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miR-302 cluster inhibits angiogenesis and growth of K562 leukemia cells by targeting VEGFA

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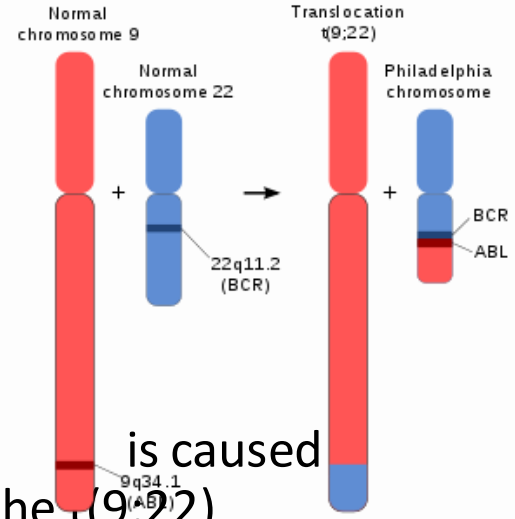
ORIGINAL RESEARCH

miR-302 cluster inhibits angiogenesis and growth of K562 leukemia cells by targeting VEGFA

- miR-302 cluster has been reported as a tumor suppressor in many human cancers;
- yet, its function in chronic myeloid leukemia (CML) tumorigenesis remains largely unclear
- The study was aimed to explore the functional roles of miR-302 cluster in CML progression.

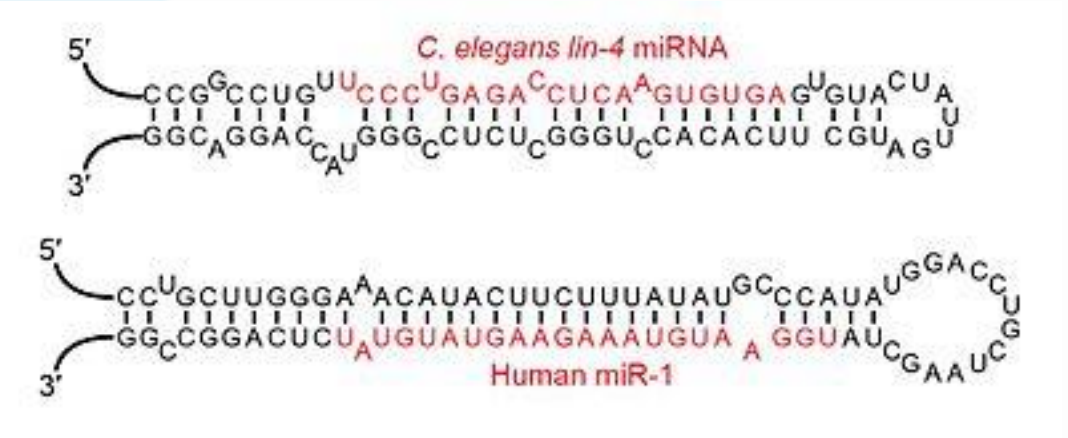
Introduction



- Chronic myeloid leukemia (CML), a clonal hematopoietic stem cell disorder, is caused by the constitutively active BCR–ABL tyrosine kinase resulting from the t(9;22) (q34;q11) reciprocal translocation (the Philadelphia translocation).
- With the introduction of imatinib, a small-molecule BCR–ABL-specific tyrosine kinase inhibitor,the 5-year survival rate of CML patients has greatly improved.
- Unfortunately, the prognosis of some patients who are resistant to imatinib therapy still remains poor. Therefore, a better understanding of how CML initiates and progresses will be pivotal to the development of new therapeutic strategies.
- miR-302 cluster was initially identified in human embryonic stem cells
- Several studies have also indicated the potential roles of miR-302 cluster in human cancers.
- For example, miR-302 inhibited cell growth by targeting MTDH in hepatocellular carcinoma

- *A microRNA* (abbreviated miRNA) is a small non-coding RNA molecule
- containing about 22 nucleotides
- found in plants, animals and some viruses
- that functions in RNA silencing and **post**-transcriptional **regulation of gene expression**
- miRNAs function via base-pairing with complementary sequences within mRNA molecules

- As a result, these mRNA molecules are silenced, by one or more of the following processes:
- (1) **Cleavage** of the mRNA strand into two pieces
- (2) Destabilization of the mRNA through **shortening** of its **poly(A) tail**
- (3) **Less efficient translation** of the mRNA into proteins by ribosomes



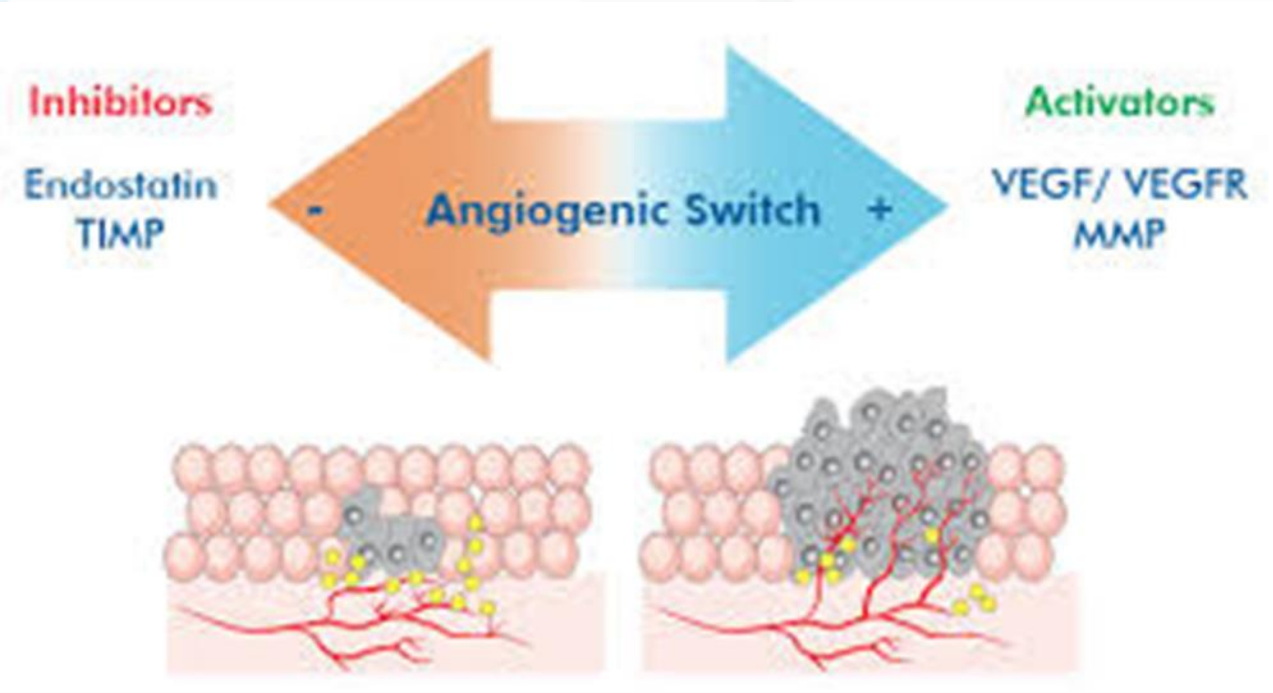
Targets

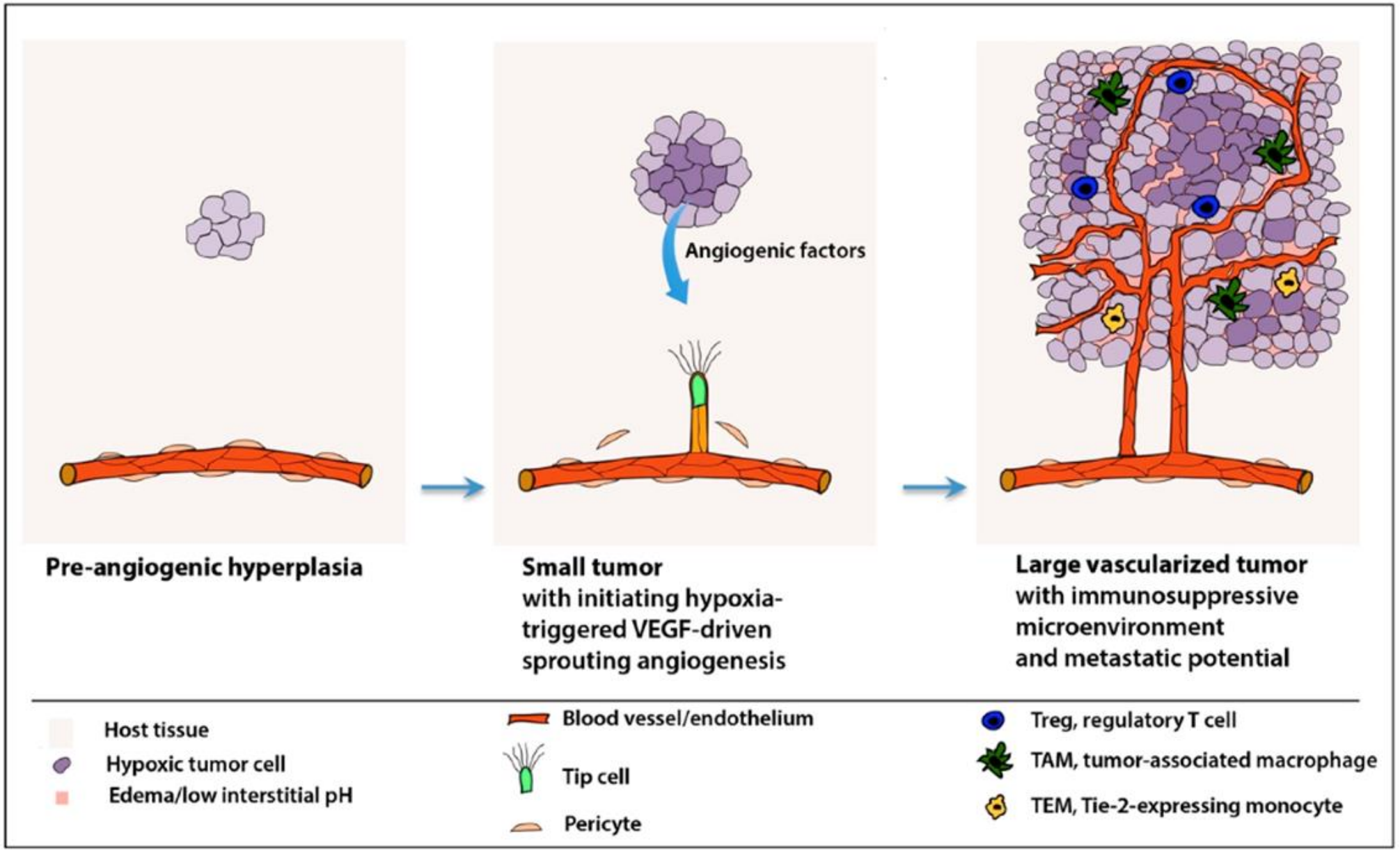
- **Plant** miRNAs usually have **near-perfect pairing** with their mRNA targets, which induces gene repression through **cleavage** of the target transcripts.
- In contrast, **animal** miRNAs are able to **recognize** their target mRNAs **by** using as few as 6–8 nucleotides (the **seed region**) at the 5' end of the miRNA,
- which is **not enough** pairing to **induce cleavage** of the target mRNAs
- Combinatorial regulation is a feature of miRNA regulation in animals
- A given **miRNA** may have **hundreds of different** mRNA **targets**, **and** a given **target** might be regulated by **multiple miRNAs**.
- The **first human disease** discovered to be **associated** with **deregulation** of **miRNAs** was chronic lymphocytic leukemia. Other B cell malignancies followed.

MicroRNA and Angiogenesis Regulation

- **miRNA's** ability to **target multiple genes** within a **signaling pathway** makes them **promising** target for the development of **second generation anti-angiogenesis drugs**.
- A wide range of regulators and signalling molecules, including
- Vascular endothelial growth factor-A (VEGF-A),
- Fibroblast growth factor (FGF),
- Epidermal growth factor(EGF),
- Interferon,
- Matrix metalloproteinase (MMP-1/9)

are **associated with angiogenesis**




Angiogenic factors
Pre-angiogenic hyperplasia
Small tumor
with initiating hypoxia-
triggered VEGF-driven
sprouting angiogenesis
Large vascularized tumor
with immunosuppressive
microenvironment
and metastatic potential
Host tissue
Hypoxic tumor cell
Edema/low interstitial pH
Blood vessel/endothelium
Tip cell
Pericyte
Treg, regulatory T cell
TAM, tumor-associated macrophage
TEM, Tie-2-expressing monocyte

- Various **angiogenic stimulators** such as
- Angiogenin,
- Angiopoietin,
- Transforming
- growth factor α/β (TGF α/β)

are engaged in keeping the balance in angiogenic switch,
along with the **angiogenic inhibitors** like

- Angioarrestin ,
- Interleukin 12,
- Fibronectin fragment
- and Angiostatin.

all the known **angiomiRs** basically **function** in either of **two ways**:

(1) By **targeting** the **negative regulators** of **angiogenesis** and promoting **vascularization**
or
(2) by targeting **positive regulators** of angiogenesis and **inhibiting angiogenesis**.

The first group is known as
pro-angiomiRs
while the second one is termed
as anti-angiomiRs

Type of miRNA	miRNA	Target	Function	Reference
Pro-angiogenic	miR-126	SPRED-1, PIK3R2, VECAM-1	Promotes VEGF-dependent AKT and ERK signalling derepressing the p85	[36] [12]
	miR-210	Ephrin-A3	Stimulation of capillary-like formation and EC chemotaxis in response to VEGF	[37]
	miR-10b and 9b	HOX Pathway	Enhance endothelial cell proliferation in response to VEGF	[38]
	Let-7b, 7f	Let-7b: TIMP1 Let-7f: TSP-1	Regulate sprout formation	[8]
	miR-132	p120RasGAP	Facilitates endothelial cell proliferation by downregulating p120RasGAP	[39]
	miR-378	Sufu, Fus1	Promotes endothelial cell migration, tube formation, and tumor angiogenesis *in vivo*	[40]
	miR-17-92 cluster	TIMP1, TSR, VEGF	Promote endothelial cell division, migration	[41]
Anti-angiogenic	miR-24	GATA4, PAK 2	Inhibits angiogenesis	[42]
	miR-195	VEGF, VAB2, CDC 42	Suppresses angiogenesis and metastasis in hepatocellular carcinoma	[43]
	miR-221/miR-222	c-kit, eNOS	Inhibit EC migration and proliferation	[44]
	miR-328	CD 44	Reduces formation of capillary structure	[45]
	miR-15b/miR-16	VEGF	Induce cell apoptosis	[46]
	miR-31	[illegible]	Globally downregulates angiogenic pathway	[47]

Targeting **VEGF Signaling Pathway** Directly

miR-126

- Response of endothelial cells to VEGF-A, is largely mediated by miR-126. It was shown that miRNA-126 alone can regulate angiogenesis and vascular integrity
- VEGFA is known to promote new blood vessel formation by proliferating

the stalk cells, in an angiogenic sprout and also by
inducing direct migration of the cells at the tip.

- VEGF-A mediated phosphorylation of ERK and AKT

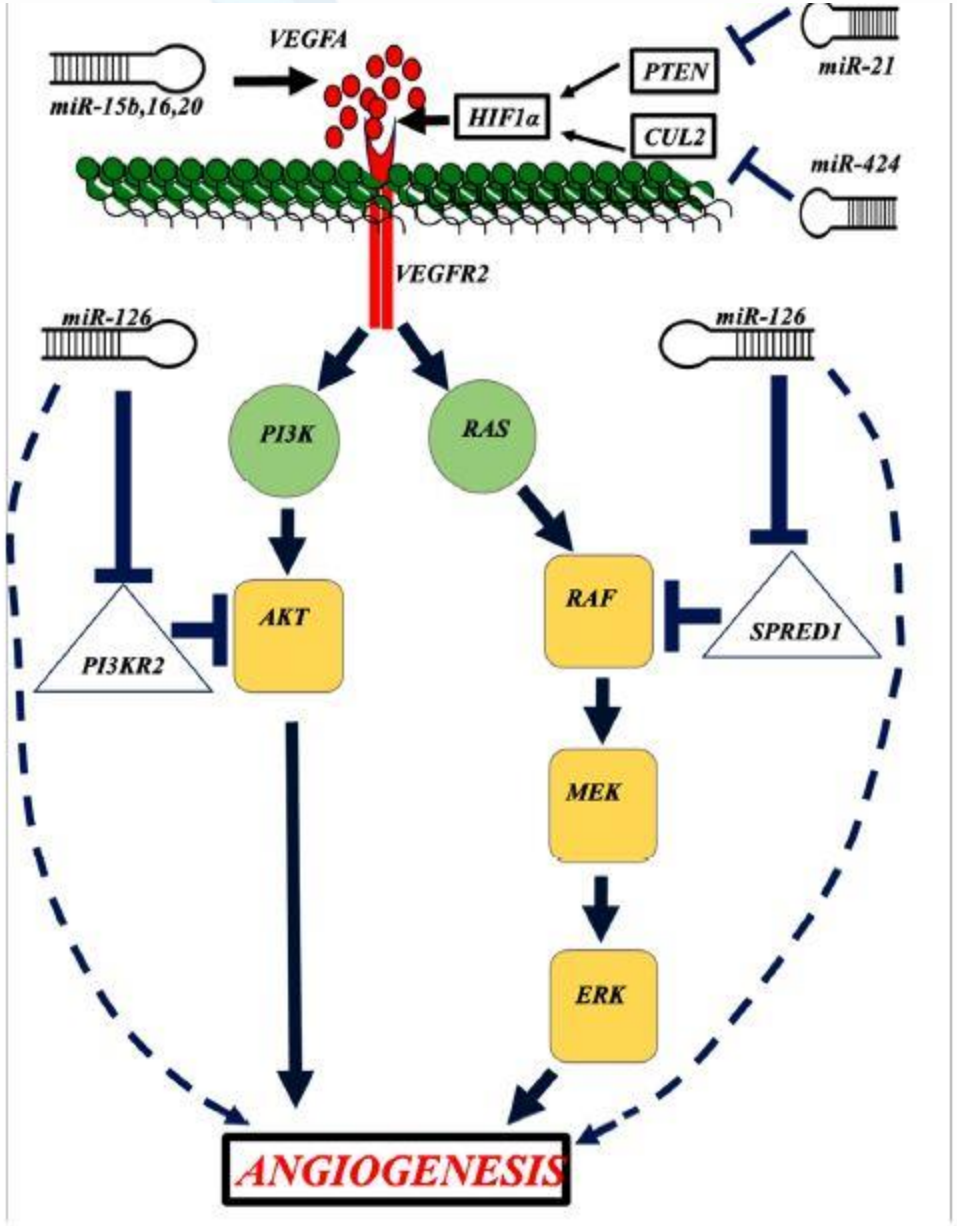
was attenuated in miR-126 knockdown cells indicating the
involvement of miR-126 in SPRED1 and PIK3R2 mediated VEGF-A pathway

- cells with reduced levels of miR-126 were found to be less responsive to

VEGF-A and other growth factors, as SPRED1 inhibits the activity of RAF1 kinase

- These findings, that miR-126 directly targets SPRED1 and PIK3R2, provide important

evidence that VEGF-A pathway can be regulated at multiple levels by a micro RNA

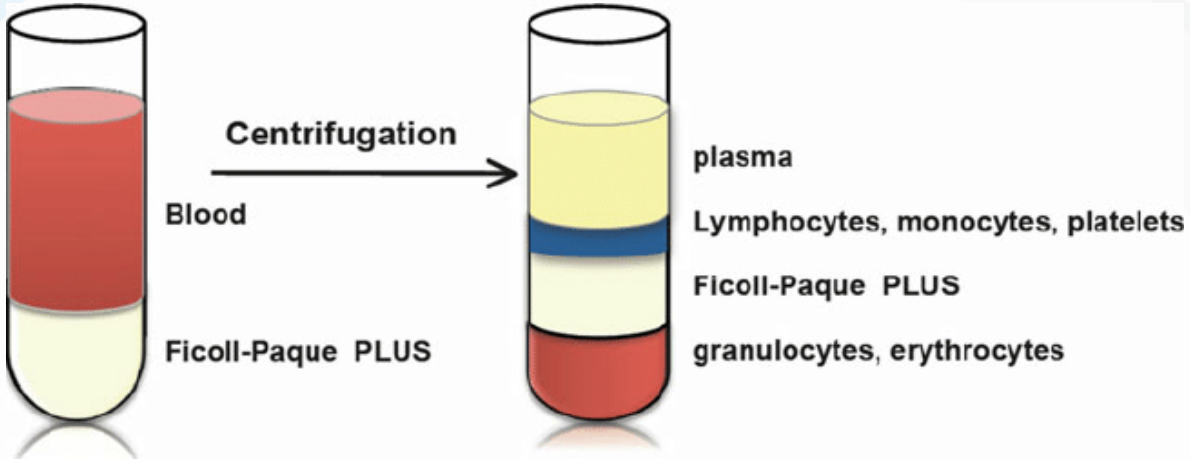

VEGFA
miR-15b,16,20
HIF1α
PTEN
CUL2
miR-21
miR-424
VEGFR2
miR-126
miR-126
PI3K
RAS
AKT
RAF
PI3KR2
SPRED1
MEK
ERK
ANGIOGENESIS

Materials and methods

Patient samples

- Bone marrow mononuclear cells were collected from 70 CML patients and 20 healthy age-matched controls at the Affiliated Hospital of Xuzhou Medical University (Xuzhou, China) between January 2014 and March 2016.

- Bone marrow mononuclear cells were isolated by Ficoll Histopaque density gradient method.



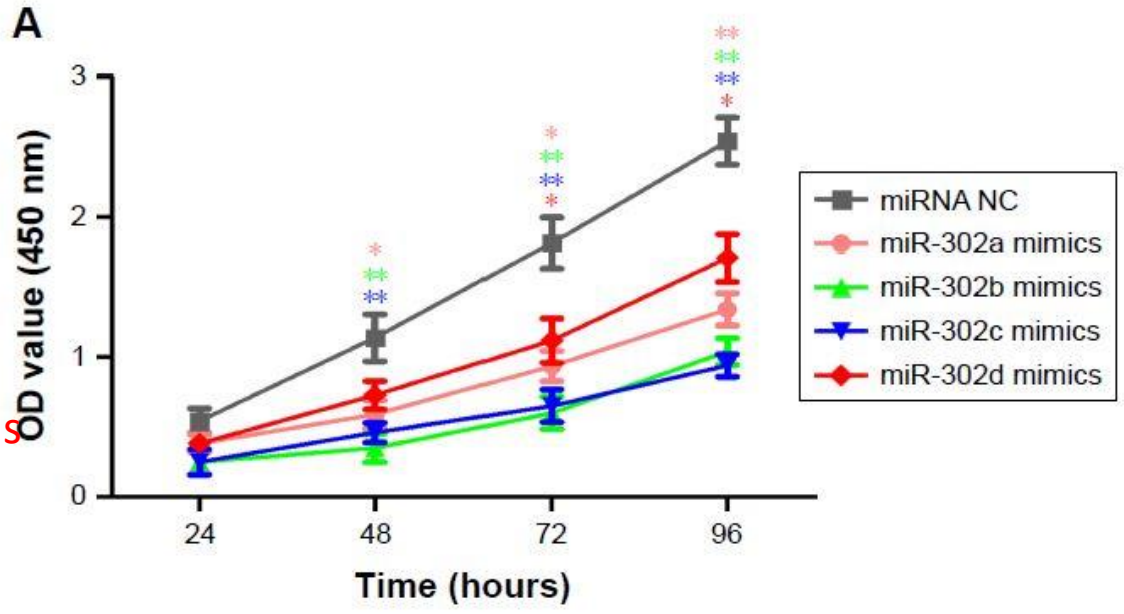
Cell culture and transfection

- Three CML cell lines (K562)
- Human umbilical vein endothelial cell lines (HUVECs) for *capillary tube formation*
- K562 cells were transiently transfected with
- miR-302 cluster mimics and miRNA negative control (GenePharma, Shanghai, China),
- Wild type (WT) and mutant type (Mut) of VEGFA reporter vector

Cell Counting Kit-8 (CCK-8) assay

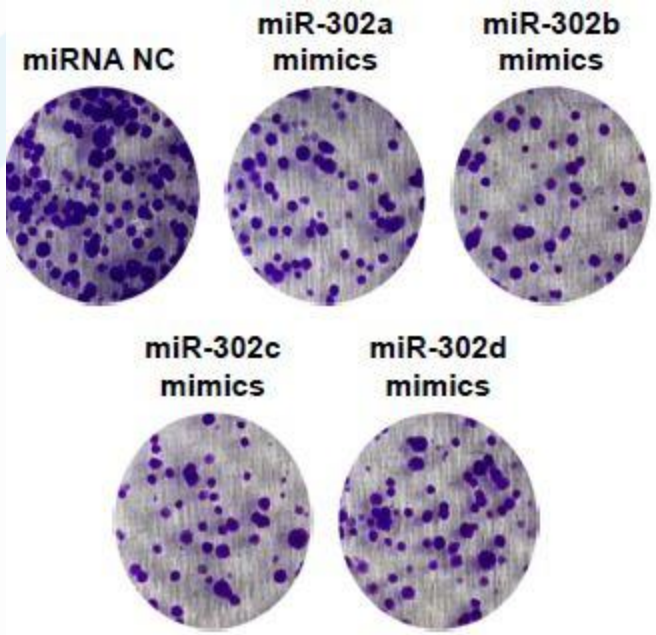
- After 24 hours of transfection, K562 cells were seeded in 96-well culture plate.
- At 24, 48, 72 and 96 hours, 100 µL of 10% CCK-8 reagent (v/v) was added to each well, and cells were cultured for 1 hour at 37°C.
- The **number of viable cells** was assessed by measurement of absorbance at 450 nm using an Enzyme Immunoassay Analyzer

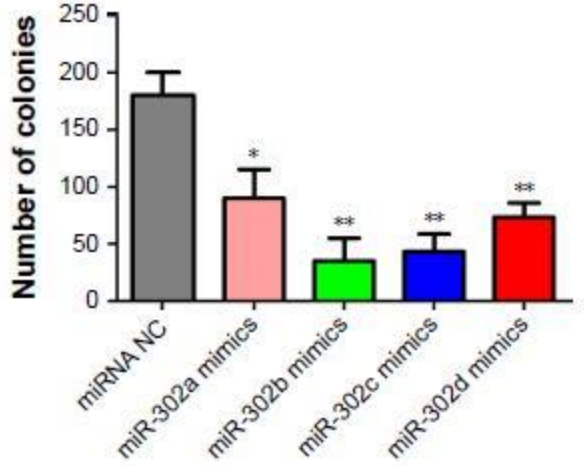
Overexpression of miR-302 cluster
inhibits cell growth, colony formation
and angiogenesis {indicate that miR-302 cluster functions
as a tumor suppressor
in CML carcinogenicity}



Colony formation assay

- After 24 hours of transfection, 150 cells were plated in 6-well plates and grown for 2 weeks.
- Cells were fixed with acetic acid:methanol (1:4) and stained with dilute crystal violet (1:30).
- The number of visible colonies was counted manually.
- All samples were assayed in triplicate.



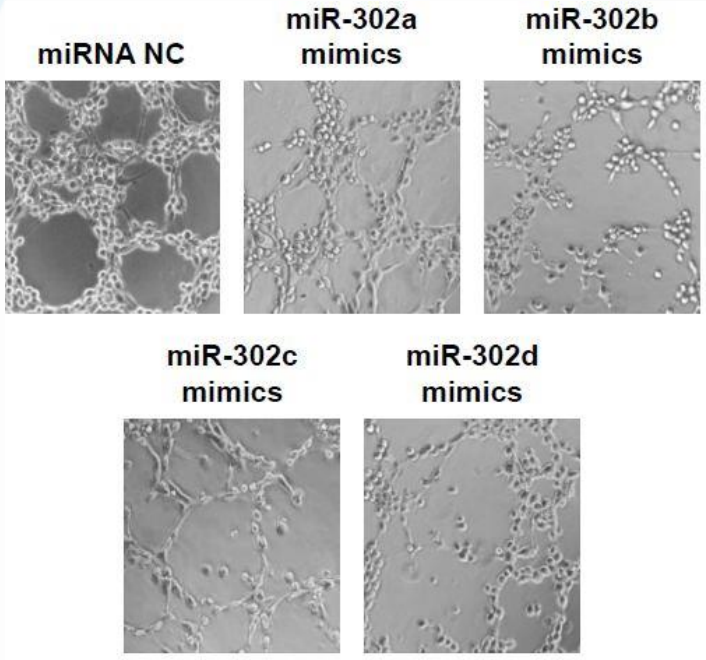


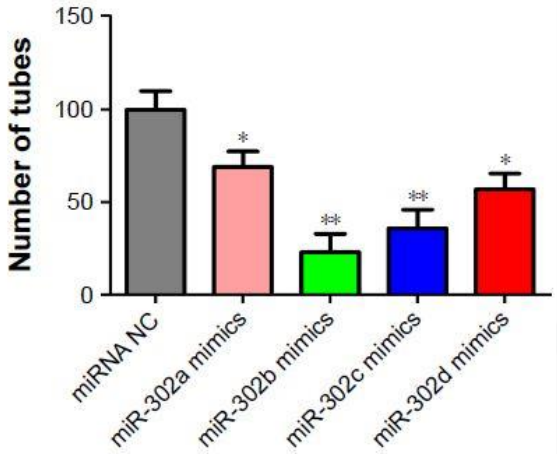
Overexpression of miR-302 cluster inhibits cell growth, colony formation and angiogenesis {indicate that miR-302 cluster functions as a tumor suppressor in CML carcinogenicity}

HUVEC capillary tube formation

- After 24 hours of transfection, K562 cells were incubated with serum-free medium for 2 days. The medium was then collected as conditioned medium.
- HUVECs at a density of 5×103 per well were grown with conditioned medium in a 24-well plate precoated with 200 µL Matrigel (BD Biosciences, San Jose, CA, USA) and then incubated at 37°C for 6 hours.
- The formation of capillary-like structures was captured under a light microscope. The number of connected tubes was counted.

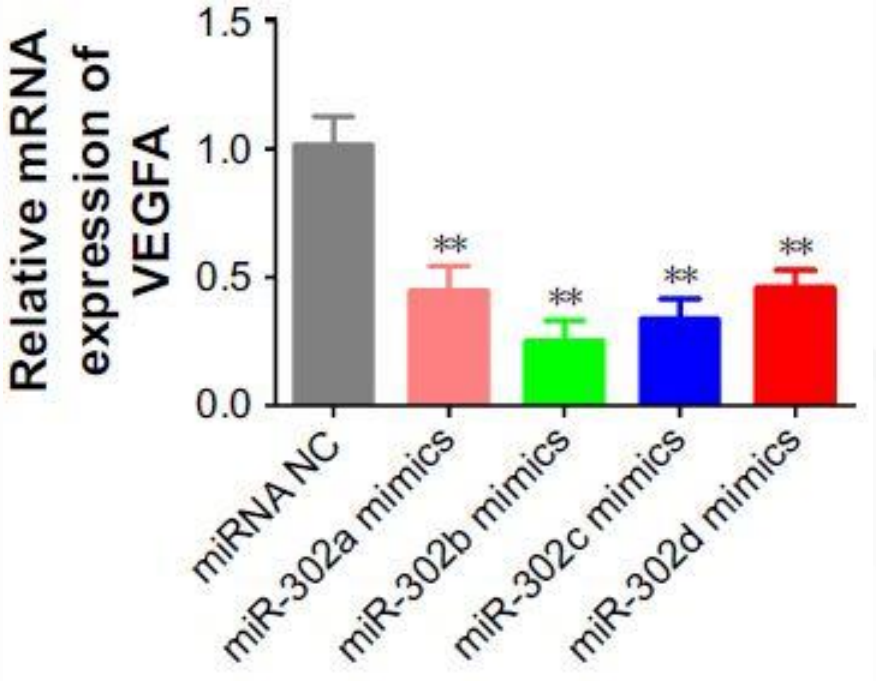
Overexpression of miR-302 cluster inhibits cell growth, colony formation and angiogenesis {indicate that miR-302 cluster functions as a tumor suppressor in CML carcinogenicity}





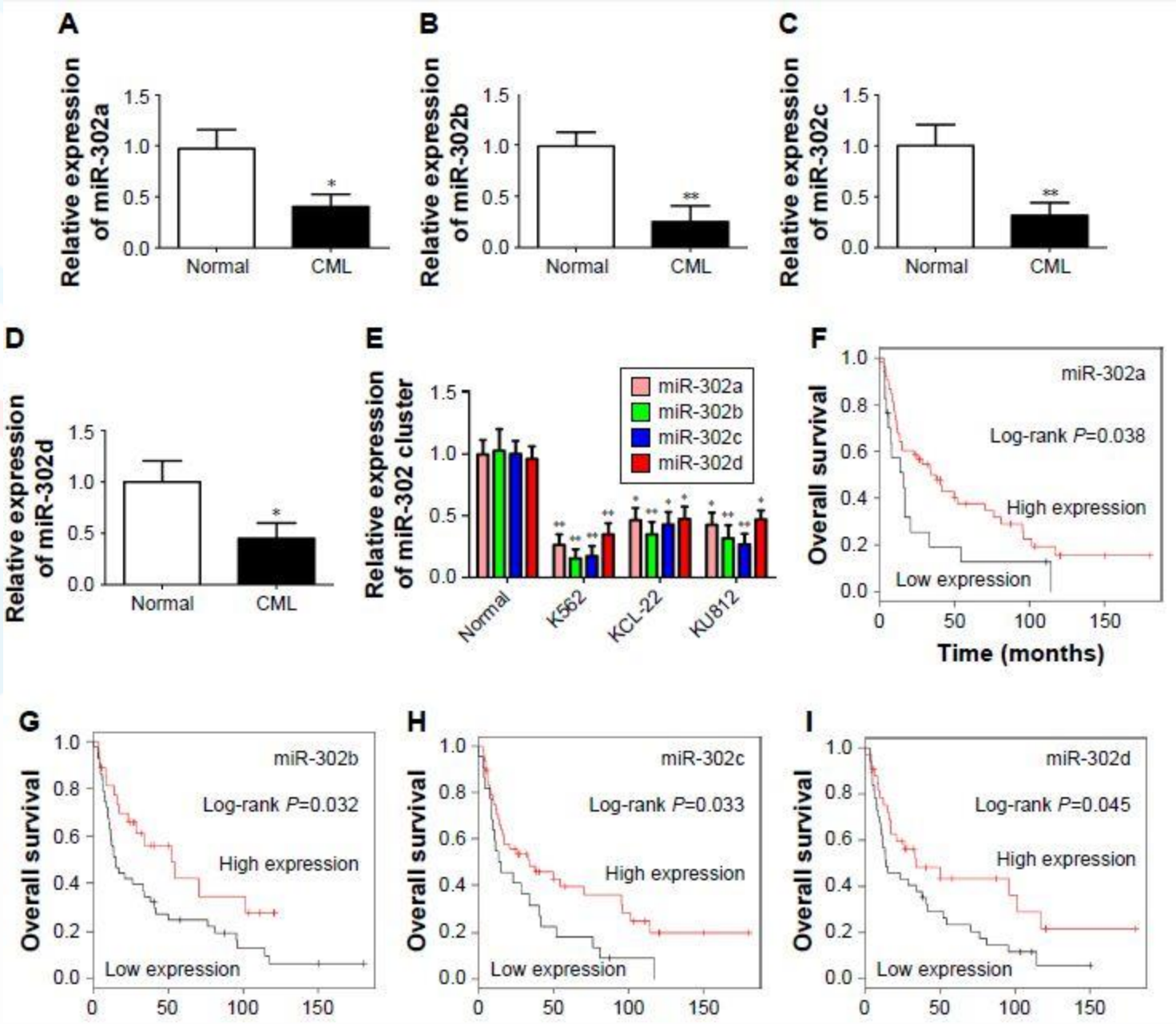
RNA isolation and quantitative reverse transcriptase PCR (qRT-PCR)

- Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific).
- The expression of miR-302 cluster was evaluated using Taqman miRNA assays (Thermo Fisher Scientific).
- The mRNA level of VEGFA was determined using SYBR Green PCR master mix (Thermo Fisher Scientific).
- β-actin was used as an endogenous control.

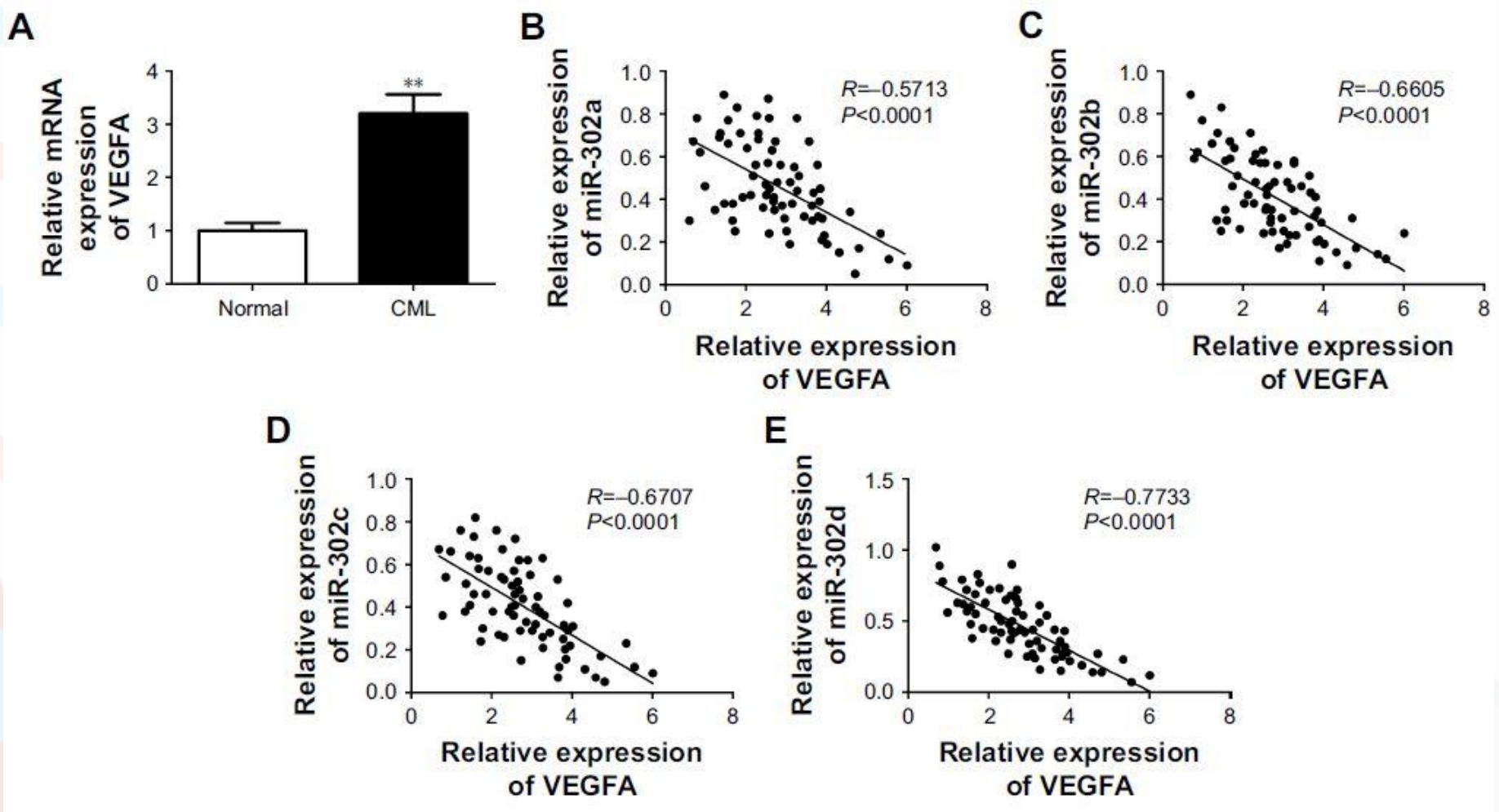


miR-302 cluster mimics suppressed VEGFA mRNA expression in K562 cells, which was determined by qRT-PCR.

- Downregulated miR-302 cluster expression is associated with poor overall survival of CML patients



Relationship between miR-302 cluster and VEGFA in CML samples

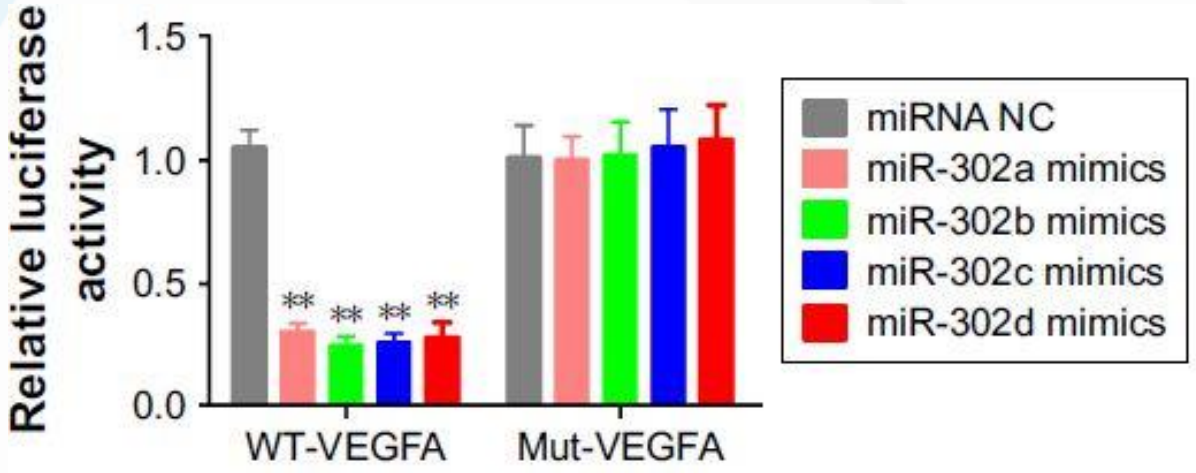


Expression level of miR-302 cluster is significantly negatively associated with VEGFA mRNA expression in CML patients.

Luciferase reporter assay

- The 3’UTR of VEGFA containing the miR-302 cluster binding site was synthesized and cloned into the pGL3 vector (Promega Corporation).
- pGL3-VEGFA-WT or pGL3-VEGFA-Mut was co-transfected with miR-302 cluster mimics or miRNA negative control, respectively.
- After 48 hours of transfection, luciferase activity was measured using Dual Luciferase Assay (Promega Corporation).

VEGFA is target gene of miR-302 cluster



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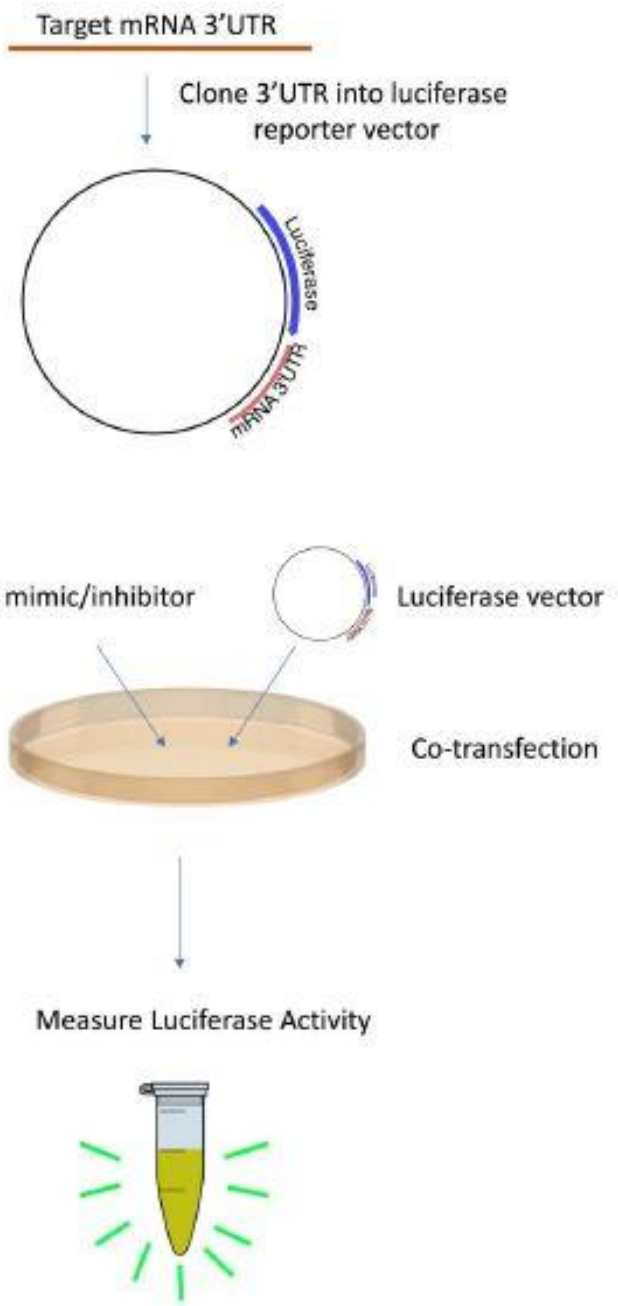
Luciferase reporter assay



Prediction of target genes

- Potential target genes that interacted with miR-302 cluster were analyzed with TargetScan 7.2 and starBase v3.0 software

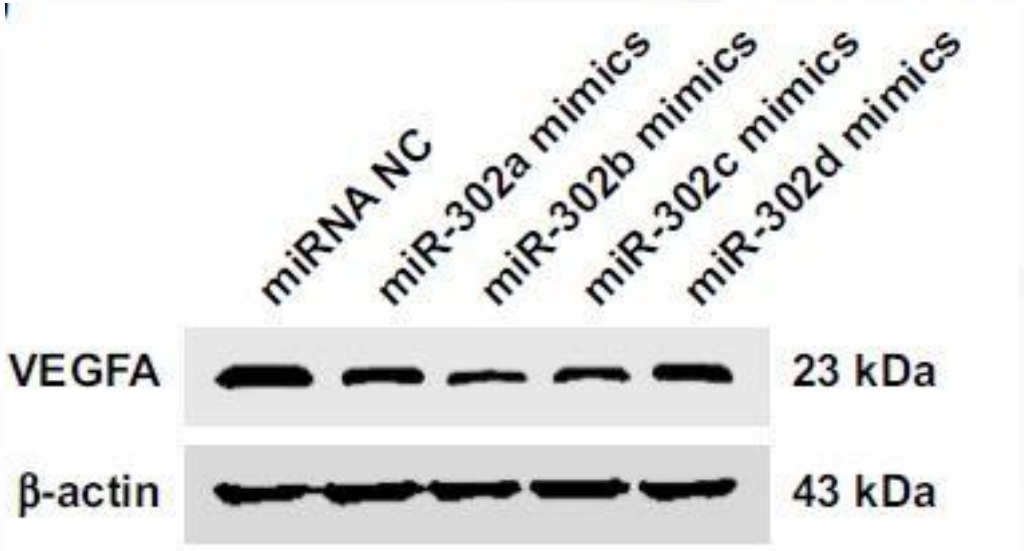
VEGFA is target gene of miR-302 cluster

Western blot

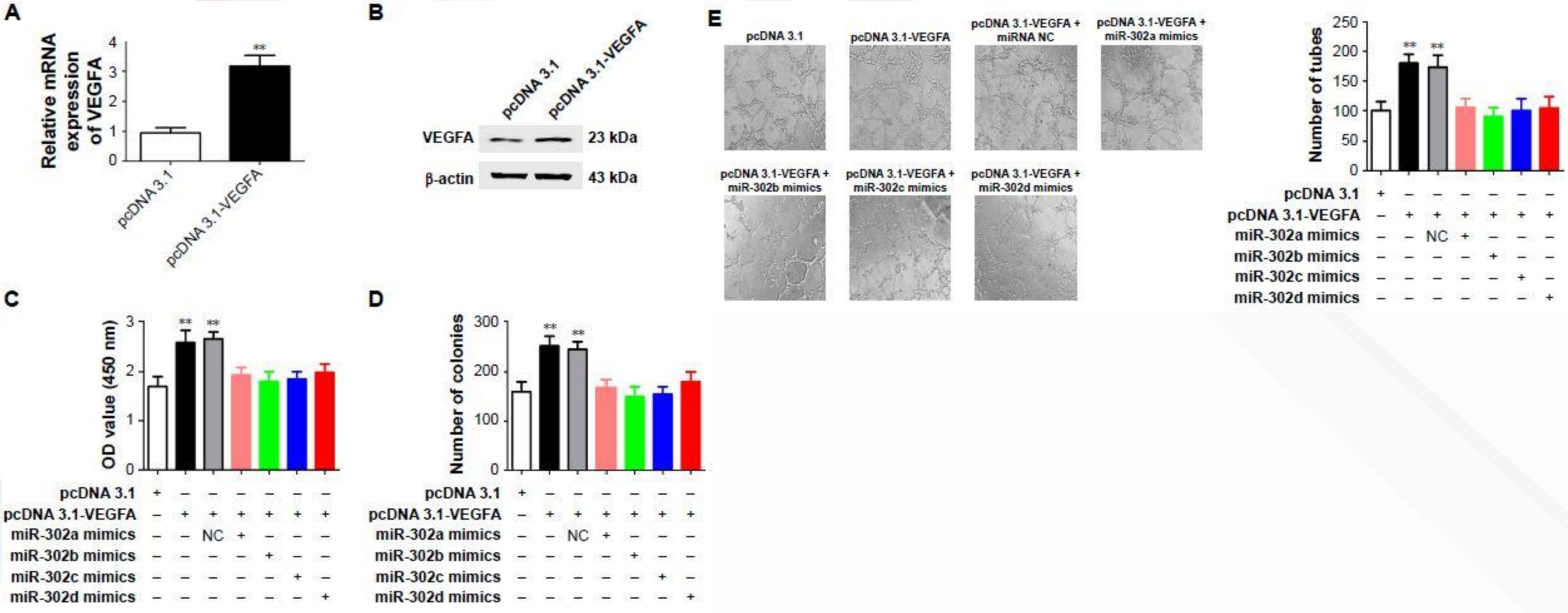
- Cells were lysed in RIPA lysis buffer (Cell Signaling) with protease inhibitor.
- Western blot analysis was performed using rabbit monoclonal anti-VEGFA and mouse monoclonal β-actin .
- β-Actin was used as an internal control.

VEGFA is target gene of miR-302 cluster

miR-302 cluster mimics suppressed VEGFA protein expression
in K562 cells, which was determined by Western blot



Overexpression of VEGFA abates the inhibition of miR-302 cluster on cell growth and angiogenesis



Discussion

- this study,
- first determined miR-302 cluster expression levels in CML samples and cell lines and found that miR-302a, miR-302b, miR-302c and miR-302d were frequently downregulated.
- High expression level of miR-302 cluster was significantly associated with good prognosis of CML patients.
- miR-302 cluster mimics could significantly suppress cell growth, colony formation and angiogenesis of K562 cells compared with miRNA negative control.
- Findings were consistent with a previous study in which Qin et al reported that miR-302a inhibited hepatocellular carcinoma cell proliferation and invasion through targeting VEGFA.
- further revealed that there was a negative correlation between miR-302 cluster and VEGFA mRNA level in CML patients.

Conclusion

- the present study revealed for the first time that frequently downregulated miR-302 cluster was associated with poor prognosis in CML patients.
- Also demonstrated that miR-302 cluster inhibited growth and angiogenesis of K562 cells by targeting VEGFA.
- Thus, miR-302 cluster may be a potential prognostic and therapeutic target in CML.

references

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- **Overview of MicroRNA Biology**
- **miR-302 cluster inhibits angiogenesis and growth**
- **of K562 leukemia cells by targeting VEGFA , Cao et al , 2019**
- **کتاب میکرو RNAاز پایه تا بالین**

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