SUPPLEMENTAL MATERIAL

TABLES

Table S1.

microRNAs differentially expressed (high glucose versus normal glucose). Total RNA extracted from HG-HUVEC and LG-HUVEC-LG was used for detection of miRNA differentially expressed as already described [1].

Briefly, total RNA (2 µg) was labelled and manually hybridized to Exiqon miRCURY^{TM} LNA Array 8.0, following the manufacturer's protocol. Differential labelling of total RNA samples with dyes spectrally equivalent to Cy3TM and CyTTM fluorophores allowed comparison of miRNA expression patterns of HG-HUVECs and LG-HUVECs. The labelling method allows selective labelling of miRNAs out of the total RNA sample. The hybridised microarrays were scanned using a GenePix 4000B instrument and data were acquired and analysed using GenePix Pro software. Data were normalized with print-tip Loess method by CARMAweb application developed at Institute for Genomics and Bioinformatics of Graz University of Technology [2].

microRNA	*Z score*
hsa-miR-325	-2,0666
hsa-miR-184	2,0596
hsa-miR-493-5p	2,2321
hsa-miR-542-3p	2,2736
hsa-miR-452	2,4130
hsa-miR-335	2,4929

hsa-miR-487b	2,6023
hsa-miR-320	2,6462
hsa-miR-375	2,6580
hsa-miR-125a-5p	2,6923
hsa-miR-373*	2,9835
hsa-miR-494	3,0677
hsa-miR-299-3p	3,2511
hsa-miR-492	4,9014

Table S2. p- value of miR-492 pull-out genes.

Gene name	p- value	Reference
PDPK1	4.6465e-05	[3]
DUSP	0.000250645	[4]
BRAF	0.000354541	[5]
PITPNA	0.000420569	[6]
MMP10	0.000520498	[7]
SP1	0.00069935	[8]
MAP3K1	0.00679158	[9]
MCL1	0.00766584	[10]
PRKCA	0.00875125	[11]

Table S3. Binding sites proposed by Miranda between miR-492 and pull-out targets

Gene name	gene_start	gene_end	align_len	miRNA_3_5 alignment	ID %	Similar %	lenght 3'UTR
MMP10	80	101	19	uucUUAGAACAGGGCGUCCAGGa gttAATTTT-TCCTGCATGTTCt	73.68	89.47	290
PDPK1	1375	1404	27	uuCUUA----GA--ACAGGG-CGUCCAGGa gaGAGTGCTCCTGGTGGCCTGGCAGGTCTg	59.26	70.37	5421
PDPK1	3700	3722	20	uuCUUAGAACAGGGCGUCCAGGa caGAATCTTGTACTTCGTGTCCa	75	85	5421
PDPK1	3935	3957	11	uucuuagaacaGGGCGUCCAGGa gtggggccccaCCTTCAGGTCTt	72.73	90.91	5421
PDPK1	1668	1690	14	uucuuagaACAGGGCGUCCAGGa ggcagagaTGTTCCCCAGGCCCt	78.57	85.71	5421
PDPK1	624	646	18	uucuUAGAACAGGGCGUCCAGGa tgctGTGTTGGCAGGCAGGTTTg	61.11	77.78	5421
PDPK1	1499	1526	24	uucUUAGAACAGG-----GCGUCCAGGa ctcAACTGTGTCCACCCTCCCTGGTCCc	58.33	62.5	5421
PDPK1	1554	1580	24	uuCUUAGA-ACAGGG--C-GUCCAGGa gtGACCCTGTGTGCCAGGCCAGATCCa	66.67	66.67	5421
PDPK1	2622	2642	14	uucuuagaACAGGGCGUCCAGGa ggcctaggTGTCC--CAGGTGCc	78.57	78.57	5421
PDPK1	1920	1942	23	uuCUUAGA-ACAGGGCGUCC--AGGa agGAATTTCTGTT---CAGGCTTCCt	65.22	73.91	5421
PDPK1	375	394	19	uucUUAGAACAGGGCGUCCAGGa cccAACTGTGT---GCTGGTCCt	63.16	68.42	5421
PDPK1	4069	4095	24	uuCUUA-GAACAG---GGCGUCCAGGa caGATTGCCTGGCTGGCAGCAAGTCCa	62.5	62.5	5421
PDPK1	3467	3489	19	uucUUAGAACAGGGCGUCCAGGa cttAGTTGTGTCTTACAGATTCt	57.89	84.21	5421
BRAF	316	346	27	uucUUAGAACA--GG------GCGUCCAGGa gcaACTTTTGTTGCCAGCTATCACATGTCCa	55.56	59.26	584
MAP3K1	1944	1964	20	uuCUUAGAACAGGGCGUCCAGGa ttGAGT-TTGT-TTGCAGTTCCc	70	85	2471
MAP3K1	824	846	18	uucUUAGAACAGGGCGUCCAGga accACTCTTATTGTGCAGGTTaa	66.67	83.33	2471
MAP3K1	442	465	21	uuCUUAGAAC--AGGGCGUCCAGga caGTATC-TGCCTCTTTTAGGTCag	61.9	76.19	2471
PITPNA	907	931	19	uucuuAGA-AC-AGGGCGUCCAGGa ttttcTCTGTGCTTTCTTAGGTCCc	68.42	84.21	2572
PITPNA	524	546	19	uucUUAGAACAGGGCGUCCAGGa agcAATGTGGTTTTGCAGCTCTg	63.16	84.21	2572
PITPNA	44	63	21	uuCUUAGAACAGGG-CGUCCAGGa agGAA----GACCCAGCAGCTCCa	66.67	66.67	2572
PITPNA	1559	1581	6	uucuuagaacagggcgUCCAGGa gcgctgaccttcattgAGGTCCc	100	100	2572
PITPNA	2316	2338	6	uucuuagaacagggcgUCCAGGa gccttctcatgcagagAGGTCCg	100	100	2572

Table S3 (continue).

SP1	1093	1117	24	uuCUUAGAACAGGGC----GUCCAGGa agGAA--ATGACTCGTAGTCAGGTTCa	58.33	66.67	5202
SP1	4583	4610	25	uuCUUAGAACA---G--GGCGUCCAGGa ttGAACATTGTGGACAAGGGCAGGTCTt	60	64	5202
SP1	1275	1294	20	uuCUUAGAACAGGGCGUCCAGGa gtGAATC-TGTTT--CAGGTTTc	65	85	5202
SP1	3650	3672	20	uuCUUAGAACAGGGCGUCCAGGa aaGAGTTTTGGAGATCAGGTACc	60	70	5202
SP1	789	807	19	uucUUAGAACAGGGCGUCCAGGa gtcAATTTTTT----CAGGACCt	63.16	68.42	5202
SP1	1217	1245	23	uucuuAGAAC----AGGG--CGUCCAGGa acttcTTTTGAACATCCCCACTAGGTTCt	56.52	69.57	5202
SP1	1908	1933	22	uucUUAG---AACAGGGCGUCCAGGa tcaGATCAAAATGCCTTTCAGGCCCa	54.55	68.18	5202
MCL1	1343	1369	20	uucuuaGAACA----GGGCGUCCAGGa ggcttgCTTGTTACACACACAGGTCTa	65	70	2816
MCL1	1692	1718	23	uuCUUAGAAC-AGGG----CGUCCAgga acGAA-CTTGATCCTGTTAGCAGGTggt	69.57	73.91	2816
MCL1	903	928	22	uucUUAGA--ACAGG-GCGUCCAGGa acaAATCTGATAACTATGCAGGTTTa	59.09	77.27	2816
MCL1	824	848	21	uucUUAGAACAGGGC--GUCCAGGa ctaAACTTTGTTCTGTTCAGTTCTa	61.9	80.95	2816
DUSP3	10	32	6	uucuuagaacagggcgUCCAGGa accaccucugcucgagAGGUCCg	100	100	3505
DUSP3	330	352	6	uucuuagaacagggcgUCCAGGa ccuaagaugcucacagAGGUCCc	100	100	3505
DUSP3	509	531	6	uucuuagaacagggcgUCCAGGa gcccucucacgucuugAGGUCCg	100	100	3505
DUSP3	1034	1056	6	uucuuagaacagggcgUCCAGGa caagcagggaagugaaAGGUCCc	100	100	3505
DUSP3	1375	1097	6	uucuuagaacagggcgUCCAGGa acucugggcaaagagaAGGUCCu	100	100	3505
DUSP3	2888	3010	6	uucuuagaacagggcgUCCAGGa aucuagaagaccucuaAGGUCCc	100	100	3505
PRKCA	2047	2069	6	uucuuagaacagggcgUCCAGGa gagacacccccaugcaAGGUCCu	100	100	6724
PRKCA	3085	3107	6	uucuuagaacagggcgUCCAGGa gccgcagccacgcaggAGGUCCc	100	100	6724

SUPPLEMENTAL MATERIAL

FIGURES

Figure S1.

Transfection efficiency of HUVEC. Exponentially growing HUVECs were transfected with 80nM oligo-FITC using Gene Silencer transfectant. After 24 hours HUVECs were collected and analyzed with BD-FACscalibur. FACS analysis showed that approximately 98% of cells were FITC-positive (Figure S3) and therefore we can reasonably assume that the various RNA molecules under testing were always efficiently transfected.

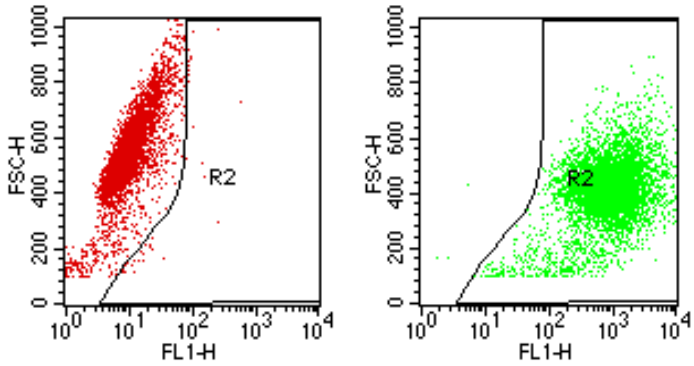


Figure S1. Scattergram analysis of HUVEC untransfected (left panel) and transfected (right panel) with oligo FITC. Transfection efficiency was ~98%.

Figure S2.

Expression of miR-492 in human tissues. Total RNA was extracted from $1x10^6$ HUVEC cells using miRNAeasy mini kit (Qiagen) following the manufacturer's recommendation. Total RNA from prostate, colon and lung tissue were purchased from Ambion (FirstChoise Human Total RNA). Total RNA were reverse transcribed using miScript Reverse Transcription kit (Qiagen) and miR-492 were quantified by qRT-PCR carried out with Rotor-Gene Q 2-Plex using QuantiTect SYBER Green PCR kit (Qiagen).

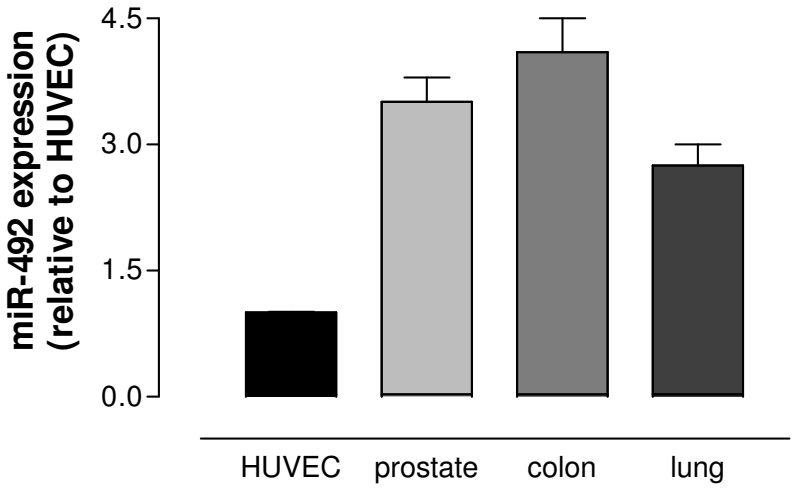
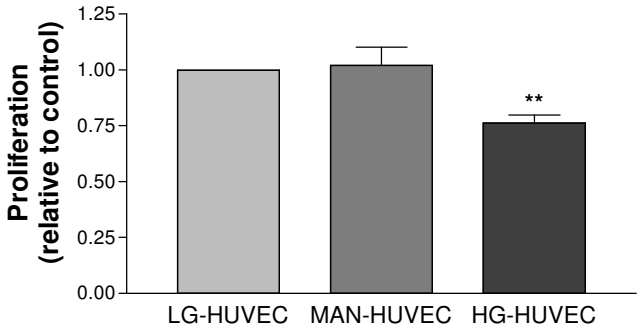


Figure S2. miR-492 expression level in HUVEC and human tissues

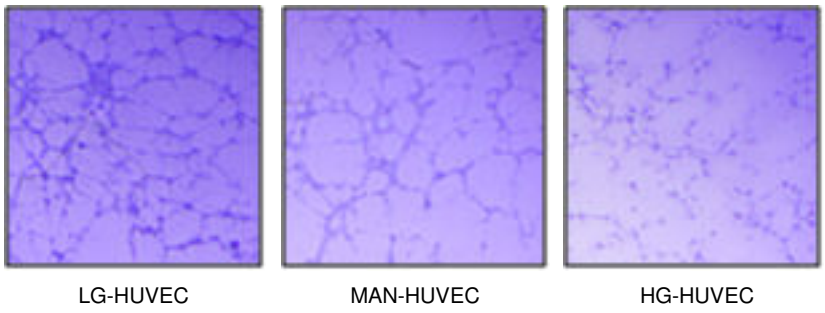
Figure S3.

Effects of high glucose concentration (30 mM) on HUVECs. We cultivated HUVEC in either high glucose-(HG-HUVEC, 30mM) or low glucose-(LG-HUVEC, 5mM) containing medium for 72 hours. HG-HUVEC were less proliferating than LG-HUVEC (Figure S3A), showed reduced ability to form tubes (Figure S3B) and down-regulation of eNOS, a key regulator of angiogenesis (Figure S3C).

A



B



C

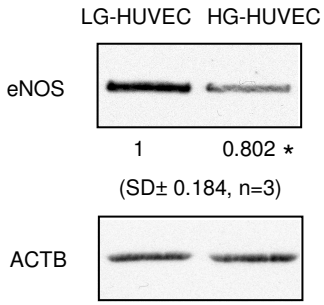


Figure S3. (A) Relative proliferation measured by MTS assay; **(B)** tube formation on matrigel; **(C)** eNOS protein expression. Mannitol 30 mM (MAN-HUVEC) was used as osmotic pressure control.

Figure S4.

Zebrafish/tumor xenograft angiogenesis assay. The zebrafish/tumor xenograft angiogenesis assay was performed as described by Nicoli and Presta [12]. DU-145 prostate cancer cells were transfected with 80 nM double stranded miR-492 mimic (miR-492) or negative control (ds-nc) using Polyfect Transfection Reagent (Qiagen) according to the manufacturer's instruction. After 48 hours cells were collected, and suspended in Cultrex BME (Basement Membrane Extract (R&D System). Tg(kdrl:eGFP)^{s843} zebrafish were raised in standard laboratory conditions (Westerfileld M zebrafish book) in a ZEBTEC Zebrafish Housing System (Tecniplast, Varese, Italy). Embryos were injected 48h post-fertilization and analyzed 24h post injection. For imaging embryos were treated with 0,01mM tricaine solution (Sigma-Aldric) and embedded in 1,2% low melting agarose medium. Stacks were acquired with a Leica TCS-SL DM IRE 2 confocal microscope and image stacks were processed with FIJI-WIN32 by projection.

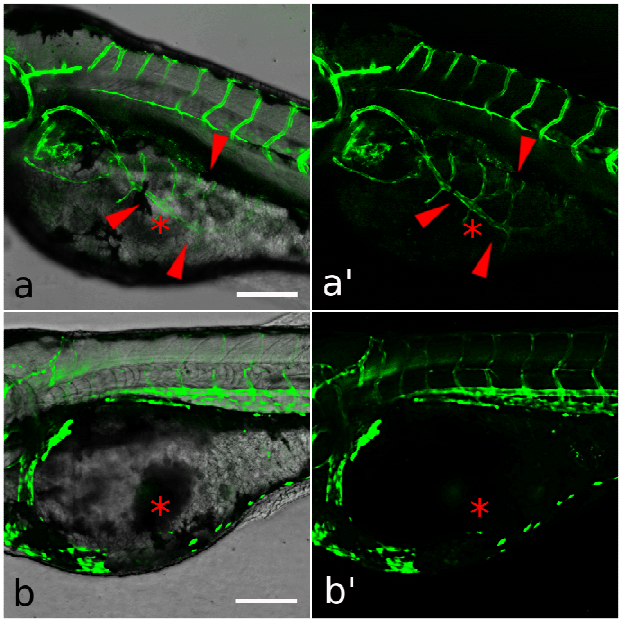


Figure S4. SX: zebrafish/tumor xenograft angiogenesis assay performed in Tg(kdrl:eGFP)^{s843} zebrafish strain: **(a)**, **(a')** 72hpf embryos injected with DU-145 cells transfected with ds-nc show new vessels (n= 22/22 embryos). **(b)**, **(b')** 72hpf embryos injected with DU-145 cells transfected with miR-492 show no angiogenetic response (n=17/20 embryos). Red star: site of injection, red arrows: new vessels, white scalebar: 150 µm.

Figure S5.

BRAF protein expression after miR-492 transfection. The luciferase assay ruled out the interaction of miR-492 with the cloned mRNA 3'UTR BRAF. Nevertheless, western blot verified BRAF down-regulation by miR-492.

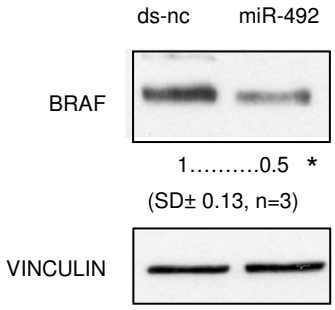


Figure S5. BRAF protein expression level in HUVEC transfected with either miR-492 or ds-nc.

Figure S6.

Set up and transfection of VEGF-HUVEC

Exponentially growing HUVEC p5 were seeded at cell density of $2x10^4/cm^2$ in EBM2 (Lonza) plus modified bullet kit (without VEGF) and containing 0.4% FBS. After 24 hours different concentrations of VEGF were added and 24 hours later the proliferation was measured as following. 7.5 10^3 cells per 24 well multiplate were seeded and at specified time points cells were fixed in 2% paraformaldehyde in PBS and subsequently stained with 0.1% crystal violet dissolved in 20% methanol and let dry at room temperature. Then, cells were lysed with acetic acid 10% and the optical density (OD 590 nm) of the solution, detected with Plate Reader apparatus (SpectraCount, Packard), was used to measure cell proliferation.

From dose-response curve (Figure S6A) we selected 10ng/ml VEGF and after 24 hours exposure to VEGF we measured expression level of miR-492, eNOS and its phosphorylated form by Western blot and found that miR-492 was downregulated (Figure S6B) whereas eNOS and p-eNOS were upregulated (Figure S6C and S6D). Finally we tested the susceptibility of VEGF-HUVEC to be transfected by using an oligoFITC. The FACScan analysis showed that approximately 70% of VEGF-HUVEC were fluorescent thus indicating that VEGF-HUVEC retained the ability to be transfected (Figure S6E).

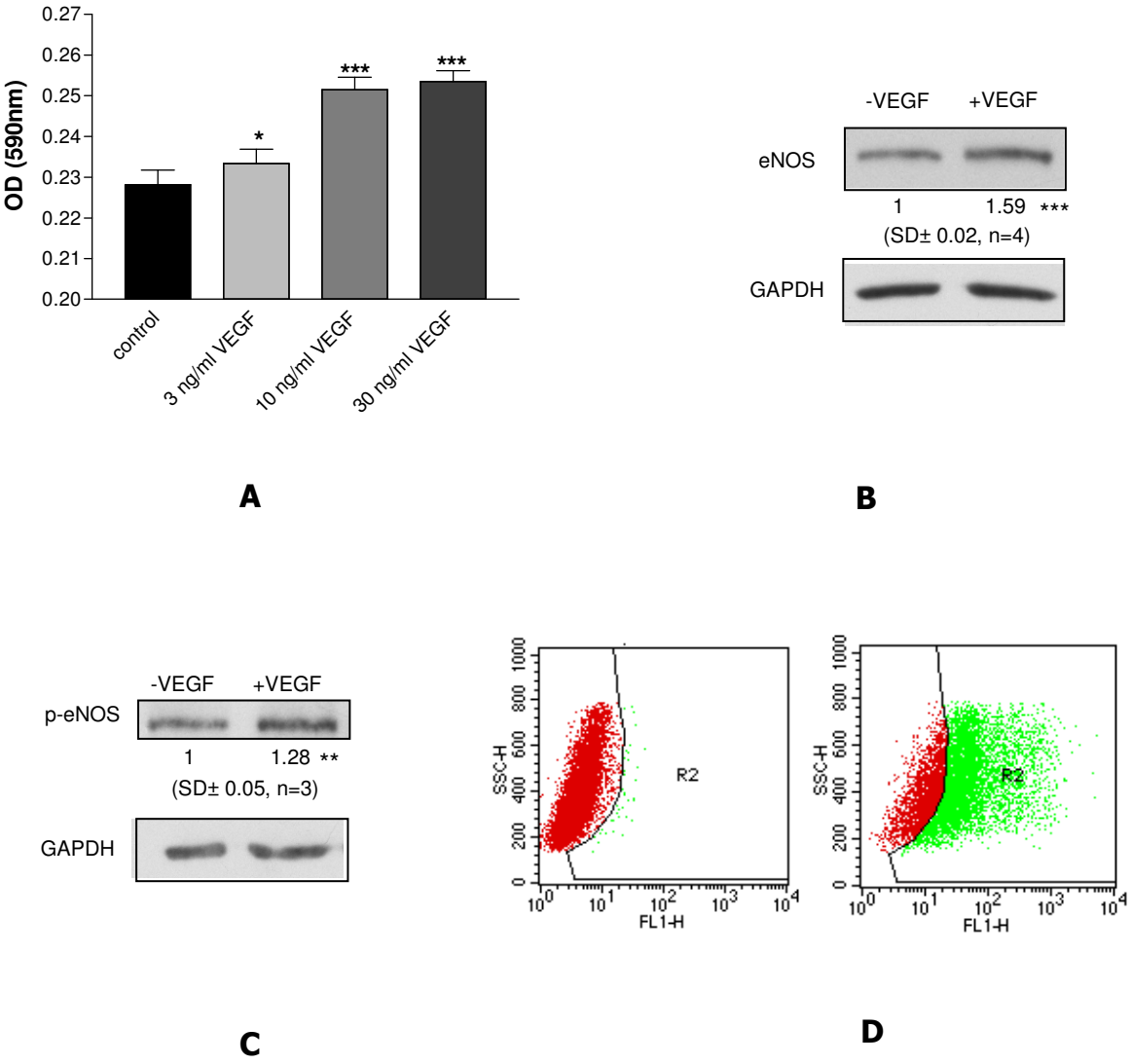


Figure S6. (A) VEGF dose-response curve; eNOS **(B)** and p-eNOS **(C)** quantification; **(E)** Scattergram analysis of VEGF-HUVEC untransfected (left panel) and transfected (right panel) with oligo FITC. Transfection efficiency was ~70%.

SUPPLEMENTAL REFERENCES

1. **Pitto L, Rizzo M, Simili M, et al.** miR-290 acts as a physiological effector of senescence in mouse embryo fibroblasts. *Physiological genomics*. 2009 Nov 6;39(3):210-8.
2. **Rainer J, Sanchez-Cabo F, Stocker G, Sturn A, Trajanoski Z.** CARMAweb: comprehensive R- and bioconductor-based web service for microarray data analysis. *Nucleic Acids Res*. 2006 Jul 1;34(Web Server issue):W498-503.
3. **Wilhelm SM, Carter C, Tang L, et al.** BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res*. 2004 Oct 1;64(19):7099-109.
4. **Heo SH, Choi YJ, Ryoo HM, Cho JY.** Expression profiling of ETS and MMP factors in VEGF-activated endothelial cells: role of MMP-10 in VEGF-induced angiogenesis. *J Cell Physiol*. Sep;224(3):734-42.
5. **Townsend KJ, Trusty JL, Traupman MA, Eastman A, Craig RW.** Expression of the antiapoptotic MCL1 gene product is regulated by a mitogen activated protein kinase-mediated pathway triggered through microtubule disruption and protein kinase C. *Oncogene*. 1998 Sep 10;17(10):1223-34.
6. **Wojnowski L, Zimmer AM, Beck TW, et al.** Endothelial apoptosis in Braf-deficient mice. *Nat Genet*. 1997 Jul;16(3):293-7.
7. **Arnoldussen YJ, Saatcioglu F.** Dual specificity phosphatases in prostate cancer. *Mol Cell Endocrinol*. 2009 Oct 15;309(1-2):1-7.
8. **Zhang R, Min W, Sessa WC.** Functional analysis of the human endothelial nitric oxide synthase promoter. Sp1 and GATA factors are necessary for basal transcription in endothelial cells. *The Journal of biological chemistry.* 1995 Jun 23;270(25):15320-6.
9. **Primo L, di Blasio L, Roca C, et al.** Essential role of PDK1 in regulating endothelial cell migration. *J Cell Biol*. 2007 Mar 26;176(7):1035-47.
10. **Partovian C, Simons M.** Regulation of protein kinase B/Akt activity and Ser473 phosphorylation by protein kinase Calpha in endothelial cells. *Cellular signalling.* 2004 Aug;16(8):951-7
11. **Kauffmann-Zeh A, Thomas GM, Ball A, et al.** Requirement for phosphatidylinositol transfer protein in epidermal growth factor signaling. *Science.* 1995 May 26;268(5214):1188-90.
12. **Nicoli S, Presta M**.The zebrafish/tumor xenograft angiogenesis assay. *Nature Protocols* 2007 (2): 2918 - 2923.