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Upregulated microRNA-21 drives proliferation of lymphatic malformation endothelial cells by inhibiting PDCD4

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To the editor,

Lymphatic malformations (LMs) are congenital anomalies of the lymphatic vasculature that affect approximately 1:4000 live births and consist of dilated lymphatic channels surrounded by hypertrophic stroma, resulting in a compressible lesion with a mass effect. Somatic activating mutations in *PIK3CA*, which encodes the p110α catalytic subunit of PI3K, have been identified in approximately 80% of LMs and are localized to lymphatic malformation endothelial cells (LM-ECs) (Boscolo et al., 2015). Targeted therapeutics such as sirolimus have been used to suppress the hyperactivation of PI3K/AKT/mTOR signaling in patients

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AUTHOR CONTRIBUTIONS

Writing original draft: RS; Investigation: RS, HZ, SM, HB, JS, CS, AB, AL, JB, DJ, VD; Methodology: CS, JK, GS. Resources: GR, CW, SB, JP, CS, JK; Software: CW, SB. Writing: GS, RS; Supervision: GS; Conceptualization: GS

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CONFLICT OF INTEREST

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with LMs and can reduce disease burden (Adams et al., 2016). While promising, symptom relief is variable and significant side effects have been reported (Rossler et al., 2021). The mechanisms underlying how *PIK3CA* mutations drive the LM phenotype are unclear, and whether epigenetic dysregulation contributes to LM pathogenesis has not been investigated.

MicroRNAs (miRNAs) regulate lymphangiogenesis and endothelial cell function (Chen et al., 2016) and drive cellular proliferation through regulation of PI3K (Catanzaro et al., 2018). While miRNA dysfunction has been identified in other vascular anomalies such as hemangiomas (Strub et al., 2016), the contribution of miRNAs to LM pathogenesis is unknown. In this study, we identified the upregulation of the oncogenic miRNA-21 in LMs containing *PIK3CA* mutations, and conducted further studies to determine if miR-21 inhibition may be a viable adjuvant molecular therapy for LMs.

LM and normal skin/subcutaneous tissue samples were collected during resection from 18 pediatric patients after written, informed consent and approval by the University of Arkansas for Medical Sciences Institutional Review Board (IRB # 114012). Macrocystic components of the LMs including the cyst wall were dissected free of surrounding tissue and collected as LM samples. Once the entirety of the lesion was removed, skin including subcutaneous tissue and subdermal lymphatics was collected from an adjacent site as control tissue. Digital droplet PCR (ddPCR) detected *PIK3CA* mutations (p.E542K, E545K and H1047R) in 13 of the 18 LM samples. RT-PCR of miRNAs known to regulate PI3K and/or normal lymphangiogenesis demonstrated that miR-21 was significantly upregulated in the LM tissue samples containing *PIK3CA* mutations when compared to normal skin/subcutaneous tissue from the same patients, despite similar lymphatic endothelial density as demonstrated by equivocal podoplanin (PDPN) mRNA expression (Figure 1a). *In situ* hybridization probes targeting miR-21 demonstrated concentration of miR-21 in the lymphatic endothelium of 2 patient samples analyzed (Figure 1b). Due to the limitations of this technique and its inability to quantitate endothelial miR-21 expression, lymphatic endothelial cells were isolated using podoplanin-conjugated magnetic beads from both LM tissue and normal control tissue from 2 patients and their levels of miR-21 were compared using RT-PCR (Figure 1c). miR-21 was enriched in PDPN+ cells isolated from LM tissue when compared to PDPN− cells, and miR-21 expression was upregulated in LM-ECs when compared to LECs isolated form control tissues. To confirm successful isolation of $PIK3CA^{mut}$ LM-ECs, purified LM-ECs were cultured as previously described (Wu et al., 2015) and analyzed at successive passages by ddPCR, which demonstrated a $PIK3CA^{mut}$ variant allele frequency of 50% after 1-2 passages (Figure 1d). A proliferation assay comparing $PIK3CA^{mut}$ LM-ECs to normal human lymphatic endothelial cell lines (HDLECs) demonstrated significantly higher proliferative rates in $PIK3CA^{mut}$ cells (Figure 1e). Taken together, these experiments demonstrate increased miR-21 expression and higher proliferative rates in $PIK3CA^{mut}$ lymphatic endothelial cells when compared to wildtype.

miR-21 is an oncogenic miRNA which silences the expression of several tumor suppressors, and its upregulation has been demonstrated to increase cellular proliferation in multiple cell types (reviewed by (Arghiani and Matin, 2021)). To determine whether upregulation of miR-21 in LM-ECs resulted in inhibition of endothelial tumor suppressors, we compared the expression of 5 miR-21 targets (*PDCD4* (programmed cell death protein 4) (Xu et al., 2017),

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SPRY-1 (Sprouty 1) (Zhuang et al., 2020), *TIMP-3* (tissue inhibitor of metalloproteinase 3) (Hu et al., 2016), *RhoB* (Ras homolog family member B) (Sabatel et al., 2011), and PTEN (phosphatase and tensin homolog) (Luo et al., 2017) by RT-PCR and western blot in HDLECs and LM-ECs (Figure 2a-b). PDCD4, SPRY-1, TIMP-3, and RhoB mRNA and protein levels were all significantly lower in LM-ECs compared to HDLECs, while PTEN mRNA expression was equivocal.

To determine whether upregulation of miR-21 was responsible for the attenuation of PDCD4/SPRY-1/TIMP-3/RhoB protein expression observed in LM-ECs, HDLECs and LM-ECs were transfected with miR-21 antisense RNA (mir-21−), miR-21 mimic RNA (miR-21+), and scrambled miR-21 RNAs as controls (Figure 2c). In HDLECs, inhibition of miR-21 led to an approximate 10% increase in PDCD4 protein levels, while overexpression of miR-21 reduced PDCD4 protein levels by approximately 70% (Figure 2d). Conversely, in LM-ECs, miR-21 inhibition increased PDCD4 protein levels by approximately 60%, while miR-21 overexpression modestly reduced PDCD4 protein levels (~ 20%) (Figure 2d). Transient transfection with miR-21 inhibitors or mimics did not affect SPRY-1/TIMP-3/RhoB protein levels in either cell type (data not shown).

Because the miR-21-PDCD4 axis is known to regulate endothelial cell proliferation (Hua et al., 2020), we compared the proliferative rates of HDLECs and LM-ECs in the presence of miR-21 inhibition or overexpression (Figure 2e). Overexpression of miR-21 increased the proliferative rates of both HDLECs and LM-ECs. In the presence of miR-21 inhibition, LM-EC proliferation was dramatically reduced when compared to the effect of miR-21 inhibition in HDLECs. These results suggest that in *PIK3CA*^{mut} LM-ECs that express abnormally high levels of miR-21, inhibition of miR-21 can reverse their hyperproliferative phenotype.

In summary, this study identifies the contribution of an aberrantly expressed miRNA in LM-ECs that drives their hyperproliferative phenotype and is reversible with miRNA inhibition, which to our knowledge has not been reported. In addition, this study demonstrates downregulation of the tumor suppressors PDCD4, SPRY-1, TIMP-3, and RhoB in LM-ECs, potentially identifying additional molecular targets for treatment. miRNA-based therapeutics are emerging as methods of targeting aberrantly expressed genes in specific cell types. A recent study demonstrated that transdermal nanocarrier delivery of a miR-21 mimic in a murine wound healing model could inhibit PDCD4 expression and increase cellular proliferation (Wang et al., 2020). A similar delivery of miR-21 inhibitors to LMs may have the opposite effect, representing a viable and minimally invasive method of reducing LM-EC proliferation *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY STATEMENT

All original data can be obtained by contacting the corresponding author at gstrub@uams.edu. No datasets were generated or analyzed during the current study.

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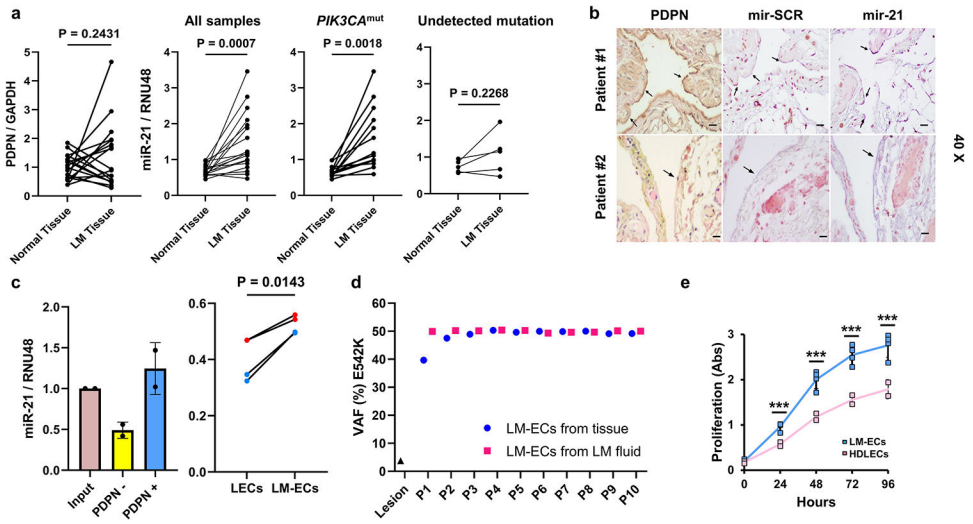

Figure 1: miR-21 is upregulated in LM tissue and hyperproliferative endothelium.

A total of 18 patients underwent surgical excision of LM tissue and adjacent normal tissue and ddPCR was performed to detect *PIK3CA* mutations. (**a**) miR-21 and PDPN expression levels were analyzed and compared between normal tissue and LM tissue. miRNA levels were normalized to RNU48, and mRNA levels were normalized to GAPDH. Paired two-tailed T tests were performed to determine statistical significance ($P < 0.05$ was considered significant) and significantly up- or downregulated miRNAs or mRNAs are illustrated on the figure. Error bars represent standard deviation and individual values are represented as white dots. (**b**) Adjacent paraffin sections of LM tissue from 2 patients were stained with anti-PDPN or *in situ* hybridization probes to miR-21 or a scrambled miR-21 control. Black arrows indicate the same cellular location on adjacent slides. Scale bar = 100 μm. (**c**) A single cell suspension of LM cells was subjected in triplicate to PDPN+ magnetic bead pulldown, and miR-21 expression in the input, PDPN−, and PDPN+ populations were determined by qRT-PCR. Simultaneously from two additional patients, triplicate single cell suspensions of both normal control tissue (LEC) and LM tissue (LM-EC) were subjected to PDPN+ bead pulldown, and miR-21 expression was determined with qRT-PCR, demonstrating increased miR-21 expression in LM-ECs ($P = 0.0143$, paired two-tailed T test). (**d**) ddPCR for *PIK3CA* hotspot mutations was performed on LM tissue (E542K mutation detected with VAF of 3.78%) and LM-ECs from 10 subsequent passages of isolated cells. (**e**) WST-8 proliferation experiments on 4 isolated p.Glu542Lys LM-EC cultures were compared to HDLECs (2 independent experiments).

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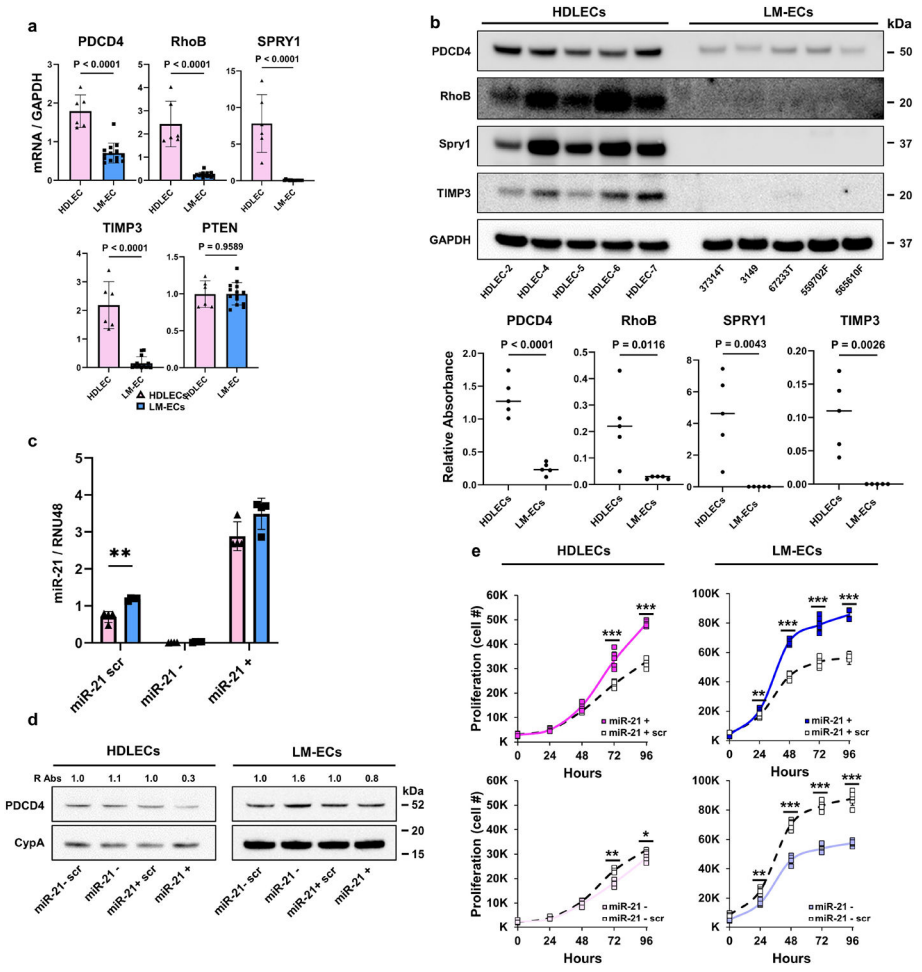

Figure 2: The miR-21 target PDCD4 is down regulated in LM endothelial cells and is restored with miR-21 inhibition, reducing endothelial proliferation.

(**a**) LM-EC cultures (n=14) were isolated from LM tissue or cyst fluid and were analyzed with RT-PCR for the miR-21 targets PDCD4, SPRY1, TIMP-3, RhoB, and PTEN. HDLEC cell lines (n=6) from different patients were used as controls. (**b**) HDLEC (n=5) and LM-EC (n=5) cell pellets from different patients were collected and protein was isolated and analyzed by western blot with primary antibodies for PDCD4, SPRY-1, TIMP-3, and RhoB. β-actin was used as a loading control and for quantitation. Relative absorbance is plotted to illustrate quantitation of western blots. Error bars represent standard deviations. (**c**) HDLEC (n=4) and p.Glu542Lys LM-EC (n=4) cell cultures were transfected with miR-21 inhibitor (miR-21−), mimic (miR-21+), or scrambled control (miR-21 scr) and analyzed by RT-PCR for miR-21 expression. (**d**) Transfected HDLEC and p.Glu542Lys LM-EC cultures were analyzed by western blot for PDCD4 protein expression. CypA was used as loading control/ quantitation for PDCD4. R Abs = relative absorbance of target protein/loading control. Error bars represent standard deviations. Included blot is one of 9 independent experiments. (**e**) Transfected HDLEC (n=6) and p.Glu542Lys LM-EC (n=6) cultures were analyzed in a CCK proliferation assay over the course of 96 hours post-transfection. Unpaired T tests were

performed at each time point comparing scrambled miRNA control to mimic or inhibitor. Error bars represent standard deviations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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