MicroRNA-132 mediated loss of p120RasGAP activates endothelium to facilitate pathological angiogenesis Sudarshan Anand, Bharat K. Majeti, Lisette M. Acevedo, Eric A. Murphy, Rajesh Mukthavaram, Lea Scheppke, Miller

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Days/Treatment hsa-let-7a-4373169 hsa-let-7b-4373168 hsa-let-7c-4373167 hsa-let-7d-4373166 hsa-let-7e-4373165 hsa-let-7f-4373164 hsa-let-7g-4373163 hsa-miR-1-4373161 hsa-miR-100-4373160 hsa-miR-101-4373159 hsa-miR-103-4373158 hsa-miR-105-4373157 hsa-miR-106b-4373155 hsa-miR-107-4373154 hsa-miR-10a-4373153 hsa-miR-10b-4373152 hsa-miR-122a-4373151 hsa-miR-124a-4373150 hsa-miR-125a-4373149 hsa-miR-125b-4373148 hsa-miR-126-4373269 hsa-miR-126-4378064 hsa-miR-127-4373147 hsa-miR-128b-4373170 hsa-miR-129-4373171 hsa-miR-130a-4373145 hsa-miR-130b-4373144 hsa-miR-132-4373143 hsa-miR-133a-4373142 hsa-miR-133b-4373172 hsa-miR-134-4373141 hsa-miR-135a-4373140 hsa-miR-135b-4373139 hsa-miR-137-4373174 hsa-miR-139-4373176 hsa-miR-140-4373138 hsa-miR-141-4373137 hsa-miR-142-3p-4373136 hsa-miR-142-5p-4373135 hsa-miR-143-4373134 hsa-miR-145-4373133 hsa-miR-146a-4373132 hsa-miR-146b-4373178 hsa-miR-147-4373131 hsa-miR-148a-4373130 hsa-miR-148b-4373129 hsa-miR-149-4373128 hsa-miR-151-4373179 hsa-miR-152-4373126



Days/Treatment hsa-miR-155-4373124 hsa-miR-15a-4373123 hsa-miR-15b-4373122 hsa-miR-16-4373121 hsa-miR-17-3p-4373120 hsa-miR-17-5p-4373119 hsa-miR-181b-4373116 hsa-miR-181c-4373115 hsa-miR-181d-4373180 hsa-miR-182-4373271 hsa-miR-182-4378066 hsa-miR-183-4373114 hsa-miR-184-4373113 hsa-miR-185-4373181 hsa-miR-186-4373112 hsa-miR-187-4373111 hsa-miR-189-4378067 hsa-miR-18a-4373118 hsa-miR-18b-4373184 hsa-miR-190-4373110 hsa-miR-191-4373109 hsa-miR-192-4373108 hsa-miR-193a-4373107 hsa-miR-193b-4373185 hsa-miR-194-4373106 hsa-miR-195-4373105 hsa-miR-196a-4373104 hsa-miR-196b-4373103 hsa-miR-197-4373102 hsa-miR-198-4373101 hsa-miR-199a-4373272 hsa-miR-199a-4378068 hsa-miR-199b-4373100 hsa-miR-19a-4373099 hsa-miR-19b-4373098 hsa-miR-200a-4373273 hsa-miR-200a-4378069 hsa-miR-200b-4381028 hsa-miR-200c-4373096 hsa-miR-202-4373274 hsa-miR-202-4378075 hsa-miR-203-4373095 hsa-miR-204-4373094 hsa-miR-205-4373093 hsa-miR-206-4373092 hsa-miR-208-4373091 hsa-miR-20a-4373286 hsa-miR-20b-4373263 hsa-miR-21-4373090 hsa-miR-210-4373089



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hsa-miR-153-4373125

Days/Treatment

hsa-miR-211-4373088 hsa-miR-213-4373086 hsa-miR-214-4373085 hsa-miR-215-4373084 hsa-miR-216-4373083 hsa-miR-217-4373082 hsa-miR-218-4373081 hsa-miR-219-4373080 hsa-miR-22-4373079 hsa-miR-220-4373078 hsa-miR-221-4373077 hsa-miR-222-4373076 hsa-miR-223-4373075 hsa-miR-224-4373187 hsa-miR-23a-4373074 hsa-miR-23b-4373073 hsa-miR-24-4373072 hsa-miR-25-4373071 hsa-miR-26a-4373070 hsa-miR-26b-4373069 hsa-miR-27a-4373287 hsa-miR-27b-4373068 hsa-miR-28-4373067 hsa-miR-296-4373066 hsa-miR-299-3p-4373189 hsa-miR-299-5p-4373188 hsa-miR-29a-4373065 hsa-miR-29c-4373289 hsa-miR-301-4373064 hsa-miR-302a-4373275 hsa-miR-302a-4378070 hsa-miR-302b-4373276 hsa-miR-302b-4378071 hsa-miR-302c-4373277 hsa-miR-302c-4378072 hsa-miR-302d-4373063 hsa-miR-30a-3p-4373062 hsa-miR-30a-5p-4373061 hsa-miR-30b-4373290 hsa-miR-30c-4373060 hsa-miR-30d-4373059 hsa-miR-30e-3p-4373057 hsa-miR-30e-5p-4373058 hsa-miR-31-4373190 hsa-miR-32-4373056 hsa-miR-320-4373055 hsa-miR-323-4373054 hsa-miR-324-3p-4373053 hsa-miR-324-5p-4373052 hsa-miR-325-4373051



Days/Treatment

hsa-miR-326-4373050 hsa-miR-328-4373049 hsa-miR-329-4373191 hsa-miR-33-4373048 hsa-miR-330-4373047 hsa-miR-331-4373046 hsa-miR-335-4373045 hsa-miR-337-4373044 hsa-miR-338-4373043 hsa-miR-339-4373042 hsa-miR-340-4373041 hsa-miR-342-4373040 hsa-miR-345-4373039 hsa-miR-34a-4373278 hsa-miR-34b-4373037 hsa-miR-34c-4373036 hsa-miR-361-4373035 hsa-miR-362-4378092 hsa-miR-363-4380917 hsa-miR-365-4373194 hsa-miR-367-4373034 hsa-miR-368-4373033 hsa-miR-369-3p-4373032 hsa-miR-369-5p-4373195 hsa-miR-371-4373030 hsa-miR-372-4373029 hsa-miR-373-4373279 hsa-miR-373-4378073 hsa-miR-374-4373028 hsa-miR-375-4373027 hsa-miR-376a-4373026 hsa-miR-376a-4378104 hsa-miR-376b-4373196 hsa-miR-378-4373024 hsa-miR-379-4373023 hsa-miR-380-3p-4373022 hsa-miR-380-5p-4373021 hsa-miR-381-4373020 hsa-miR-382-4373019 hsa-miR-383-4373018 hsa-miR-409-5p-4373197 hsa-miR-410-4378093 hsa-miR-411-4381013 hsa-miR-412-4373199 hsa-miR-422a-4373200 hsa-miR-422b-4373016 hsa-miR-423-4373015 hsa-miR-424-4373201 hsa-miR-425-4373202 hsa-miR-425-5p-4380926



Days/Treatment

hsa-miR-429-4373203 hsa-miR-432-4373280 hsa-miR-432-4378076 hsa-miR-433-4373205 hsa-miR-448-4373206 hsa-miR-449-4373207 hsa-miR-449b-4381011 hsa-miR-450-4373208 hsa-miR-451-4373209 hsa-miR-452-4373281 hsa-miR-452-4378077 hsa-miR-453-4373210 hsa-miR-484-4381032 hsa-miR-485-3p-4378095 hsa-miR-485-5p-4373212 hsa-miR-486-4378096 hsa-miR-487b-4378102 hsa-miR-488-4373213 hsa-miR-489-4373214 hsa-miR-490-4373215 hsa-miR-491-4373216 hsa-miR-492-4373217 hsa-miR-493-4373218 hsa-miR-494-4373219 hsa-miR-496-4373221 hsa-miR-497-4373222 hsa-miR-500-4373225 hsa-miR-501-4373226 hsa-miR-502-4373227 hsa-miR-503-4373228 hsa-miR-504-4373229 hsa-miR-505-4373230 hsa-miR-506-4373231 hsa-miR-507-4373232 hsa-miR-508-4373233 hsa-miR-509-4373234 hsa-miR-510-4373235 hsa-miR-511-4373236 hsa-miR-512-3p-4381034 hsa-miR-512-5p-4373238 hsa-miR-513-4373239 hsa-miR-514-4373240 hsa-miR-515-3p-4373241 hsa-miR-515-5p-4373242 hsa-miR-516-5p-4378099 hsa-miR-517-4378078 hsa-miR-517a-4373243 hsa-miR-517b-4373244 hsa-miR-517c-4373264 hsa-miR-518a-4373186



Days/Treatment

hsa-miR-518b-4373246 hsa-miR-518c-4373247 hsa-miR-518c-4378082 hsa-miR-518d-4373248 hsa-miR-518e-4373265 hsa-miR-518f-4378083 hsa-miR-519b-4373250 hsa-miR-519c-4373251 hsa-miR-519d-4373266 hsa-miR-519e-4373267 hsa-miR-520a-4373268 hsa-miR-520b-4373252 hsa-miR-520c-4373253 hsa-miR-520d-4373254 hsa-miR-520e-4373255 hsa-miR-520f-4373256 hsa-miR-520g-4373257 hsa-miR-520h-4373258 hsa-miR-521-4373259 hsa-miR-522-4373245 hsa-miR-523-4373260 hsa-miR-524-4378087 hsa-miR-526b-4378080 hsa-miR-532-4380928 hsa-miR-542-5p-4378105 hsa-miR-544-4380919 hsa-miR-545-4380918 hsa-miR-548a-4380948 hsa-miR-548b-4380951 hsa-miR-548c-4380993 hsa-miR-548d-4381008 hsa-miR-549-4380921 hsa-miR-550-4380954 hsa-miR-551a-4380929 hsa-miR-551b-4380945 hsa-miR-552-4380930 hsa-miR-553-4380931 hsa-miR-554-4380932 hsa-miR-555-4380933 hsa-miR-556-4380934 hsa-miR-558-4380936 hsa-miR-562-4380939 hsa-miR-563-4380940 hsa-miR-564-4380941 hsa-miR-565-4380942 hsa-miR-566-4380943 hsa-miR-569-4380946 hsa-miR-570-4380947 hsa-miR-572-4381017 hsa-miR-575-4381020



Days/Treatment

hsa-miR-576-4381021 hsa-miR-578-4381022 hsa-miR-579-4381023 hsa-miR-580-4381024 hsa-miR-585-4381027 hsa-miR-586-4380949 hsa-miR-587-4380950 hsa-miR-588-4380952 hsa-miR-589-4380953 hsa-miR-591-4380955 hsa-miR-593-4380957 hsa-miR-594-4380958 hsa-miR-596-4380959 hsa-miR-597-4380960 hsa-miR-599-4380962 hsa-miR-600-4380963 hsa-miR-601-4380965 hsa-miR-603-4380972 hsa-miR-606-4380974 hsa-miR-607-4380975 hsa-miR-608-4380976 hsa-miR-609-4380978 hsa-miR-613-4380989 hsa-miR-614-4380990 hsa-miR-615-4380991 hsa-miR-616-4380992 hsa-miR-617-4380994 hsa-miR-618-4380996 hsa-miR-622-4380961 hsa-miR-624-4380964 hsa-miR-626-4380966 hsa-miR-627-4380967 hsa-miR-629-4380969 hsa-miR-630-4380970 hsa-miR-631-4380971 hsa-miR-633-4380979 hsa-miR-639-4380987 hsa-miR-642-4380995 hsa-miR-644-4380999 hsa-miR-645-4381000 hsa-miR-646-4381002 hsa-miR-647-4381003 hsa-miR-649-4381005 hsa-miR-650-4381006 hsa-miR-651-4381007 hsa-miR-652-4380927 hsa-miR-653-4381012 hsa-miR-654-4381014 hsa-miR-656-4380920 hsa-miR-657-4380922







	hES Vascular Differentiation	
Days/Treatment	14	21
hsa-miR-658-4380923		
hsa-miR-659-4380924		
hsa-miR-660-4380925		
hsa-miR-661-4381009		
hsa-miR-662-4381010		
hsa-miR-7-4373014		
hsa-miR-9-4373285		
hsa-miR-9-4378074		
hsa-miR-92-4373013		
hsa-miR-93-4373012		
hsa-miR-95-4373011		
hsa-miR-96-4373010		
hsa-miR-98-4373009		
hsa-miR-99a-4373008		
hsa-miR-99b-4373007		

HUVECs

FGF

VEGF



Supplementary Figure 1. Heatmap showing miR profiles of hES cell vascular differentiation at day 14, 21 and HUVECs treated with VEGF and FGF

microRNA	hES cell vasculogenesis	HUVEC VEGF/FGF	Combined Rank
miR-132	8	19	1
miR-501	27	1	2
miR-181c	20	26	3
miR-646	14	40	4
miR-575	18	43	5

Supplementary Figure 2. Ranking of miRs in angiogenesis screens.

The raw Ct (Cycle threshold) values from the TaqMan panel were filtered to include only Ct values below 32 to eliminate false positives based on the manufacturer's recommendation. miR fold upregulation was calculated on the basis of miR Ct values compared to the Ct values of the housekeeping small RNA RNU48. The miRs were ranked according to their fold upregulation compared to the untreated control for HUVECs and day 0 values for hES differentiation. Combined Rank was obtained by averaging the rank of both the hES cell and HUVEC screens.



Supplementary Figure 3. miR-132 is rapidly upregulated upon growth factor treatment in HUVECs. (a) HUVECs were starved overnight and treated with 40 ng/ml recombinant human VEGF-165. At the indicated time points the cells were harvested lysed and assayed for phosphorylation of CREB (Ser133). The numbers indicate band intensities relative to 0 hour. (b) HUVECs were starved overnight and treated with either 40ng/ml recombinant human VEGF-165 (grey bars) or 100 ng/ml bFGF (black bars). RNA was isolated and RT-PCR was performed at the indicated time points. Bars reflect mean change of miR-132 levels normalized to the expression of the housekeeping small RNA RNU-48. (c) HUVECs were starved overnight and treated with either complete DMEM or conditioned DMEM from tumors and RT-PCR was performed as described in (b). (d) HUVECs were starved overnight and treated with Vatalanib or vehicle for 30 minutes and subsequently either conditioned media or DMEM was added. 15 minutes later the cells were lysed and assayed for phosphorylated CREB.



Supplementary Figure 4. Expression and knockdown of miR-132 in HUVECs. (a) Representative confocal image of FITC-lectin labeled HUVECs transfected with 30nM Cy3-labeled miR or anti-miR showing the perinuclear localization of miR or anti-miR. Scale bar = 50μ m (b) HUVECs were transfected with 30nM control miR or miR-132 or control anti-miR or anti-miR-132. 48 hours later the cells were harvested and RT-PCR was performed. Fold change with respect to RNU48 is shown. One of three independent experiments is shown.

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Supplementary Figure 5. Anti-miR-132 has minimal effect on established

vasculature in the retina. a) Representative images of the superficial plexus from P12 retinas of mice injected with either a control anti-miR or Anti miR-132 and stained with CD31-FITC. Bar graph depicts mean + s.e.m of CD31 area as measured by pixel intensity. **b)** Representative images from retinas of adult mice stained with CD31 and SMA. Scale Bar = 100 microns

ank		Alternative names	Binding Energy (kcal/mol)	Synergistic sites
1	RasGAP	Rasa1, p120RasGAP	-23.0 and -21.1	Yes
2	TMEFF1	tomoregulin-1	-22.6	No
3	LRRFIP1	GCF2, TRIP	-20.4	No
4	ZNF644	zinc-finger protein 644	-19.8	No
5	тімм9	TIM9	-17.2	No

Cow ----- AGAAUUUAACUGGAUCACA<mark>GACAG</mark>--UUCUUACUGCAAUUUCUUCU------ AUGAACAUGACCAUUUGACUG-UUCACUGACU Supplementary Figure 6. In silico identification of p120RasGAP as a putative miR-132

target.(a) Venn diagram of the predictions of human miR-132 targets by three different algorithms yielding 5 common hits. **(b)** Ranking of the 5 common targets based on the RNA hybrid scores and presence of synergistic sites¹⁷. **(c)** Schematic illustration of RNAhybrid models showing binding of miR-132 (green) with human p120RasGAP 3' untranslated region (UTR). **(d)** p120RasGAP 3'UTR sequences from different species showing a high degree of homology. Shaded regions represent the binding site of the miR-132 seed sequences

Supplementary Figure 7. miR-132 transfection does not downregulate some of the top predicted targets. HUVECs were transfected with either a control miR or miR-132. 48 hours later the cells were lysed and the lysates were blotted for the indicated proteins (left panel) or RNA was isolated and RT-PCR was performed as described. The levels of TIMM9A were normalized to β -actin.

Ctrl AM-132 1 1.7 p120RasGAP β-actin

Supplementary Figure 8. Anti-miR-132 upregulates p120RasGAP in vitro and in vivo. (a) HUVECs were transfected with either scrambled control anti-miR or 30nM anti-miR-132 and assayed for p120RasGAP expression. Numbers indicate fold increase normalized to loading control. (b) and (c) B6 mice were injected with bFGF containing matrigel plugs. On day 3 and day 4, mice were injected i.v. with either 20 μ g of scrambled anti-miR or anti-miR-132. Mice were euthanized on day 5 and the plugs were harvested. Representative confocal images (b) and mean % overlap between CD31 and p120RasGAP (c) is shown. n=3 mice per group. Scale bar = 50 μ m.

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Supplementary Figure 9. miR-132 functions through the regulation of the MEK pathway in HUVECS. a) HUVECs were transfected with either a control anti-miR or anti miR-132. 24 hours later, the cells were starved overnight and treated with recombinant human VEGF-165 (50 ng/ml). 10 minutes later the cells were lysed and pMEK levels were measured using a Pathscan ELISA kit. Mean + s.e.m. of duplicate samples are shown. b) HUVECs were transfected with either a control miR or miR-132 in serum free media. 24 hours later the cells were treated with a MEK inhibitor at the indicated concentrations and 30 minutes later treated with 50 ng/ml recombinant humanVEGF-165. Subsequently the cells were pulsed with BrdU and the proliferation was measured using a BrdU ELISA.

Supplementary Figure 10. miR-132 regulates p120RasGAP in mouse endothelioma cells. (a) b.End3 cells were starved overnight and stimulated with 50 ng/ml recombinant human VEGF-165. RNA was isolated 6 hours later and RT-PCR was performed for miR-132 and snoRNA202. miR-132 levels normalized to snoRNA202 are shown. (b) b.End3 cells were transfected with 50 nM control miR or miR-132. 48 hours later, the cells were lysed and probed for p120RasGAP expression by western blot. (c) b.End3 cells were transfected as in (b) and proliferation was measured 48 hours later by BrdU incorporation. Bars show mean + s.e.m. * P<0.05

Supplementary Figure 11. Reciprocal expression of miR-132 and p120RasGAP on tumor endothelium vs normal endothelium. Tissue sections from a normal mouse pancreas or an orthotopic pancreatic carcinoma stained with CD31 and p120RasGAP (a) or stained for miR-132 expression by in situ hybridization (b). One representative image of atleast three mice is shown. Scale bar (a) = 100 microns, (b) = 25 microns.

Normal Tissue

Hemangiomas

Hemangiomas

Normal Tissue

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Supplementary Figure 12. Reciprocal expression of miR-132 and p120RasGAP on hyperproliferative hemangiomas but not on the normal endothelium. (a) Representative images from immunohistochemical staining for p120RasGAP on normal tissue array and hemangioma array. Arrows indicate blood vessels staining positive and * indicates vessels staining negative for p120RasGAP. Scale bar = 100 microns.(b) In-situ hybridization of human hemangioma tissue array or normal human tissue slides. Nuclei stained with TOPRO are pseudo-colored green. Scale bar = 100 microns (c) Representative images from immunohistochemical staining for p120RasGAP and (d) in situ hybridization for miR-132 on breast tumor and adjacent normal samples from a tissue array. * indicates vessels. Scale bar = 100 microns

Supplementary Figure 13. Delivery of anti miR-132 using nanoparticles to tumor vasculature. Nude mice were injected subcutaneously with 2x10⁶ M21 human melanoma cells. 14 days later mice were injected i.v with BODIPY labeled RGD-nanoparticles containing Cy-3 labeled anti-miR-132. The mice were euthanized 6 hours later and the tumors were harvested, sectioned and imaged using confocal microscopy.

CD31-FITC

Supplementary Figure 14. Targeted delivery of anti-miR-132 decreases

angiogenesis in tumors. a) C57BL/6 mice were injected subcutaneously with 50,000 VEGF-expressing, GFP+, ID-8 ovarian carcinoma cells in matrigel. Two days later, treatment was initiated with either 10 μ g scrambled anti-miR or anti-miR-132 in RGD-nanopartcles and repeated every two days. On day 10 the mice were injected with Alexafluor-647 lectin, euthanized, plugs were harvested, lysed in RIPA and the lectin content was quantified on a spectrophotometer (right panel). Bars show mean + s.e.m of atleast 4 mice per group. Scale bar = 100 microns. b) Nude mice were implanted with 2 x 10⁶ MDA-MB 231 cells in the mammary fat pad. 8 days later, the tumors were measured and mice with palpable tumors of similar volumes were randomly assigned to three groups. Mice received 50 μ g of scrambled anti-miR or anti-miR-132 every 48 hours until day 24. Representative images from sections stained for CD31 expression are shown.

Supplementary Figure 15. Anti miR-132 upregulates expression of p120RasGAP in LYVE1 positive cells. Nude mice were implanted with 2×10^6 MDA-MB 231 cells in the mammary fat pad. 8 days later, the tumors were measured and mice with palpable tumors of similar volumes were randomly assigned to treatment groups receiving either 50 µg of scrambled anti-miR or anti-miR-132 every 48 hours until day 24. Tumor sections were stained for LYVE-1 and p120RasGAP expression (a) and the LYVE1 p120RasGAP colocalization was quantified using Metamorph (b). Scale bar = 100 microns. Bars show mean + s.e.m of 3 sections from atleast 3 mice per group

Supplementary Figure 16. Deletion of p120RasGAP increases the angiogenic response to bFGF in Matrigel plugs. p120RasGAP^{fl/fl} Cre⁺ mice were left untreated or treated with Tamoxifen (2mg/mouse in corn oil) every three days for a total of nine days. They were then implanted with subcutaneous matrigel plugs containing bFGF. Five days later, the mice were injected with FITC-lectin and the plugs were harvested, homogenized and the lectin content was quantified. Bars show mean+ s.e.m of atleast 3 plugs per group.