FINAL REPORT

Apelin, a ligand of the G-protein-coupled APJ receptor plays an important role in tumor angiogenesis and enhance tumor growth in various experimental systems.[1-9] Apelin is overexpressed in several human cancers and high tumor and blood levels are associated with unfavourable prognosis.[2, 7, 10-16]

Our team demonstrated that apelin is a new angiogenic factor in non-small cell lung cancer, and high apelin protein expression in the case of patients with this disease was found to be a significant independent factor for predicting poor prognosis.[2] We also showed that apelin enhances the migration of lymphatic endothelial cells (LECs), protects them against apoptosis, increases their spheroid numbers in 3D culture and stimulates their capillary-like tube formation in vitro. Moreover, apelin promotes the invasive growth of lymphatic microvessels in Matrigel plug assay in vivo. We also demonstrate that apelin overexpression in malignant cells is associated with accelerated in vivo tumor growth and with increased intratumoral lymphangiogenesis and lymph node metastases.[1]

We are also interested in studying whether transfection of tumor cells with apelin expression constructs results in an increase in angio- and lymphangiogenesis of metastases in vivo. Our studies to address this goal will involve experiments with B16 murine melanoma cell lines expressing murine apelin or control vectors. These stable transfected cells are generous gift from Dr. Bernard Masri (Institute of Cardiovascular and Metabolic Diseases, INSERM U1048-University Toulouse III, Toulouse, France) and have been described in Ref [7]. For in vitro studies, we also developed stably transfected human melanoma cell lines (A375) with control or apelin-encoding pcDNA3.1 vector based on Ref [2].

Overexpression of apelin was validated by RT-qPCR. B16 Ap and A375 Ap cells showed 303 and 290-fold overexpression of apelin, respectively, compared to their control vector-transfected counterparts. (Fig 1A) We performed sulforhodamine B (SRB) cell proliferation studies to determine whether apelin overexpression alters the growth the B16 or A375 cell lines in monolayer cell culture. Our results show that increasing apelin expression by gene transfer had no effect on the 2D growth of B16 and A375 cells, regardless of the initial cell density. (Fig 1B-C)

A growing body of evidence has suggested that 3D cell culture environment is more similar to the in vivo solid tumors' environment than the 2D cell cultures, and the cells in spheroids differ morphologically and physiologically from cells in the monolayer cell culture.

[17, 18] We also tested the effects of apelin overexpression on the formation of multicellular spheroid structures by melanoma cells. Notably, apelin overexpression significantly increased the diameter of B16 or A375 tumor spheres ($p<0.05$; Fig 1D-E).

In our further in vitro experiments, we studied the effect of apelin overexpression on the migratory and invasive activity of melanoma cells. Previous reports have shown that apelin stimulates migration in various cell types, including oral and gastric cancer cell lines, vascular smooth muscle cells, retinal pigment epithelial cells and, on the basis of our studies, lymphatic endothelial cells as well.[1, 11, 12, 19, 20] In our experiments, apelin overexpression significantly enhanced the migration of melanoma cells independently of cell density (90 vs. 30 vs. 10 cells/mm^2) on non-coated tissue culture dishes and on collagen I-coated surfaces as well. (Fig 2A-C)

The three-dimensional tumor spheroid invasion assay is a useful method to assess tumor cell invasion in vitro, because the protein expression pattern may change in tumor spheroids which can promote the invasion. When the spheroid aggregates are placed into collagen I type gel as ECM substrates, the tumor cells will spread out from the spheroid body and extend into the extracellular-like environment.[21, 22] In our experiments, we estimated the extent of invasion from perimeter/area ratio of spheroids using ImageJ software. We found, that apelin overexpression increased the perimeter/area ratio of spheroids in both the B16 and A375 cells ($p<0.05$; Fig 2D-G).

In order to investigate the effect of apelin overexpression on the growth of experimental lung metastases in vivo, apelin overexpressing and control B16 cells were injected intravenously into C57Bl/6 mice. Lungs were removed 3 weeks after injection (Fig 3A-B) and the metastases were counted and measured with ocular micrometer. Our results show that apelin overexpression significantly increased the volume of lung metastases ($p<0.05$; Fig 3C), but did not influence the number of them ($p>0.05$; Fig 3D). Next, we studied the association of apelin expression with angiogenesis and lymphangiogenesis. Histological analysis of the lung metastases revealed that the blood and lymph vessel densities were significantly higher in the tumors formed by apelin overexpressing B16 cells (Fig 4A-F). In line with these findings, the ratio of BrdU positive cells was also increased in the B16 Ap metastases. (Fig 4G-H)

Our in vitro and in vivo results suggest that apelin enhances the growth and vascularization of melanoma. Therefore, we planned to clarify the clinical significance of apelin/APJ signalling in this disease. We investigated if melanoma patients have elevated circulating apelin levels (vs. healthy controls). We found, that melanoma patients had significantly higher

blood apelin concentrations vs. the healthy controls (n=80; 0.5630-1.253 ng/ml vs. n=21; 0.00744-2.164 ng/ml, p=0.0047; Fig 5). To the best of our knowledge, this is the first report that plasma apelin levels are significantly higher in patients with melanoma.

We presented our data on the EACR-AACR-SIC 2017 Special Conference, 24-27 June 2017, Florance, Italy (Berta J, Drozdovszky O, Török Sz, Tóvári J, Paku S, Masri B, Döme B, László V. The role of apelin signaling in the malignant behavior of cutaneous melanoma, conference abstract). Next, we plan to evaluate the prognostic significance of blood apelin levels in melanoma. We will compare the blood apelin levels with clinicopathological parameters. If we get possession of these data, our manuscript will be ready to submit to an international journal for review.

As a side project, we measured apelin levels in serum samples from a cohort of 55 renal cell carcinoma (RCC) patients that were treated for 3-5 months with sunitinib as a single agent (Fig 6A) and evaluated their progression-free survival (PFS). RCC patients with lower apelin levels indeed had a significantly longer PFS (median survival = 459.5 days) than patients with higher apelin levels (median survival = 280 days; Fig 6B). We also evaluated the serum levels of both VEGF and apelin in our cohort of sunitinib-treated RCC patients and categorized them into high or low expression by the median. (Fig 6C) Patients with both low apelin and low VEGF serum levels had a significantly higher PFS (median survival = 623 days), whereas patients with high serum levels of both proteins had the lowest median survival (167 days). Low levels of only apelin or VEGF showed a median survival of 340.5 and 343 days, respectively. (Fig 6C) Taken together, these results indicate that high apelin levels in serum samples correlate with worse prognosis of renal cancer patients treated with approved and clinically utilized anti-angiogenic therapy. Our results are under review by EMBO Molecular Medicine.

FIGURE LEGEND

Figure 1. Increasing apelin mRNA levels by gene transfer to melanoma cells has no effect on the proliferation of cell lines in vitro in monolayer cell cultures, but it increased the spheroid diameter in three-dimensional spheroid cultures. (A) Apelin mRNA was detected at a significantly higher level in the case of the apelin overexpressing B16 and A375 cells compared to the cells transfected with control vectors. (B-C) For sulforhodamine B assay, the melanoma cells were cultured in serum-free medium. No significant difference in proliferation was found by 96 hours when comparing B16 (B) and A375 cells (C) stably transfected with control or apelin expression vectors. (D-E) For three-dimensional spheroid formation assay, the melanoma cells were plated in serum-free medium in non-adherent plate. After 96 hours incubation, all spheroids were photographed. Apelin overexpression significantly increased the diameter of B16 (D) and A375 (E) spheroids compared to cells stably transfected with control vector. Experiments were replicated three times for each cell lines. 5000, 2500 and 1250 mean the initial cell numbers; bars, standard deviation. *$p<0.05$

Figure 2. Apelin overexpression enhanced the migration of melanoma cell lines and the melanoma spheroid invasion. (A) Apelin overexpression increased the 2D motility independently of the cell densities (1/3/9 K/cm^2) and surface (TC plastic vs. collagen I) in both cell lines (B16 and A375). Columns show the average speed of two experiments and three densities at 48 hours +/- sem. (B-C) The time-dependent average speed has been increased in apelin overexpressing B16 (B) and A375 (C) melanoma cell lines independently of the surface (non-precoated vs. collagen I coated) at 9 K /cm^2 cell density. Experiments were repeated twice for each cell line. (D-G) Representative images (D-E) and quantification of tumor cell invasion (F-G) are shown. Apelin overexpression significantly increased the perimeter and area ratio of spheroids in the case of both melanoma cell lines (F-G) compared to spheroids formed from the cells transfected with control vector. Columns, mean for three experiments; bars, standard deviation. *$p<0.05$

Figure 3. Effect of apelin overexpression on lung metastases in mice. (A-B) Representative pictures show the lung metastases (black narrows) of apelin overexpressing (A) or control (B) B16 melanoma cells in mice. (C) Apelin overexpression enhanced significantly the volume of lung metastases compared to the metastases of control melanoma

cells. (D) Apelin overexpression did not influence the number of metastases in mice compared to metastases of control melanoma cells. *p<0.05

Figure 4. Effect of apelin overexpression on the proliferating tumor cells and angio- and lymphangiogenesis in lung metastases in mice. (A-F) Apelin overexpression enhances the angiogenesis and lymphangiogenesis in lung metastases of mice. Paraffin-embedded sections of lung metastases of apelin overexpressing B16 (B,D) and control B16 (A,C) cells were stained for the endothelial cell marker CD31 (A-B) and lymphatic endothelial cell marker LYVE-1 (C-D). (E-F) Apelin overexpression increased significantly the microvessel (E) and lymph vessel (F) density in lung metastases. (G) Representative image of lung metastases derived from a mouse injected with apelin overexpressing B16 cells. BrdU-postive cells are visualized with 3-amino-9-ethylcarbazole (AEC) peroxidase substrate solution (Dako). (H) The ratio of BrdU-positive tumor cells and total tumor cell number was increased significantly in the case of lung metastases derived from the apelin overexpressing B16 cells compared to the metastases from the B16 cells transfected with control expression vector.*p<0.05

Figure 5. Apelin plasma levels in treatment-naive patients with melanoma vs. healthy group. Plasma apelin levels are significantly elevated in patients with melanoma (n=80) compared to the healthy group (n=21) (0.5630-1.253 ng/ml vs. 0.00744-2.164 ng/ml; p=0.0047). *p<0.05

Figure 6. High apelin levels correlate with poor prognosis of patients on anti-VEGFR therapy. (A) Experimental set up for clinical study in RCC (renal cell carcinoma) patients that received sunitinib anti-angiogenic therapy, as a single agent. (B) Kaplan-Meier plot for progression-free survival stratifying RCC patients with high and low APELIN serum levels 3-5 months after the start date of sunitinib treatment. *p=0.0367; Log rank test. (C) Kaplan-Meier plots for progression-free survival in RCC patients stratified into groups of high or low levels of APELIN and VEGF. Cut-off levels were set by the median. Serum was analyzed 3-5 months after the start date of sunitinib treatment. *p<0.05, **p<0.01; Log rank test.

FIGURES

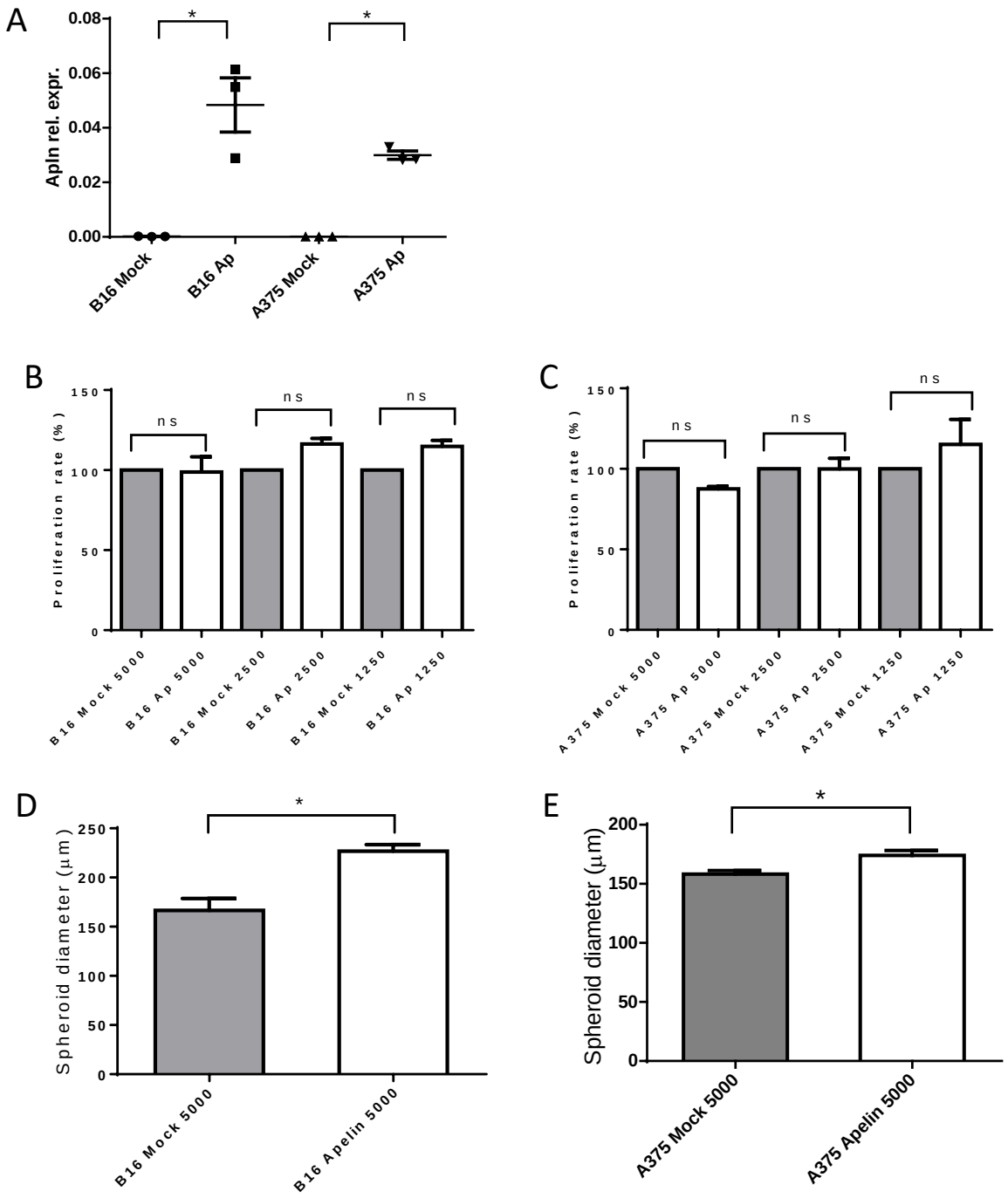
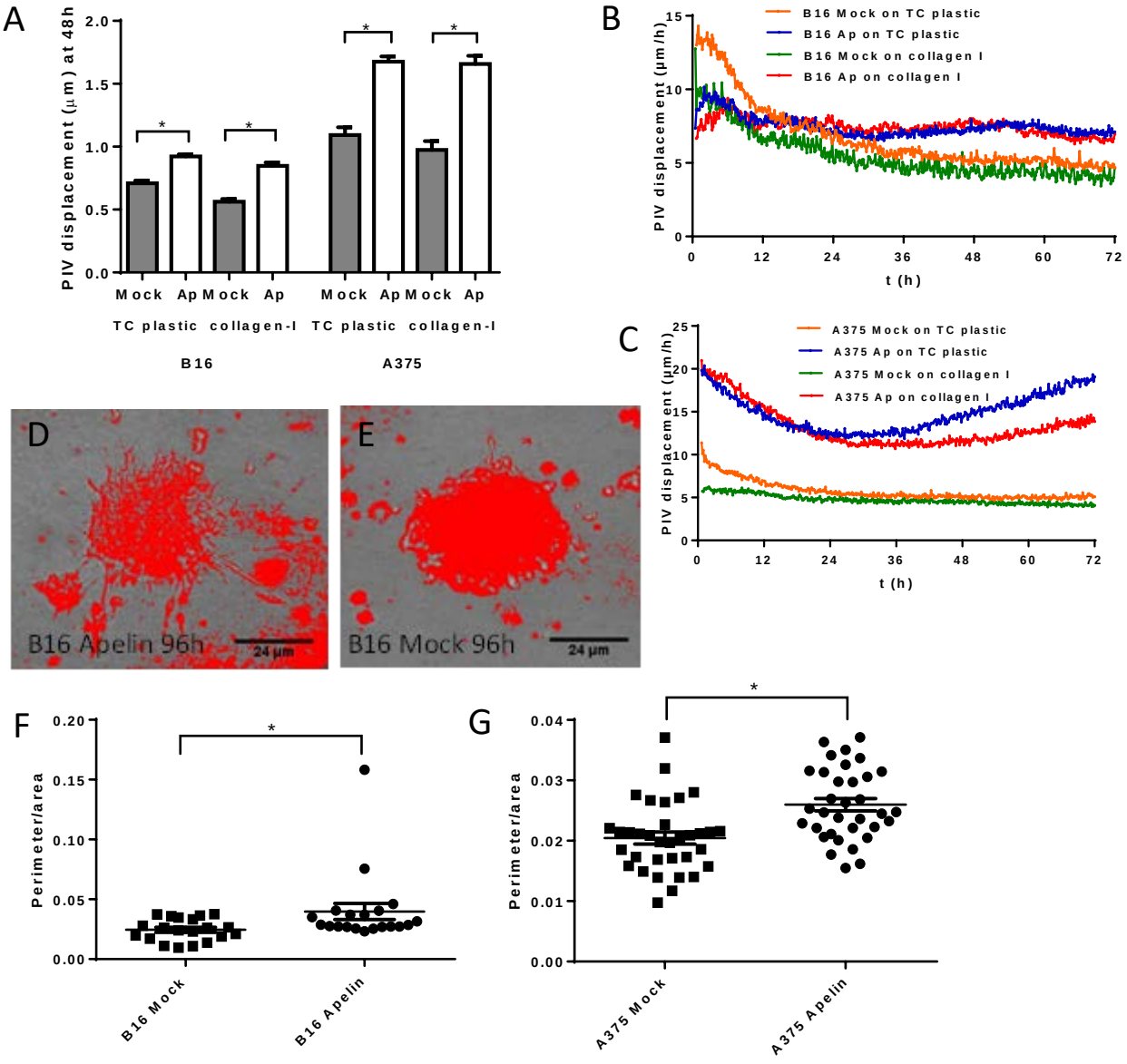


Figure 1


A
PIV displacement (µm) at 48h
2.0
1.5
1.0
0.5
0.0
Mock Ap Mock Ap Mock Ap Mock Ap
TC plastic collagen-I TC plastic collagen-I
B16
A375
B
B16 Mock on TC plastic
B16 Ap on TC plastic
B16 Mock on collagen I
B16 Ap on collagen I
PIV displacement (µm/h)
t (h)
C
A375 Mock on TC plastic
A375 Ap on TC plastic
A375 Mock on collagen I
A375 Ap on collagen I
PIV displacement (µm/h)
t (h)
D
B16 Apelin 96h
24 µm
E
B16 Mock 96h
24 µm
F
Perimeter/area
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B16 Apelin
G
Perimeter/area
A375 Mock
A375 Apelin

Figure 2

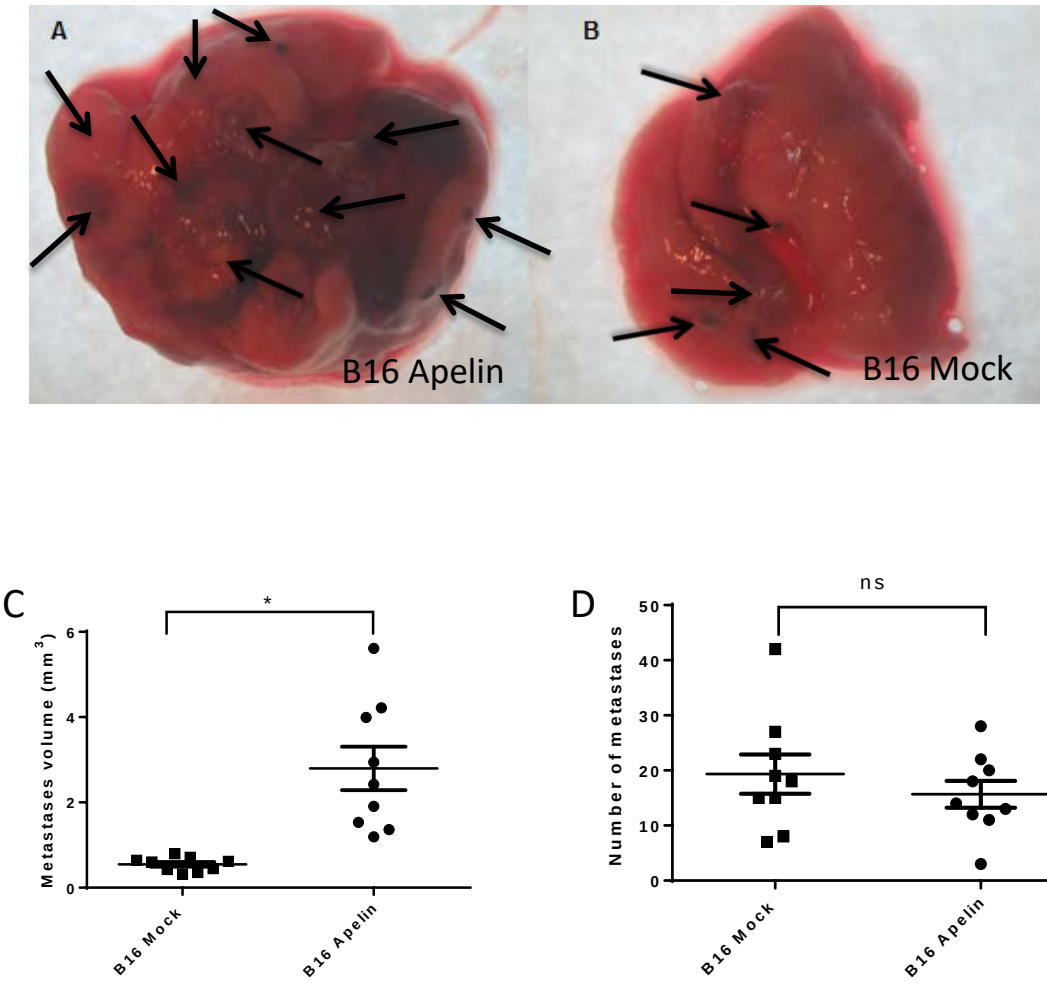
A
B16 Apelin
B
B16 Mock
C
*
Metastases volume (mm3)
0
2
4
6
B16 Mock
B16 Apelin
D
ns
Number of metastases
0
10
20
30
40
50
B16 Mock
B16 Apelin

Figure 3

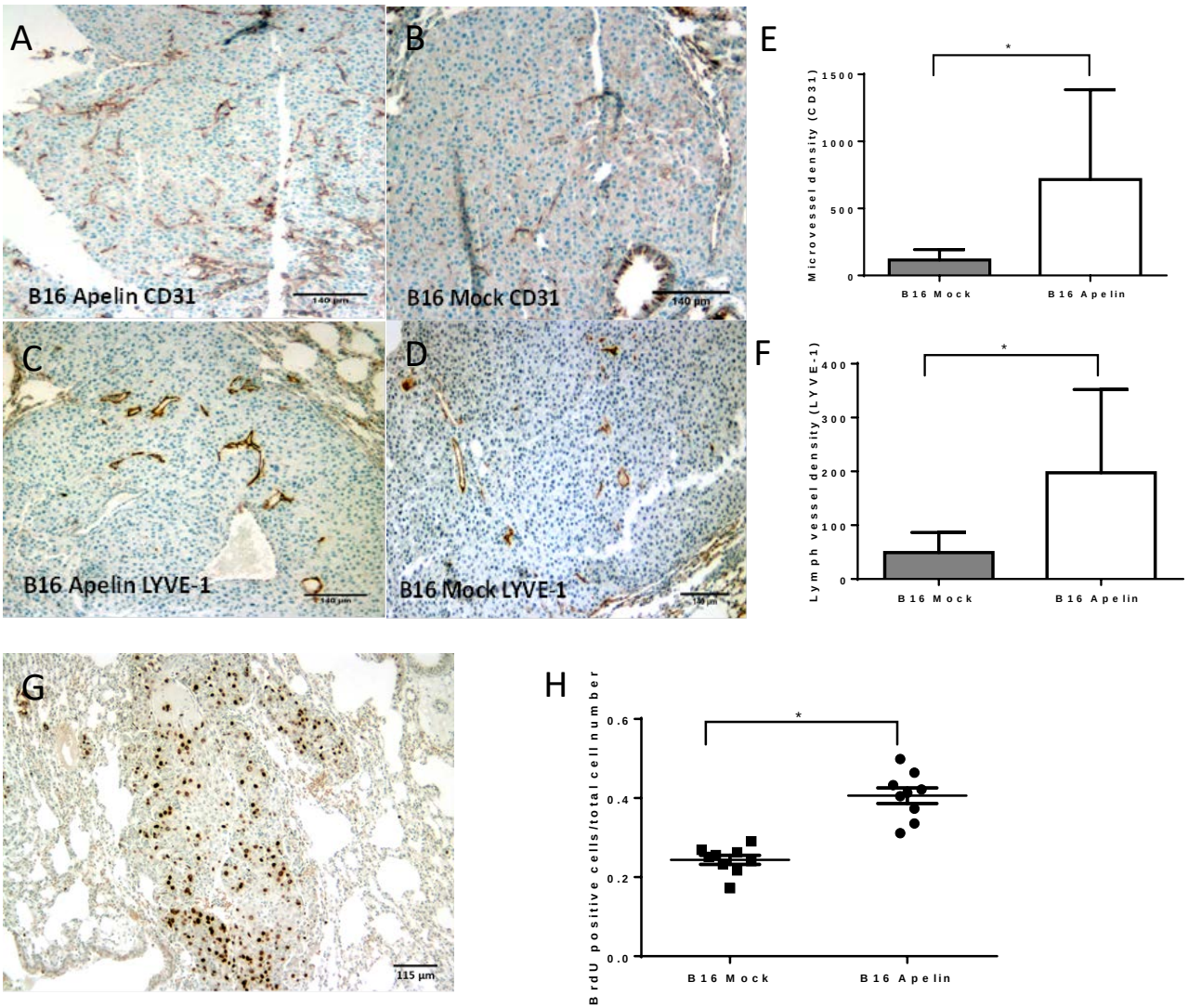

Figure 4

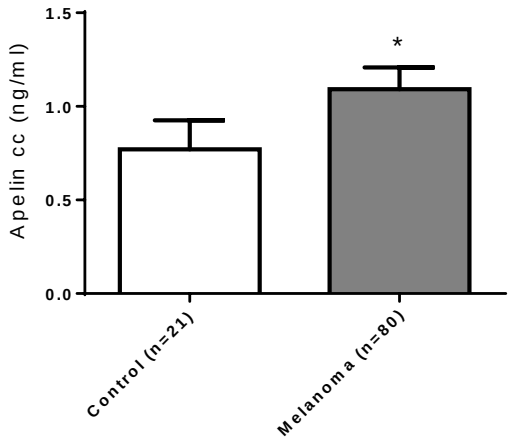

Figure 5

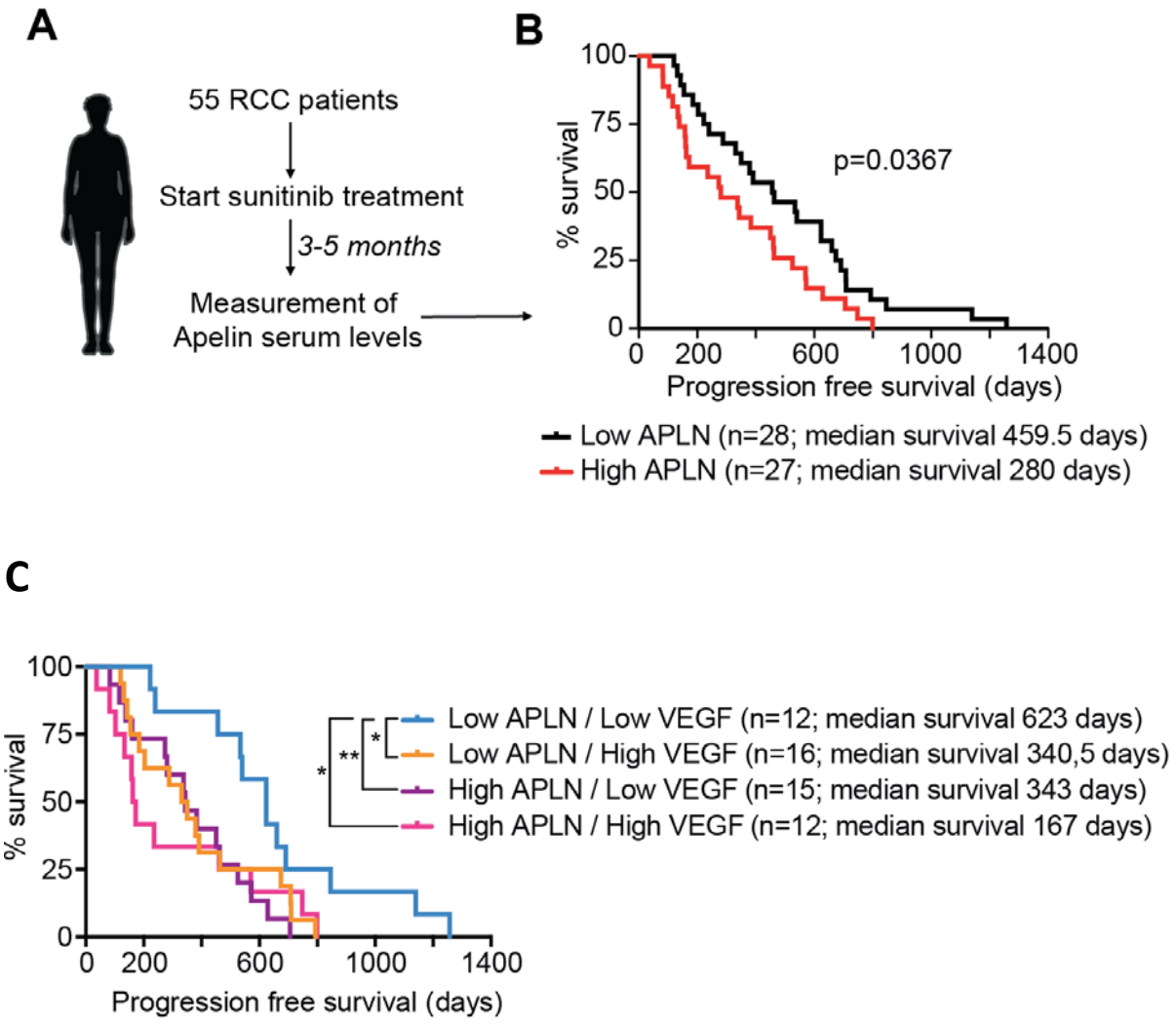
A
55 RCC patients
Start sunitinib treatment
3-5 months
Measurement of
Apelin serum levels
B
% survival
100
75
50
25
0
0
200
600
1000
1400
Progression free survival (days)
p=0.0367
Low APLN (n=28; median survival 459.5 days)
High APLN (n=27; median survival 280 days)
C
% survival
100
75
50
25
0
0
200
600
1000
1400
Progression free survival (days)
*
**
*
Low APLN / Low VEGF (n=12; median survival 623 days)
Low APLN / High VEGF (n=16; median survival 340,5 days)
High APLN / Low VEGF (n=15; median survival 343 days)
High APLN / High VEGF (n=12; median survival 167 days)

Figure 6

REFERENCES

1. Berta, J., et al., *Apelin promotes lymphangiogenesis and lymph node metastasis.* Oncotarget, 2014. **5**(12): p. 4426-37.
2. Berta, J., et al., *Apelin expression in human non-small cell lung cancer: role in angiogenesis and prognosis.* J Thorac Oncol, 2010. **5**(8): p. 1120-9.
3. Kalin, R.E., et al., *Paracrine and autocrine mechanisms of apelin signaling govern embryonic and tumor angiogenesis.* Dev Biol, 2007. **305**(2): p. 599-614.
4. Kidoya, H., et al., *The apelin/APJ system induces maturation of the tumor vasculature and improves the efficiency of immune therapy.* Oncogene, 2012. **31**(27): p. 3254-64.
5. Muto, J., et al., *The apelin-APJ system induces tumor arteriogenesis in hepatocellular carcinoma.* Anticancer Res, 2014. **34**(10): p. 5313-20.
6. Rayalam, S., et al., *Emerging role of apelin as a therapeutic target in cancer: a patent review.* Recent Pat Anticancer Drug Discov, 2011. **6**(3): p. 367-72.
7. Sorli, S.C., et al., *Apelin is a potent activator of tumour neoangiogenesis.* Oncogene, 2007. **26**(55): p. 7692-9.
8. Sorli, S.C., et al., *Therapeutic potential of interfering with apelin signalling.* Drug Discov Today, 2006. **11**(23-24): p. 1100-6.
9. Zhang, L., et al., *Apelin as a marker for monitoring the tumor vessel normalization window during antiangiogenic therapy.* Cancer Sci, 2016. **107**(1): p. 36-44.
10. Diakowska, D., et al., *Serum levels of resistin, adiponectin, and apelin in gastroesophageal cancer patients.* Dis Markers, 2014. **2014**: p. 619649.
11. Feng, M., et al., *Tumor apelin, not serum apelin, is associated with the clinical features and prognosis of gastric cancer.* BMC Cancer, 2016. **16**(1): p. 794.
12. Heo, K., et al., *Hypoxia-induced up-regulation of apelin is associated with a poor prognosis in oral squamous cell carcinoma patients.* Oral Oncol, 2012. **48**(6): p. 500-6.
13. Lacquaniti, A., et al., *Apelin beyond kidney failure and hyponatremia: a useful biomarker for cancer disease progression evaluation.* Clin Exp Med, 2015. **15**(1): p. 97-105.
14. Ni, Y.e.a., *Apelin is a novel circulating biomarker for the diagnosis of lung cancer.* Int J Clin Exp Pathol, 2017. **10(5)**: p. 5559-5565.
15. Wan, Y., et al., *Dysregulated microRNA-224/apelin axis associated with aggressive progression and poor prognosis in patients with prostate cancer.* Hum Pathol, 2015. **46**(2): p. 295-303.
16. Yang, Y., et al., *Apelin/APJ system and cancer.* Clin Chim Acta, 2016. **457**: p. 112-6.
17. Edmondson, R., et al., *Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors.* Assay Drug Dev Technol, 2014. **12**(4): p. 207-18.
18. Zanoni, M., et al., *3D tumor spheroid models for in vitro therapeutic screening: a systematic approach to enhance the biological relevance of data obtained.* Sci Rep, 2016. **6**: p. 19103.
19. Qin, D., X.X. Zheng, and Y.R. Jiang, *Apelin-13 induces proliferation, migration, and collagen I mRNA expression in human RPE cells via PI3K/Akt and MEK/Erk signaling pathways.* Mol Vis, 2013. **19**: p. 2227-36.
20. Samura, M., et al., *Combinatorial Treatment with Apelin-13 Enhances the Therapeutic Efficacy of a Preconditioned Cell-Based Therapy for Peripheral Ischemia.* Sci Rep, 2016. **6**: p. 19379.

21. De Wever, O., et al., *Modeling and quantification of cancer cell invasion through collagen type I matrices.* Int J Dev Biol, 2010. **54**(5): p. 887-96.
22. Vinci, M., C. Box, and S.A. Eccles, *Three-dimensional (3D) tumor spheroid invasion assay.* J Vis Exp, 2015(99): p. e52686.