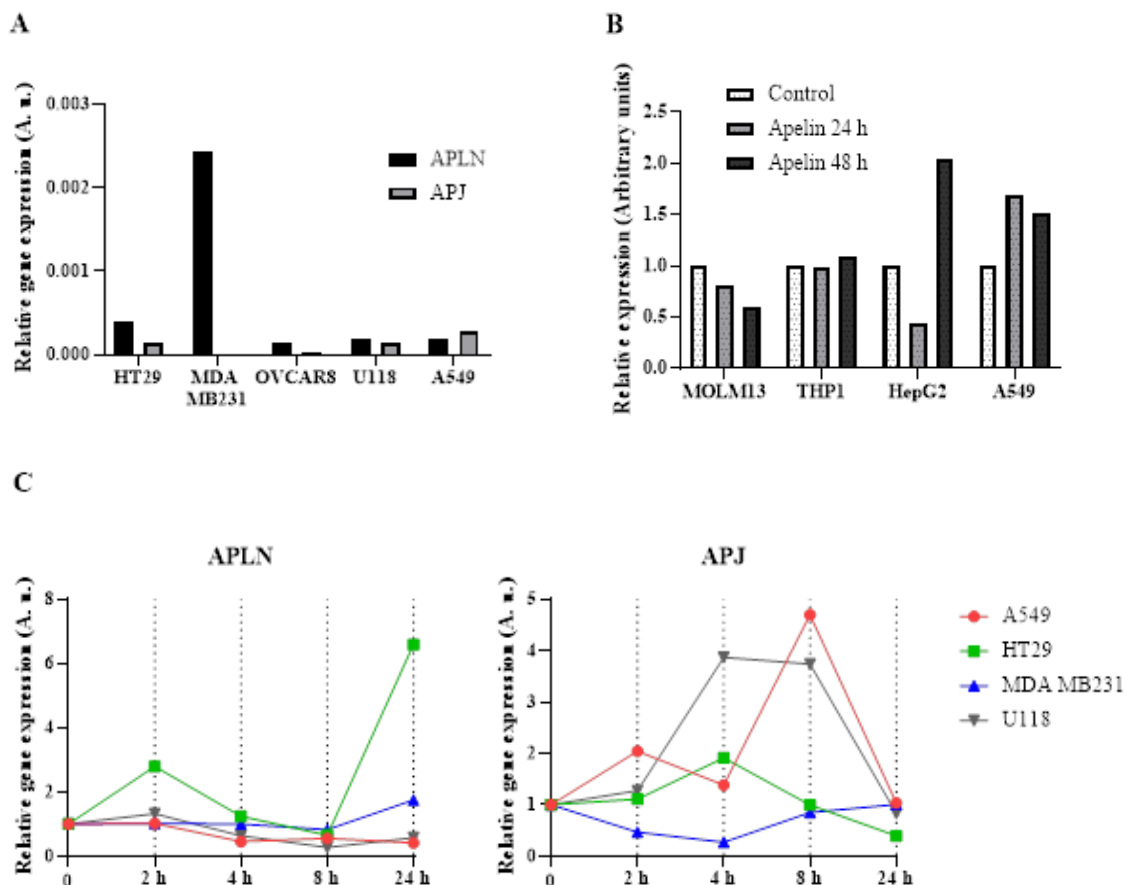


Figure 4. Apelinergic expression profile and its regulation in solid tumor cell lines. (A) The study of APLN/APJ gene expression in five solid tumor cell lines indicated low mRNA levels. (B) Gene expression after incubation of AML and solid tumor cell lines with 100 nM Apelin-13 for 24 hr and 48 hr. (C) Solid tumor cell lines were maintained under hypoxia for 24 hr. mRNA quantification was assessed after 2 hr, 4 hr, 8 hr and 24 hr. The Pfaffl approach was used to examine gene expression. All samples were examined in triplicate, and the findings (expression) were standardized to glyceraldehyde-3-phosphate dehydrogenase expression (GAPDH).

Hypoxia had little effect on APLN mRNA levels. Changes in APJ expression, on the other hand, were expected to be more impacted by hypoxic circumstances, as was the case for the A549, HT29, and U118 cell lines.

It has already been seen and reported that the investigated cell lines only exhibit modest expression of APJ and APLN, with APJ mRNA being undetectable in the TS/A breast cancer cell line and APLN mRNA being detectable only at a high cycle threshold (Sorli et al. 2007). However, in another investigation, higher expression of APLN and APJ mRNA was found in breast cancer cell line (Hs 578T), indicating a distinct situation (Wang, Greeley, and Qiu 2008). Limited expression of Apelin and APJ in cell lines, on the other hand, may be related to the assumption that tumor cell lines do not express the apelinergic system on a general level. It is also likely that certain cell types that are part of the tumor (for example, vascular cells), may display high of APLN and APJ expression levels. However, further insight into tumor endothelium gene expression profile is essential.

Certain authors found increased levels of APLN and APJ mRNA expression in colorectal cancer cell lines (Picault et al. 2014; PodgOrska et al. 2021; Podgorska, Pietraszek-Gremplewicz, and Nowak 2018), which contradicted the findings of this study. As a result, it is reasonable to conclude that the progressive reduction of apelinergic expression in cell culture is a limiting factor in accurately

reflecting *in vivo* settings. Using the pre-established “standard” cell line with the high basal expression of apelinergic system is likely to confirm this claim.

Well-vascularized tumors, as a result of antiangiogenic treatment, often become hypoxic and upregulate various genes (Crawford and Ferrara 2009; Ebos et al. 2009). Hypoxia is one of the triggering mechanisms in the angiogenesis cascade, as well as an indirect cause of metastasis onset. The goal of this research was to look at how tumor cells react to hypoxic circumstances *in vitro* in terms of apelinergic system expression, assuming that when tumor cells are upregulated, they employ the apelinergic system to communicate with the tumor microenvironment. Surprisingly, exposing cancer cells to 1% oxygen for various time periods did not result in substantial apelinergic activation in tumor cell lines. In contrast to our findings, Heo et al. demonstrated that hypoxia affects Apelin expression in oral cancer cell lines (Heo et al. 2012).

Functional analyses

Several functional *in vitro* experiments were performed to determine if functional changes in cells can occur as a result of APJ activation or inhibition. Proliferation potential in terms of viability of solid cancer cell lines after stimulation/inhibition was investigated using WST-1 assay. Surprisingly, neither cell line reacted significantly to APJ stimulation or inhibition, demonstrating no changes in cell proliferation rate (Figure 5).

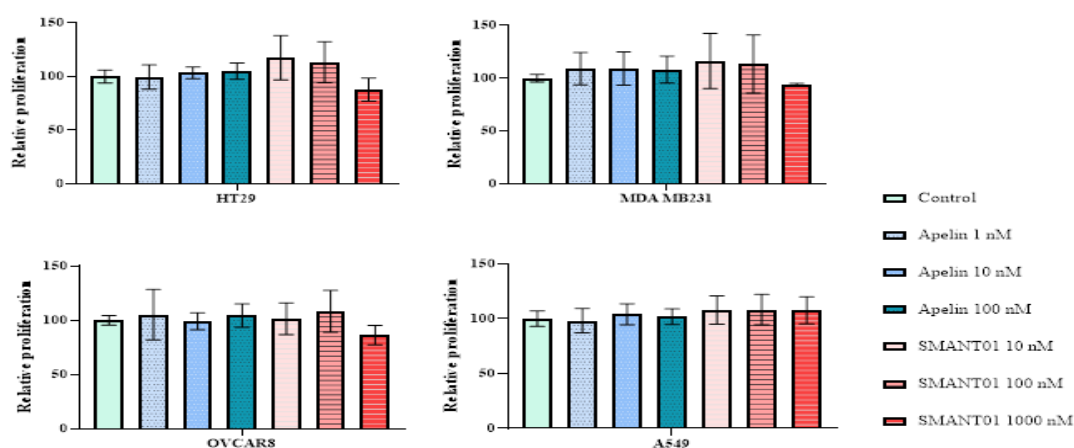


Figure 5. The rate of proliferation of solid tumor cell lines in response to APJ activation and inhibition. The WST-1 proliferation test was used to measure proliferation. Cell viability was assessed after 48 hr of treatment with Apelin-13 (1, 10, and 100 nM) and SMANT01 (10, 100, and 1000 nM). In all, two separate tests were carried out, with three replicates per group (n=2). The graphical depiction is based on the mean and standard deviation. One-way ANOVA followed by Tukey's multiple comparisons test.

Cell transduction with an APJ-overexpressing lentiviral vector system was used to surpass very low APJ endogenous expression in the majority of tumor cell lines. Following that, all cells were incubated with Apelin at three different concentrations. Apelin and SMANT01 concentrations were within the recognized EC50 and IC50 ranges, and both compounds were employed at five distinct nanomolar concentrations.

The stable expression of APJ in transgenic cell lines had a little influence on AML cell proliferation, as no significant changes between control and treated samples were identified in both wildtype and APJ-overexpressing cells (Figure 6). Despite the fact that APJ expression was steady in the cells, Apelin had no influence on cell growth. As a result, SMANT01 had no effect on cell growth at any concentration tested. Overall, the presence of APJ had no effect on transgenic cell growth.

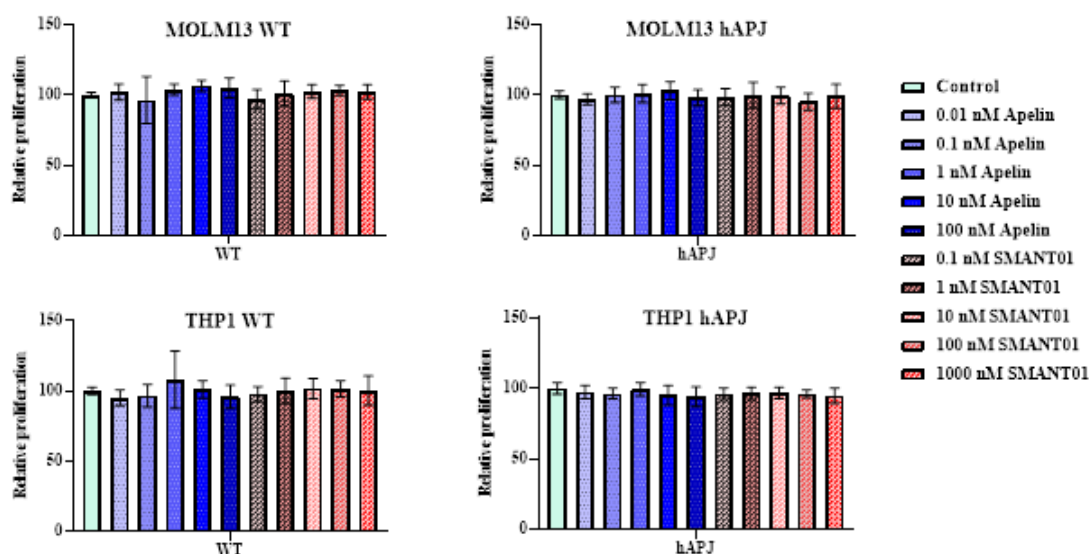


Figure 6. The effect of APJ stimulation and inhibition on AML cell growth in wildtype and APJ-overexpressing cells. Cells were cultured at a certain density and then treated in five different concentrations with Apelin-13 and SMANT01. The cell counts were determined by automated cell counting after three days of incubation. The data indicate the proliferation's mean value $\pm$ SD, normalized to the solvent control. The results were collected from a minimum of three separate, triplicated experiments. The differences between the control and single treatments were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparisons test.

Interestingly, comparable results were shown in solid tumor cell lines, with the exception of SMANT01 having a mild effect on wildtype HT29, MDA MB231 and OVCAR8 cell lines. The antagonism of APJ was shown to have a mild, but statistically insignificant, influence on the proliferation rates of these cell lines. These findings are in part consistent with prior research, which shown that inhibiting APJ leads to reduced tumor cell growth rates (Podgorska, Pietraszek-Gremplewicz, and Nowak 2018; Neelakantan et al. 2019). However, this experimental setting clearly shown that the apelinergic system is not a vital element in tumor cell survival; rather, the

involvement of this system as a helping element might be theorized.

Clonogenicity experiments were performed on two AML cell lines, MOLM13 and THP1, to get a better understanding of the Apelin/APJ system's participation in the self-renewal capabilities of acute myeloid leukemia cells. In agreement with the experimental setup from the proliferation experiments, the experiments were carried out using wildtype and APJ-overexpressing AML cell lines (Figure 7), with the goal of determining whether Apelin and APJ antagonist SMANT01 alter tumor cell clonogenicity (both wildtype and APJ-overexpressing cells).

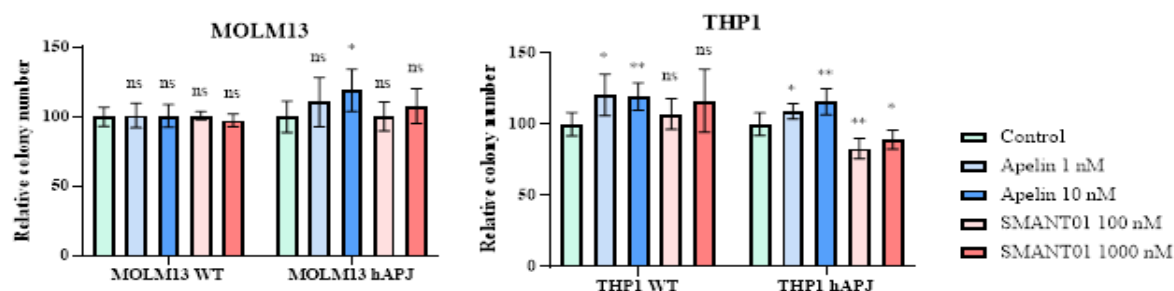


Figure 7. Clonogenicity assay for AML cell lines. MOLM13 and THP1 cells were grown on semisolid medium with Apelin-13 and SMANT01 in the presence of wildtype and APJ-overexpressing APJ. After seven days, the colony number was manually counted and adjusted to the vehicle control. The results are reported as the mean standard deviation of three separate experiments. Levels of significance: ns (non-significant); * $p < 0.05$, ** $p < 0.01$. Unpaired t-test.

The results of such functional assays indicated that APJ stimulation may have an influence on the clonogenicity of the THP1 cell line. While Apelin increased the number of colonies, APJ antagonism by SMANT01 inhibited colony formation, demonstrating that APJ antagonism has an antileukemic effect. However, this impact was only minimally observed in the case of the MOLM13 cell line, where only larger concentrations of Apelin augmented clonogenicity. In general, the effect of APJ stimulation and inhibition was more visible in

APJ-overexpressing cell lines, suggesting that APJ presence in the cells is required to impact functional features. To get a better understanding of the involvement of the apelinergic system in cell self-renewal, these findings must be replicated in additional cell lines.

The effect of APJ stimulation and inhibition on cell migration was studied in solid tumor cell lines with three distinct phenotypes: wildtype (WT), APJ-overexpressing (APJ-OE), and Apelin-overexpressing (Apelin-OE).

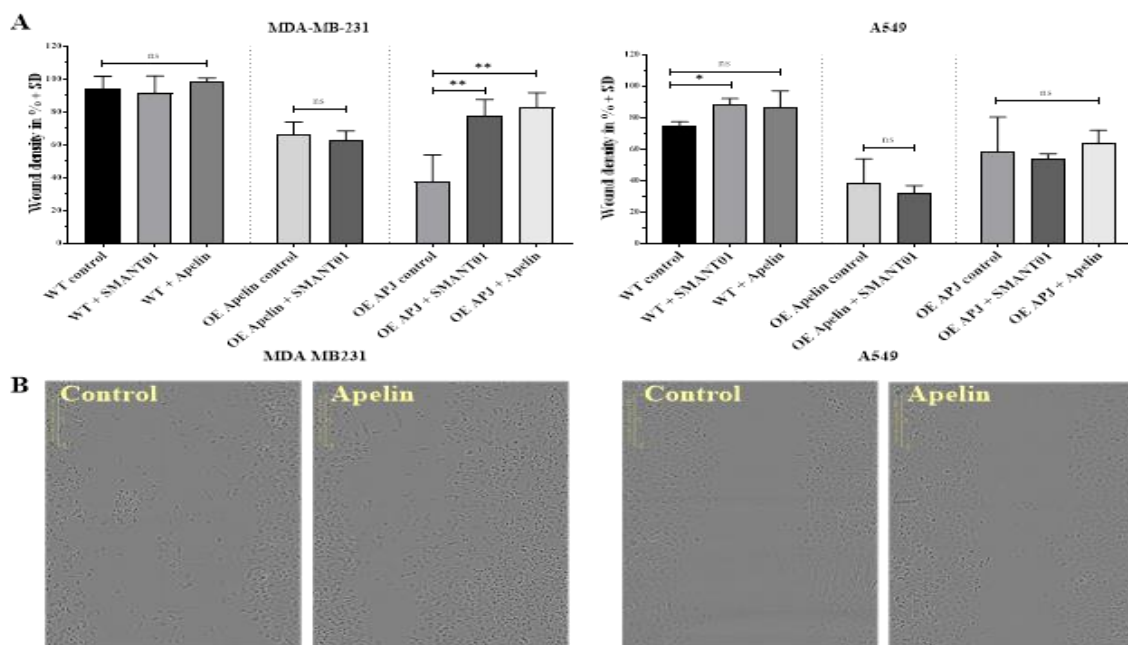


Figure 8. The effect of apelinergic activation and inhibition on the migratory capacities of solid cancer cells. (A) After incubation with Apelin (10 nM) and SMANT01, the migration rates of MDA MB231 and A549 cancer cells were continually recorded and studied (1000 nM). WT and APJ-OE cells were treated with either Apelin or SMANT01, whereas APJ-OE cells were solely treated with SMANT01. As a vehicle control, water-treated samples were used. (B) Exemplary images of APJ-OE cell lines at 12 hr (MDA MB231, left) and 10 hr (A549, right) timepoints. The results are given as the mean (wound closure) + SD of one experiment with four repetitions in each group. Significance levels: * $p < 0.05$, ** $p < 0.01$; One-way ANOVA and unpaired followed by Dunnett's multiple comparison test.

When compared to normal (untreated) cells, Apelin stimulation accelerated the speed of wound closure in APJ-overexpressing cell lines (Figure 8). Curiously, the APJ inhibitor SMANT01 had a stimulatory rather than inhibitory impact on cell migration. However, due to previously demonstrated unstable APJ expression in OE cells, this could not be detected in the A549 cell line. Furthermore, independent of APJ stimulation/inhibition, no significant changes in cell migration rate were found in wildtype cells. SMANT01 had no effect on migration rates in Apelin-OE cell lines. APJ, on the other hand, was expressed at a very low level in both the MDA-MB231 and A549 cell lines, according to gene expression analyses. Wound closure times were usually varied between the control MDA MB231 cell line (50 hr until wound closure) and the A549 cell line (20 hr until wound closure), indicating that these variances are related to the nature of cell lines. According to the results of the migration experiments, cell migration is dependent on the existence and activity of the apelinergic system. There was a clear difference in the migration of breast cancer cells following stimulation and inhibition of APJ. Cells, contrary to predictions, responded similarly to APJ activation and inhibition, increasing the rate of migration. Furthermore, it was demonstrated that there are changes in migratory rates between wildtype and overexpressing cells. It was also determined why there was no alteration in another investigated cell line, HT29. The RT-qPCR analysis found that transgenic HT29 cells lost APJ expression because its expression profile was identical to that of wildtype cells. However, this is not an isolated instance; loss of gene of interest expression in overexpressing cells is known to occur as a result of promoter silencing.

It was thought that by treating APLN-overexpressing cell lines with SMANT01, the impact of secreted Apelin would be reduced. Surprisingly, no impact was found since there was no APJ expression to begin with. Future experimental setups should be able to compensate for the loss of both Apelin and APJ. Several authors have partially corroborated the changes in cell migratory features that occur in response to modified apelinergic signaling throughout the course of this study (Podgorska, Pietraszek-Gremplewicz, and Nowak 2018), which is consistent with our findings.

Conclusion

Apelin and its putative receptor APJ were not found to have a major influence on the functional features of tumor cells in this investigation, which is assumed to be related to their overall lower expression levels in *in vitro* conditions. Several tumor cell lines were tested in order to gain a better understanding of Apelin/APJ expression. However, it seemed unlikely that the studied cell lines could reproduce the circumstances of elevated APJ expression previously observed in cancer tissue. The findings of this study reveal that the *in vitro* experimental method is insufficient to depict the possible interaction of tumor cells with a multicellular tumor microenvironment.

Overexpression has compensated the absence of APJ expression in cell lines. *In vitro* stimulation of apelinergic system APJ-overexpressed cells somewhat altered cell functional features such as cell motility and self-renewal, but had no effect on proliferation. In this sense, Apelin/APJ signaling in tumor-derived cell lines is unlikely to have a direct and critical function in cancer survival *in vitro*, but rather an indirect effect in cell migratory features. The notion is that the highest expression of the apelinergic system is found within tumor tissue, which should be reconsidered *in vivo*.

Acknowledgment

This study would not have been feasible without the assistance of Dr. Daniel J. Smit from Prof. Dr. Manfred Jücker's research group, as well as PD Dr. rer. nat. Kristoffer Riecken, who graciously contributed resources for migration experiments and lentiviral transduction, respectively. I am grateful to my mentor, Prof. Dr. Walter Fiedler, and supervisor, PD Dr. rer. nat. Jasmin Wellbrock, as well as my lab colleagues, who were always eager to help when it was needed.

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