

C-myc overexpression drives melanoma metastasis by promoting vasculogenic mimicry via c-myc/snail/Bax signaling

Xian Lin¹ · Ran Sun² · Xiulan Zhao^{1,5} · Dongwang Zhu⁴ · Xueming Zhao¹ · Qiang Gu^{1,5} · Xueyi Dong¹ · Danfang Zhang¹ · Yanhui Zhang³ · Yanlei Li¹ · Baocun Sun^{1,3,5}

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Abstract

c-Myc is a well-characterized proto-oncogene that induces cellular transformation and modulates programmed cell death. While recent studies have demonstrated high expression of c-Myc protein in advanced and metastatic melanoma, the clinical and biological implications remain to be fully elucidated. In this study, we investigated the effect of c-Myc overexpression in melanoma tumorigenesis. Clinicopathological analysis demonstrated that c-Myc expression positively correlated with the formation of vasculogenic mimicry (VM) and linearly patterned programmed cell necrosis (LPPCN). Clinically, high c-Myc expression was significantly associated with distant metastasis and poor prognosis, while biologically, c-Myc overexpression led to significant increases in cell motility,

invasiveness and metastasis. Moreover, c-Myc induced the formation of VM and promoted the expression of epithelial-mesenchymal transition (EMT)-associated protein Snail both in vivo and in vitro. High expression of c-Myc increased Bax expression in hypoxic conditions and induced cell apoptosis. Taken together, we conclude that c-Myc overexpression promotes the formation of VM by EMT and LPPCN in melanoma. Our improved understanding of the clinical and biological effects of c-Myc overexpression in melanoma highlights the incomplete understanding of this oncogene, and indicates that c-Myc is a potential therapeutic target of this disease.

Key message

- High c-Myc expression is associated with tumor metastasis and poor prognosis in human melanoma.
- c-Myc upregulates Snail expression to promote EMT via the TGF- β /Snail/Ecadherin signal pathway.
- c-Myc leads to cell death by upregulating Bax expression causing a lower Bcl2/Bax ratio under severe hypoxic conditions.
- c-Myc promotes vasculogenic mimicry and linearly patterned programmed cell necrosis.

Xian Lin and Ran Sun contributed equally to this work.

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✉ Baocun Sun
baocunsun@aliyun.com

¹ Department of Pathology, Tianjin Medical University, Tianjin 300070, China

² Department of Surgery, Tianjin Hospital of ITCWM Nankai Hospital, Tianjin 300100, China

³ Department of Pathology, Cancer Hospital of Tianjin Medical University, Tianjin 300060, China

⁴ Department of Surgery, Stomatological Hospital of Tianjin Medical University, Tianjin 300070, China

⁵ Department of Pathology, General Hospital of Tianjin Medical University, Tianjin 300052, China

Keywords c-Myc · Vasculogenic mimicry · Linearly patterned programmed cell necrosis

Introduction

Melanoma is a type of cancer with an aggressive clinical course and poor outcome associated with acquired resistance to existing therapies [1]. Tumor growth and metastasis depend on angiogenesis [2]. Inhibition of angiogenesis has become a clinical anti-cancer strategy [3–7].

Vascular mimicry (VM) represents an extension of tumor angiogenesis [8]. VM has been identified in numerous types of cancers including melanoma and is associated with metastasis and a poor prognosis [9]. However, the mechanisms leading to VM remain poorly characterized, and few investigations have evaluated the nature of the structure or formation of VM.

Emerging evidence suggests the involvement of epithelial-mesenchymal transition (EMT) in the formation of VM [10–12]. The physical constraints of the microenvironment around a rapidly growing tumor have led researchers to question how tumors are able to create sufficient space to induce VM. Recently, Sun et al. reported a previously undescribed mechanism of programmed cell death, “linearly patterned programmed cell necrosis” (LPPCN), in which distinct channel-shaped, vessel-like empty spaces are created via cell necrosis thereby providing a spatial structure for the formation of VM and endothelial-dependent vessels (EDVs) [13]. Zhang and colleagues recently highlighted the importance of LPPCN, describing a correlation between this phenomenon and both metastasis and poor prognosis in melanoma. Ultimately, the biological triggers leading to LPPCN cell death remain unknown.

c-Myc is a proto-oncogene known to promote cell proliferation, resulting in cellular transformation and modulation of programmed cell death, among a multitude of other functions [14–17]. Cellular transformation and cell death are the opposing cellular fates. In 2001, Kraehn et al. reported c-Myc amplification in advanced and metastatic melanoma [18]. However, the role of c-Myc in the complex microenvironment of melanoma has not been well described. In the present study, we evaluated the effect of c-Myc protein overexpression on the formation of VM via promotion of both cellular transformation (EMT) and cell death (LPPCN).

Materials and methods

Collection of patient samples

A total of 135 primary tumor specimens were obtained from the Tumor Tissue Bank of the Tianjin Cancer Hospital (Tianjin, China). Specimens were excised from melanoma patients who underwent surgical resection at the Tianjin Medical University Cancer Institute and Hospital, in China, between January 1999 and October 2005, and July 2006 and December 2010.

All tissue sections were reviewed by a pathologist according to the 2010 World Health Organization Classification of Tumors Pathology and Genetics of Skin Tumors. The use of patient specimens was approved by the Institutional Research Committee.

CD34 (or Endomucin for mice tissue) immunohistochemistry and periodic acid-schiff (PAS) staining

The tissue sections were deparaffinized, hydrated and rehydrated according to standard protocols. Antigen retrieval was performed, and non-specific binding sites were blocked. The sections were then incubated with the primary antibody overnight at 4 °C and the second antibody for 30 min at 37 °C. The color was developed using 3,3'-diamino-benzidine-chromogen (DAB). Finally, the sections were subjected to PAS staining and counterstained with hematoxylin.

Immunohistochemical staining methods was performed as above without PAS staining. Details of the primary antibody are shown in Supplementary Table S1.

Immunohistochemical scoring

Evaluation of sections was performed by two independent pathologists. The sections were semi-quantitatively assessed according to both the percentage of positive neoplastic cells and the immunostaining intensity of individual tumor cells (extension + intensity) [19]. For statistical analysis, a total score of 0–2 was considered as negative/low expression, while scores of 3–6 were considered as positive/high expression.

Melanoma cell lines and animals

Melanoma cell lines A375, MUM-2C, and MUM-2B were obtained from China Infrastructure of Cell Line Resources (Beijing, China). Forty male NIH BALB/c nu/nu mice (4–6 weeks of age), were obtained from HFK Bioscience Co. Ltd. (Beijing, China). The mice were housed in the Animal Center of Tianjin Medical University (Tianjin, China) at 1 week before experiments started and bred under specific pathogen-free conditions. All mice were handled according to the recommendations of the NIH Guidelines for the Care and Use of Laboratory Animals. The experiment was approved by the Institutional Animal Care and Use Committee.

c-Myc expression plasmids and c-Myc gene silencing

To functionally characterize the relationships of c-Myc with VM and LPPCN, we established stable c-Myc-expressing cells. c-Myc suppression was mediated by lentivirus infection using OmicsLink short hairpin RNA (shRNA) Expression Clones (target sequence: ACAGCTACGGAACTCTTGT, Catalog no. HSH011597-HIVmU6; GeneCopoeia) (shown in Figures as “sh-c-Myc”), whereas c-Myc overexpression was achieved by an ORF lentiviral expression clone with high expression of the c-Myc gene (Catalog no. EX-Z2845-Lv105–5) (shown in Figures as “c-Myc”). A non-silencing

shRNA sequence without the c-Myc shRNA component was used as the negative control (Catalog no. CSHCTR001-HIVmU6) (shown in Figures as “vector”). Cells were transfected using a Lenti-Pac HIV Expression Packaging Kit (Catalog no. HPK-LvTR-40) according to the manufacturer’s protocol. Stably expressing cell lines were obtained by selection with 0.2 $\mu\text{g}/\text{ml}$ puromycin. The transfection efficiency was confirmed by western blot analysis and semi-quantitative RT-PCR.

Transwell assay, wound healing assay and matrigel-based tube formation assay

Transwell assay, wound healing assay and matrigel-based tube formation assay were performed as previously mentioned [20]. The number of invading/migrating cells was counted in five fields under an inverted light microscope (Nikon) at $\times 200$ magnification. The speed of wound closure was monitored after 12 and 24 h by measuring the ratio of the distance of the wound at 0 h. The cells of matrigel-based tube assay were photographed at $\times 200$ magnification. Each experiment was performed in triplicate.

Immunofluorescence

Cells were plated onto coverslips and fixed in ice cold methanol for 10 min. The cells were blocked with 5 % FBS and incubated with the primary antibodies and the FITC-conjugated secondary antibodies. The sections were counterstained with 4,6-diamidino-2-phenylindole and observed under a fluorescence microscope (80i, Nikon) at $\times 200$ magnification.

Western blot analysis

Blots were blocked and incubated with antibodies and the enhanced chemiluminescence method was used to measure protein expression. Bands were imaged and analyzed using a C-Digit $\text{\textcircled{B}}$ Blot Scanner (Gene Company).

Co immunoprecipitation (Co-IP) assay

The Co-IP products of c-Myc/Snail were immunoprecipitated from melanoma cell lysates by a Pierce Crosslink Immunoprecipitation Kit (Thermo Fisher Scientific). Mouse IgG was used as a control. Input and immunoprecipitated proteins were analyzed by western blotting.

Luciferase reporter gene assay

The VE-cadherin, c-Myc and Snail promoters were purchased from GeneCopoeia. VE-cadherin and Snail promoters was cotransfected with c-Myc overexpression plasmids into

A375, MUM-2B cell lines by way of percutaneous ethanol injection. Transactivation assays were performed with the Secrete-PairTM Dual Luminescence Assay Kit (GeneCopoeia). Luciferase activities were measured using the Synergy 2 microplate reader system (Gene Company). TGF- β was purchased from PEPROTECH and used at 30 ng/ml.

Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was reverse transcribed using a QuantScript RT Kit (Tiangen Biotech). Semi-quantitative RT-PCR was performed according to the recommended thermal profile. The amplified products were subjected to electrophoresis in a 1 % agarose gel containing ethidium bromide (BIO-RAD Company). The expression of GAPDH was used to standardize the amount of cDNA added to each PCR tube. The primers used for semi-quantitative RT-PCR are shown in Supplementary Table S3.

Cell proliferation and apoptosis assay

Cells were plated in triplicate and analyzed over time using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay read on a Synergy 2 plate reader (Bio Tek). In addition, cells with or without Bcl2 or Bax siRNA treatment were incubated with 1 % O_2 for 12 h and then collected and stained using an Annexin V-FITC/PI Kit (KeyGen Biotech) according to the manufacturer’s instructions. Apoptotic cells were detected and analyzed using a flow cytometer (Accuri C6; BD, USA).

CoCl₂ treatments

The cells were seeded in a 6-well plate and grown to 70 % confluence in complete medium. The medium was then removed and the cells were washed with phosphate-buffered saline. The cells were treated with CoCl₂ at 0, 100, 200, 300, 400, and 800 μM and then incubated for 6, 12, 24, and 36 h. Then, semi-quantitative RT-PCR and western blotting were performed to detect mRNA and protein expression. The results were analyzed by multi-factor analysis of variance (ANOVA).

Murine xenograft model, metastasis assay, and colonization assay

Six-week-old male NIH BALB/c-null mice were housed in the animal facilities of Tianjin Medical University as approved by the Institutional Animal Care and Use Committee. All mice were handled according to the recommendations of the

Guidelines for the welfare and use of animals in cancer research [21]. Approximately 1×10^6 melanoma cells were injected into the backs of nude mice ($N = 10$ per group). Following a 12 day observation period, the mice were sacrificed. For metastasis assay, approximately 1×10^6 of melanoma cells were injected into the backs of nude mice ($N = 6$ per group). Moreover, for colonization assay, 4×10^5 cells were injected via the tail veins of mouse. All of these mice were allowed to proliferate for 10 weeks ($N = 6$ per group). The tumors, lungs and livers were harvested, embedded in paraffin, and stained with H&E.

Statistical analysis

All data were analyzed with SPSS 17.0 (SPSS, Chicago, IL, USA). Measurement data are shown as the mean \pm standard error of mean. $P < 0.05$ was considered as statistically significant. Kaplan Meier survival analysis was also performed. Differences in survival curves were assessed using the log-rank test. The Student's *t* test was performed to determine differences between two groups. Measurement data in more groups were compared by ANOVA.

Results

c-Myc expression, VM and LPPCN in melanoma and their correlation with clinicopathological parameters and prognosis

We respectively evaluated the expression of c-Myc, VM and LPPCN across a cohort of 135 melanoma specimens collected from Cancer Hospital of Tianjin Medical University. Sixty-nine (51.1 %) cases in our cohort were positive for c-Myc (Table 1 and Fig. 1A).

Tube cavities lined with PAS-positive, CD34-negative tumor cells and red blood cells observed within cavities were deemed to be VM channels (red arrows, Fig. 1B). Dual PAS and CD34 double positive channels were defined as EDVs (green arrows, Fig. 1B).

H&E staining showed LPPCN as dark cells with loss of cell-cell adhesion, concentrated cytoplasm, a nucleus exhibiting pyknosis, karyorrhexis or karyolysis, and homogeneous chromatin distributed in patterns of lines and/or networks (Fig. 1Ca-e). LPPCN cells displayed an integrated plasma membrane without an inflammatory reaction [22] (Fig. 1C-b and c). The LPPCN lines and/or networks were occasionally accompanied by neovasculogenic networks (Fig. 1C-d and e).

In 62 (45.9 %) specimens, VM formation was observed, while LPPCN was detected in 55.5 % (75/135) of the analyzed melanoma tissues. Notably, our results demonstrated that high

Table 1 The correlation of c-Myc with the clinicopathological parameter of melanoma

Variant	c-Myc expression			
	Positive(69)	Negative(66)	χ^2	P-value
Sex				
Male	43	45	0.125	0.857
Female	26	21		
Age(years)				
≥ 55	41	38	0.047	0.863
< 55	28	28		
Tumor size(cm ³)				
≥ 7.8	33	22	2.935	0.115
< 7.8	36	44		
AJCC staging				
Stage(I)	9	20	5.958	0.020*
Stage(II + III + IV)	60	46		
Classification				
Acral melanoma	30	29	0.003	1.000
Others#	39	37		
Breslow's depth				
≤ 1.5 mm	13	38	21.532	**
> 1.5 mm	56	28		
Pigments				
YES	29	36	2.117	0.170
NO	40	30		
Lymphatic metastasis				
YES	40	23	1.334	0.299
NO	29	43		
Distant metastasis				
YES	38	12	19.687	**
NO	31	54		

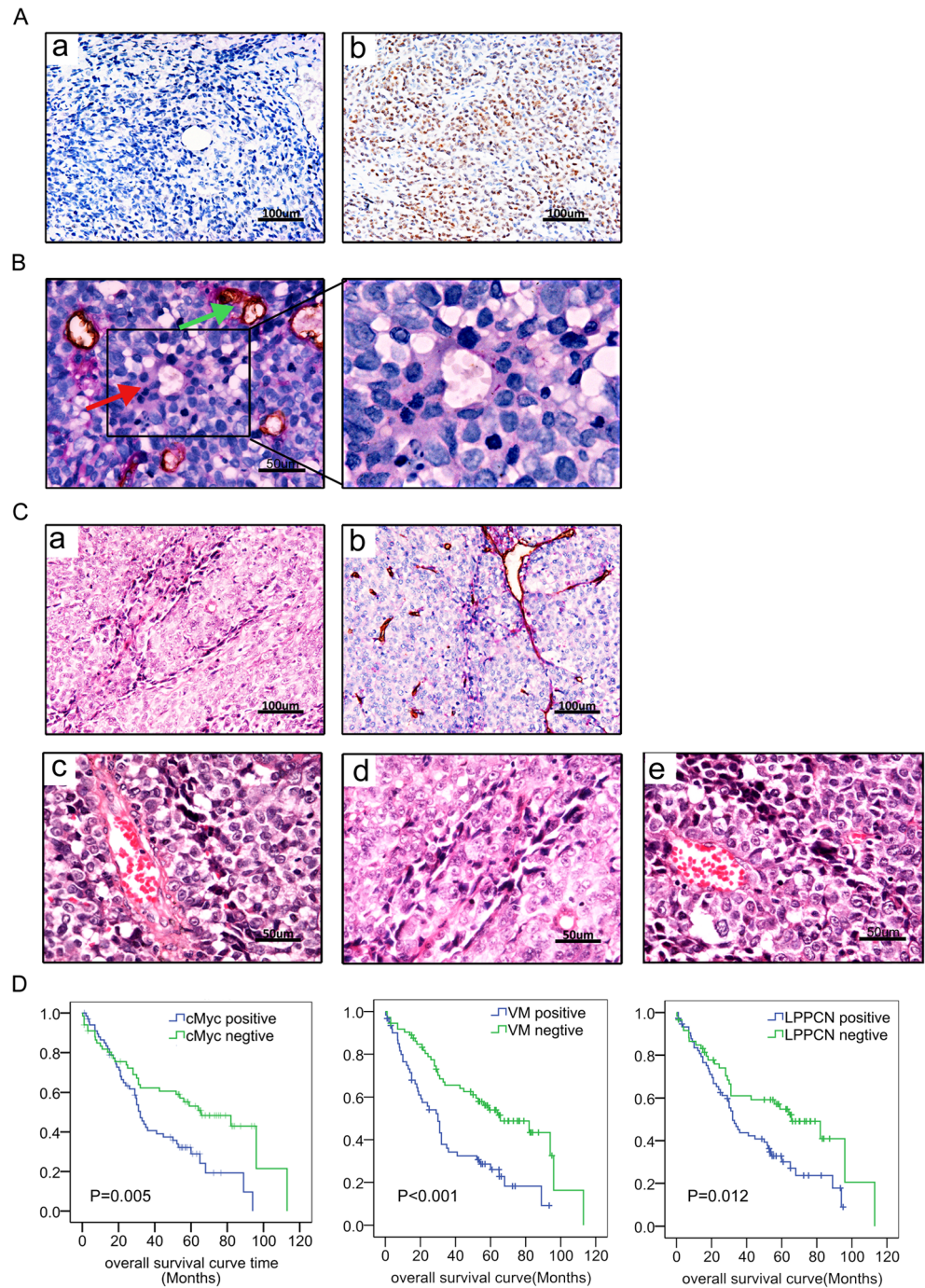
#(mucosal melanoma, CSD, non-CSD); * $p < 0.05$, ** $P < 0.001$

c-Myc overexpression, VM and LPPCN respectively correlate with the tumor's AJCC staging, Breslow's depth, metastasis of tumors, and poor prognosis ($P < 0.05$, Tables 2 and 3, and Fig. 1D).

c-Myc expression correlates to the presence of VM and LPPCN in melanoma

In c-Myc positive cases, VM was identified in 41 (59.4 %) patients, while 53 (76.81 %) melanoma cases demonstrated LPPCN. We also observed a significant association of c-Myc expression with the formation of VM and LPPCN ($P = 0.001$, and $P < 0.001$, respectively). Our results suggest that c-Myc expression positively correlates to the formation of VM and LPPCN.

Fig. 1 c-Myc expression, phenomena of VM and LPPCN in melanoma. **A** IHC staining of c-Myc *a* for negative and *b* for positive nuclear expression, ($\times 200$); **B** Phenomena of VM (red arrow, CD34-negative and PAS-positive) and EDV (green arrow, CD34-positive and PAS-positive) in melanoma specimens ($\times 400$); **C** LPPCN phenomena. Cells that underwent LPPCN are distributed in patterns of lines and/or networks (*a* and *b*; $\times 200$) among the environmental tumor cells and darkly stained (*c-e*; $\times 400$). Inflammatory cells are absent in the environmental district (*c*; $\times 400$). These networks come with neovasculogenesis networks (*b* and *d*; $\times 200$); **D** Kaplan-Meier survival curve demonstrated that high c-Myc expression, VM and LPPCN are significantly related to poor prognosis ($p < 0.05$, for all)



High c-Myc expression promotes cell migration, invasion, VM formation and proliferation in vitro

Cell migration and invasion

To evaluate the role of c-Myc in regulating the invasion and migration of melanoma cells, we performed the invasion/migration assay, and wound healing assays. Our results demonstrated that c-Myc knockdown significantly

decreased the abilities of A375 and MUM-2B cells to invade and migrate, whereas c-Myc overexpression had the inverse effect in A375 and MUM-2C cells. (Fig. 2A and B, $p < 0.05$ for all).

VM formation

To investigate the function of c-Myc in the formation of VM in vitro, we used a matrigel-based tube formation

Table 2 The correlation of VM with the clinicopathological parameter of melanoma

Variant	VM		χ^2	P-value
	Positive(62)	Negative(73)		
Sex				
Male	39	49	0.263	0.717
Female	23	24		
Age(years)				
≥ 55	37	42	0.063	0.862
<55	25	31		
Tumor size(cm ³)				
≥ 7.8	29	26	1.729	0.220
<7.8	33	47		
AJCC staging				
Stage(I)	8	21	5.002	0.035*
Stage(II + III + IV)	54	52		
Classification				
Acral melanoma	15	44	17.739	**
Others#	47	29		
Breslow's depth				
≤ 1.5 mm	15	36	9.001	0.004*
>1.5 mm	47	37		
Pigments				
YES	24	41	4.091	0.057
NO	38	32		
Lymphatic metastasis				
YES	35	28	4.411	0.040*
NO	27	45		
Distant metastasis				
YES	25	25	0.531	0.480
NO	37	48		

#(mucosal melanoma, CSD, non-CSD)* $p < 0.05$, ** $P < 0.001$

assay. c-Myc silencing in A375 cells inhibited VM formation on matrigel, whereas c-Myc overexpression facilitated the development of VM tubes. MUM-2B cells formed tube-like structures on the surface of the matrigel, whereas loss of c-Myc triggered the disappearance of these structures within 24 h. Moreover, MUM-2C cells lacking pseudopods formed VM tubes following exogenous overexpression c-Myc. ($p < 0.05$; Fig. 2C).

Cell proliferation

To further investigate the effect of high c-Myc expression on cell viability and proliferation, we performed MTT assays (Fig. 2D). The results showed that inhibition of c-Myc expression suppressed the growth of A375 and MUM-2B melanoma cells. In contrast, promotion of c-

Table 3 The correlation of LPPCN with the clinicopathological parameter of melanoma

Variant	LPPCN		χ^2	P-value
	Positive(75)	Negative(60)		
Sex				
Male	45	43	1.999	0.203
Female	30	17		
Age(years)				
≥ 55	44	35	0.002	1.000
<55	31	25		
Tumor size(cm ³)				
≥ 7.8	34	21	1.474	0.290
<7.8	41	39		
AJCC staging				
Stage(I)	11	18	4.646	0.036*
Stage(II + III + IV)	64	42		
Classification				
Acral melanoma	27	32	4.070	0.055
Others#	48	28		
Breslow's depth				
≤ 1.5 mm	18	33	13.628	**
>1.5 mm	57	27		
Pigments				
YES	36	29	0.001	1.000
NO	39	31		
Lymphatic metastasis				
YES	39	24	1.929	0.224
NO	36	36		
Distant metastasis				
YES	36	14	6.273	0.004*
NO	39	46		

#(mucosal melanoma, CSD, non-CSD)* $p < 0.05$, ** $P < 0.001$

Myc expression accelerated the growth of A375 and MUM-2C cells.

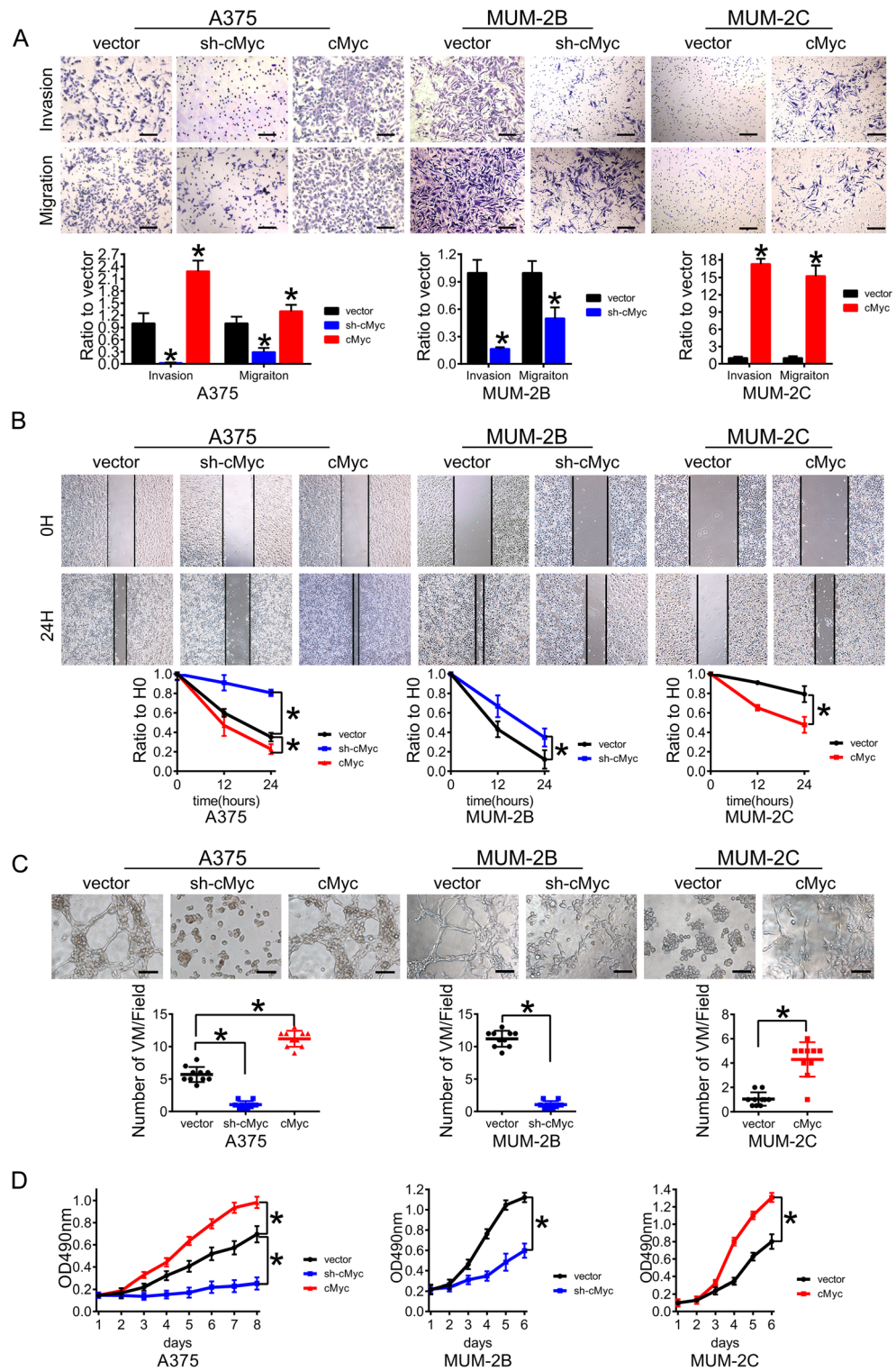
High c-Myc expression promotes EMT by the TGF- β /snail signaling pathway in vitro

c-Myc promotes expression of EMT related proteins

EMT is an important cellular program during tumor migration and invasion. To explore the molecular mechanisms by which c-Myc promotes EMT, we evaluated EMT-related indexes using western blotting, semi-quantitative RT-PCR and immunofluorescence.

Western blot (Fig. 3A) and RT-PCR (Fig. 3B) results confirmed that high c-Myc expression upregulated Snail and downregulated E-cadherin expression. The gray

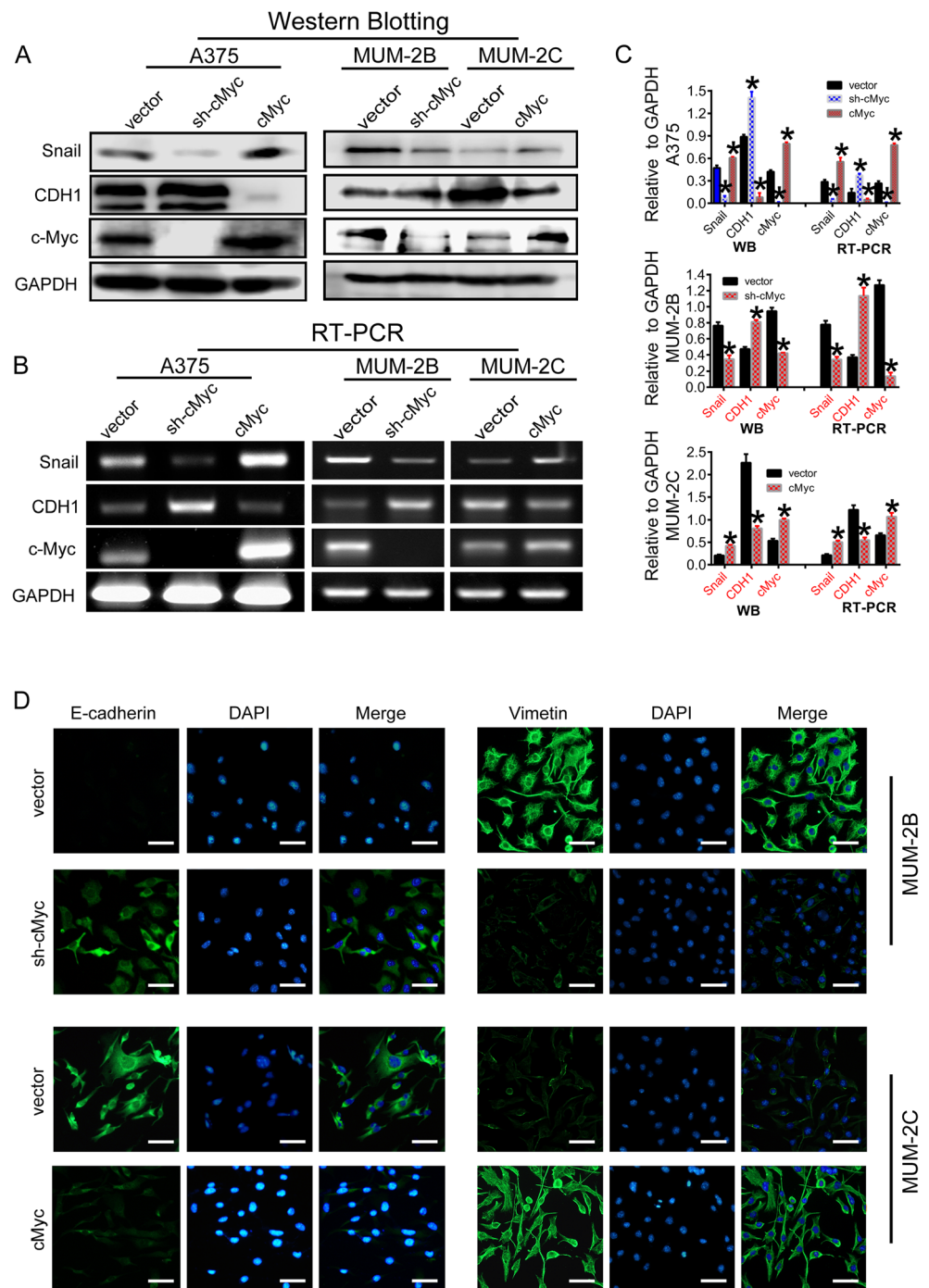
Fig. 2 High c-Myc expression promotes cell invasion, migration, VM formation. **A** The invasion and migration abilities of melanoma cells were increased following c-Myc overexpression, and decreased by c-Myc knockdown (bar 200um); **B** Wound healing assay ($\times 40$). Quantitative analysis showed a significant difference at 24 h; **C** c-Myc overexpression promoted VM formation and Knockdown of c-Myc inhibited the VM formation (bar 100um, $*P < 0.05$, $n = 3$); **D** MTT tests showed high c-Myc expressions increased cell proliferation ($*P < 0.05$, $n = 3$)



analysis showed that the differences were statistically significant (Fig. 3C). Furthermore, immunofluorescence revealed that high E-cadherin expression and down-regulation of Vimentin, a mesenchymal marker, in

MUM-2B cells occurs following c-Myc knockdown. Conversely, MUM-2C cells overexpressing c-Myc demonstrated loss of E-cadherin and up-regulation of Vimentin expression (Fig. 3D).

Fig. 3 The influence of c-Myc in EMT related index expression. **A** and **B** Knockdown c-Myc induced down-regulation of Snail expression and upregulation of E-cadherin (CDH1), the inverse change was obtained with the overexpression c-Myc; **C** showed the Gray value analysis of Western blotting and RT-PCR results; **D** Immunofluorescence staining. Overexpression of c-Myc increased the protein expression of Vimentin and inactivated the expression of E-cadherin and c-Myc silencing expression inhibited Vimentin expression and upregulated E-cadherin expression (bar, 50um)



c-Myc may promote EMT by the TGF- β /snail signaling pathway

To further analyze the signaling pathway of c-Myc in regulation of EMT, we performed western blot, luciferase reporter gene assay and Co-IP assays.

Western blot results showed that TGF- β increased the protein expression of c-Myc and Snail (Fig. 4A). The gray assay showed that the differences were statistically

significant (Fig. 4B). Luciferase reporter gene assay results showed that TGF- β (30 ng/ml) enhanced c-Myc gene expression (Fig. 4C). A375 and MUM-2B cells were cotransfected with a Snail promoter-luciferase construct and c-Myc-cDNA in an expression vector. c-Myc overexpression resulted in enhancement of transactivation of Snail promoter activity (Fig. 4D). These results indicated that TGF- β is one of the upstream signaling molecules of c-Myc, and the Snail promoter is also the target of c-Myc.

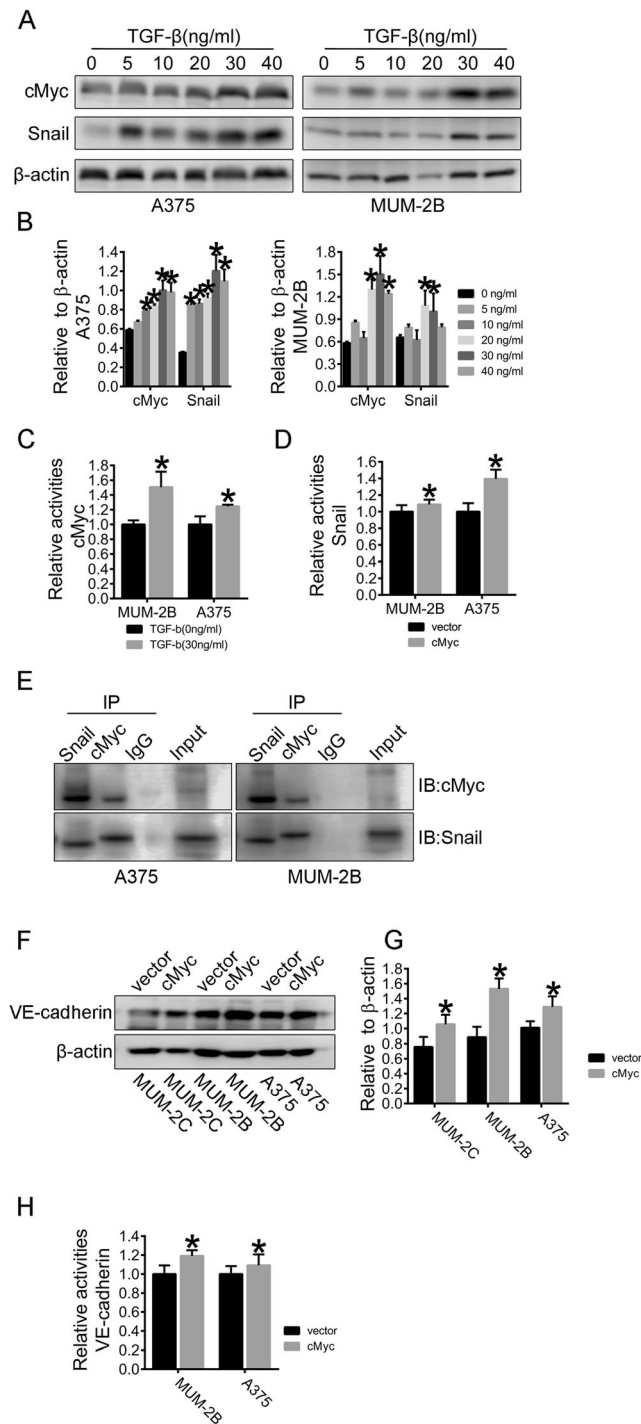


Fig. 4 c-Myc promotes EMT via TGF-β/Snail signaling pathway and VM via VE-cadherin. **A** and **B** Western Blotting assay and Gray value analysis showed TGF-β promoted c-Myc and Snail protein expression; **C** showed TGF-β promotes c-Myc gene expression; **D** showed c-Myc promotes Snail gene expression; **E** showed there is interaction between c-Myc and Snail; **F**, **G** and **H** showed c-Myc promotes VE-cadherin protein and gene expression. (**P* < 0.05)

Next, we determined whether c-Myc interacts with Snail using a Co-IP assay, and found that Snail was also be pulled down by c-Myc (Fig. 4E).

c-Myc promotes VM by upregulation of VE-cadherin expression

To further delineate the signaling pathway of c-Myc in regulation of VM, we used western blot and luciferase reporter gene assays.

Western blot results showed that c-Myc overexpression can increased VE-cadherin protein expression (Fig. 4F and G). A375, MUM-2B cells were cotransfected with a VE-cadherin promoter-luciferase construct and c-Myc-cDNA in an expression vector. Luciferase reporter gene assay results showed that c-Myc overexpression enhanced VE-cadherin promoter activity (Fig. 4H). These results indicate that the VE-cadherin promoter is one of the targets of c-Myc.

c-Myc overexpression promotes LPPCN by the upregulating Bax expression

c-Myc overexpression sensitizes cells to CoCl₂ treatment and hypoxia (1 % O₂), leading to cell apoptosis by upregulating Bax expression

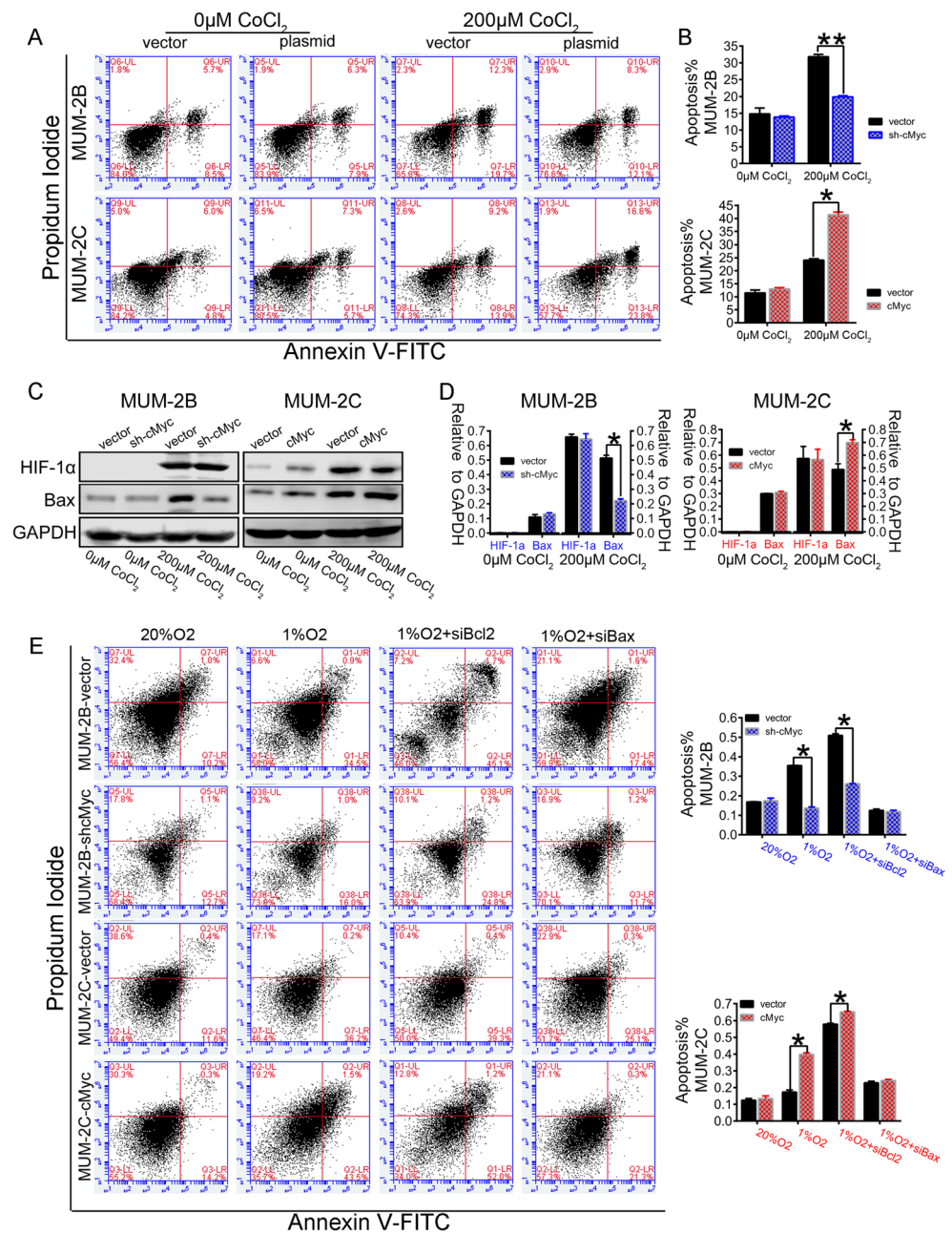
To assess the pro-apoptotic role of c-Myc in tumor cells in vivo, we treated melanoma cells with hypoxia-mimetic agent CoCl₂ (200 μM, 12 h) [23, 24] and then used an AnnexinV-FITC/PI Apoptosis Detection Kit and flow cytometry to detect cell death.

Without CoCl₂ treatments, there were no differences in the apoptotic index of high versus low-c-Myc expressing cells. However, the rate of apoptosis in MUM-2B cells transfected with sh-c-Myc was lower than that in controls, when the cells were treated with CoCl₂. The expected opposing relationship was observed when c-Myc overexpressing MUM-2C cells were compared with controls (Fig. 5A and B, *P* < 0.05).

To explore the roles of c-Myc in mediating apoptosis, we evaluated Bax protein expression in melanoma cells treated with or without 200 μM CoCl₂ for 12 h. Our data demonstrated that Bax protein expressions was linked to c-Myc expression levels. c-Myc knock-down in MUM-2B cells downregulated Bax expression, whereas c-Myc overexpression in MUM-2C cells upregulated Bax expression only with CoCl₂ treatment (Fig. 5C and D).

To further analyze the roles of Bax in the process that c-Myc sensitized cells to hypoxia (1 % O₂) by c-Myc, we silenced Bax or Bcl2 expression by siRNAs. Under hypoxic conditions, the differences in apoptosis rates were similar to cells treated with CoCl₂. Moreover, silencing the expression of Bax but not Bcl2 reduced these differences. (Fig. 5E, *P* < 0.05).

Fig. 5 **A** Melanoma cell lines treated with or without CoCl_2 ($200 \mu\text{M}$, 12 h) were stained with PI and Annexin V; **B** Early and late apoptotic cells are shown in the right quadrant. There were significant differences in cells with CoCl_2 treatment; **C** and **D** Western blotting and gray analysis showed Bax expression was significantly upregulated in cells with CoCl_2 treatment; **E** showed that it is BAX siRNA not BCL2 siRNA weakened the differences in the number of apoptotic cells under hypoxic conditions (1% O_2). * $P < 0.05$, ** $P < 0.001$



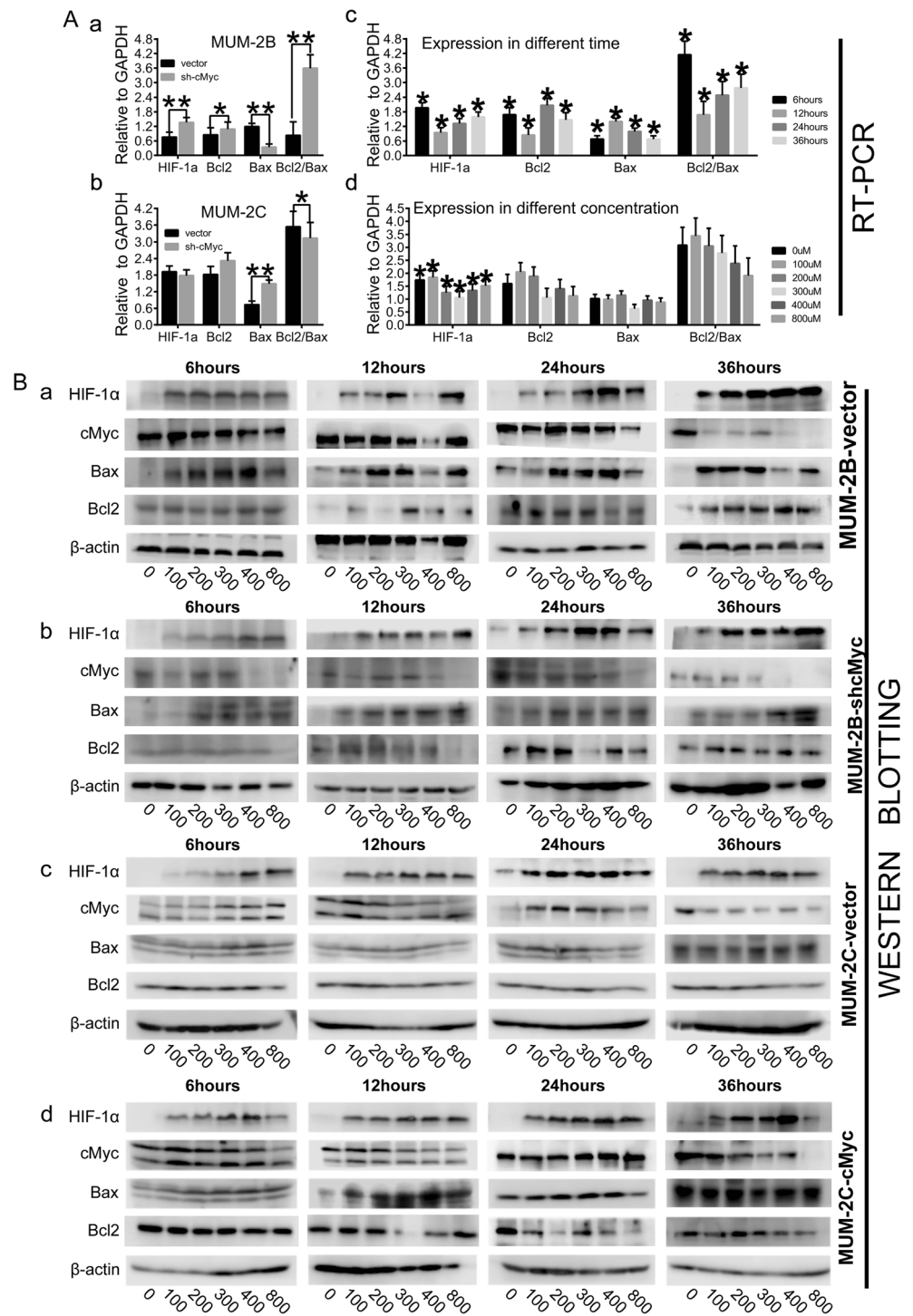
c-Myc overexpression plays different roles in cells treated with different levels of CoCl_2 by regulating the ratio of Bcl2/Bax

To explore the role of c-Myc in cells treated with CoCl_2 , we conducted experiments to modulate the concentration and time in which cells were exposed to CoCl_2 in vitro. We then evaluated the expression of apoptosis-related genes by RT-PCR and multi-factor ANOVA.

Bax expression was down-regulated by c-Myc knock-down expression, while the ratio of Bcl2/Bax was concomitantly reduced (Fig. 6A-a). The converse results were observed following the c-Myc overexpression in

MUM-2C cells (Fig. 6A-b). More specifically, the results suggested that both Bax expression and the Ratio of Bcl2/Bax were related to HIF-1 α expression, and this relationship was modulated by a concentration and time-dependent manner, relating to the exposure to CoCl_2 . Notably, Bax response to CoCl_2 treatment was most prominent at 12 h (Fig. 6A-c), while the ratio of Bcl2/Bax increased over time. The ratio of Bcl2/Bax increased at 100 μM CoCl_2 treatment and decreased upon exposure to higher concentrations of CoCl_2 (200, 300, 400 and 800 μM) (Fig. 6A-d). Detailed results of multi-factor ANOVA are shown in Supplementary Table S2.

Fig. 6 **A** *a* and *b* showed c-Myc high expression upregulated Bax expression and increased the ratio of Bcl2/Bax in cells with CoCl₂ treatment. *c* showed the ratio of Bcl2/Bax was at its lowest level at this time point 12 h; *d* the ratio of Bcl2/Bax peaked at concentration of 100 μM of CoCl₂, decreased with increasing of levels of CoCl₂; **B** Western Blotting assay showed the similar results to RT-PCR. **P* < 0.05, ***P* < 0.001, n = 3



To examine the roles of hypoxia-inducible factor 1α (HIF-1α) and c-Myc, we detected the expression of these proteins by western blotting. The results were shown in Fig. 6B. High HIF-1α expression inhibited the expression of c-Myc and Bcl2, and enhanced Bax expression. Taken together, these results suggest that c-Myc overexpression can cause cell death via the c-Myc/Bax signaling pathway by decreasing the ratio of Bcl2/Bax.

Examining the effects of c-myc using a murine xenograft model

High c-Myc expression promotes tumor growth in vivo

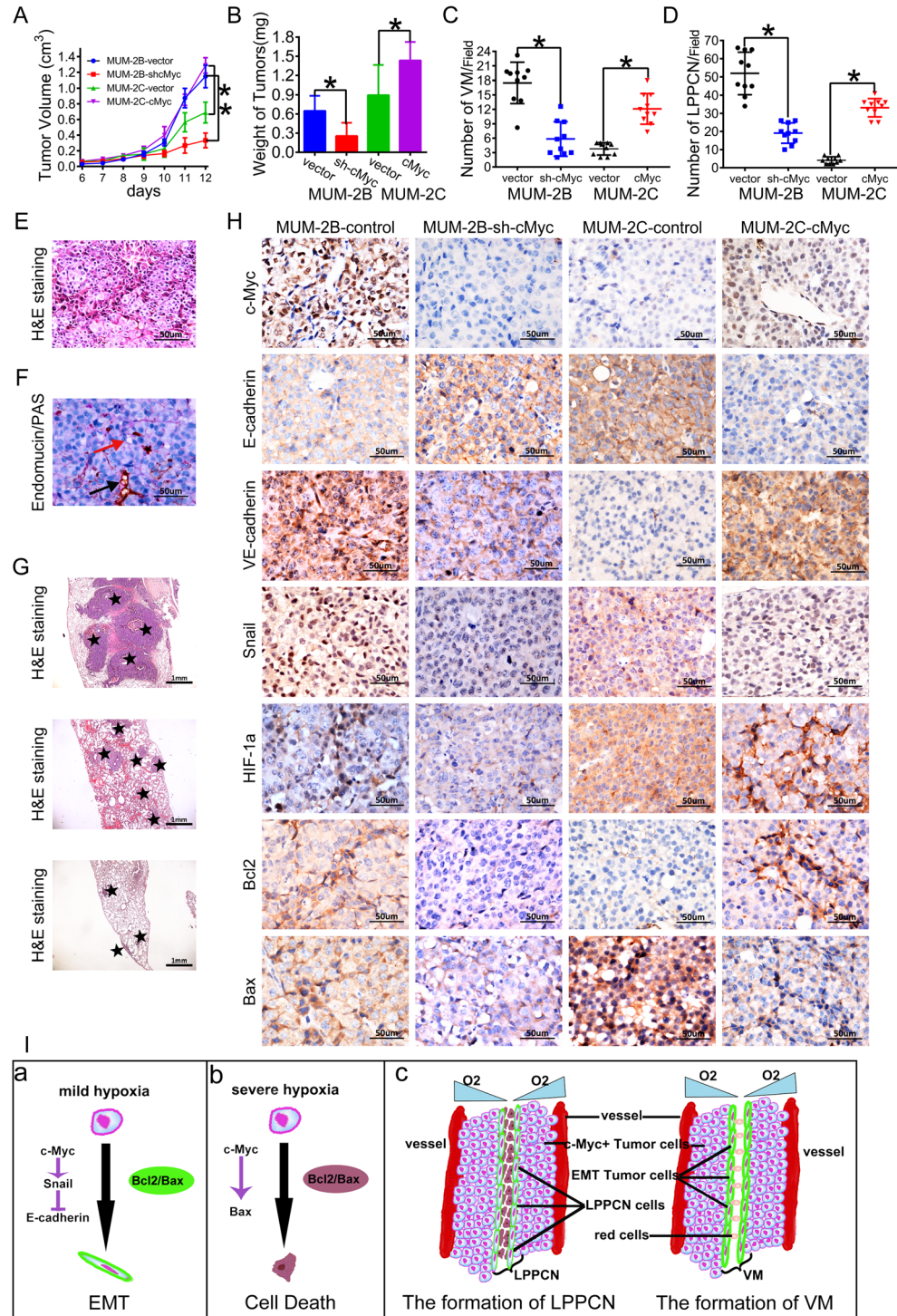
The effects of c-Myc on tumor growth in nude mice were reflected by growth curves. The growth curves showed that murine xenografted melanoma tumors with

high c-Myc expression developed faster than those with low c-Myc expression ($p < 0.05$, Fig. 7A). The weight of tumors with high c-Myc expressions was higher than that of tumors with low c-Myc expression ($p < 0.05$, Fig. 7B).

c-Myc overexpression increases the formation of VM and LPPCN in vivo

To evaluate the relationship between c-Myc expression and VM in vivo, we performed Endomucin/PAS double staining.

Fig. 7 The roles of c-Myc in melanoma murine xenograft tumor. **A** and **B** Tumor growth curve and tumor weight showed high c-Myc expression promotes tumor growth; **C** tumors with high c-Myc expression have more VM tubes; **D** high c-Myc expression increased the number of LPPCN cells; **E** LPPCN cells are darkly stained and distributed in patterns of lines and/or networks among the environmental tumor cells with no inflammation cells. ($\times 400$, bar 50um); **F** EDV with positive PAS staining and Endomucin expression (the black arrow) and VM channels lined with tumor cells and PAS-positive substances and negative Endomucin expression, and red cells remained visible at the center of the VM channels (the red arrow) ($\times 400$, bar 50um); **G** H&E staining showed lung metastases of melanoma. **H** showed that tumors with higher c-Myc expression had loss of E-cadherin proteins and elevated Snail, VE-cadherin expression. HIF-1 α , Bcl2 Bax expression exhibited in linear pattern ($\times 400$, bar 50um). **I** A model and the molecular mechanisms of c-Myc on the formation of VM and LPPCN. *a* Under mild hypoxia, the ratio of Bcl2/Bax was up-regulated and c-Myc triggered the epithelial-mesenchymal transition (EMT) and led cell survive; *b* Under severe hypoxia, the ratio of Bcl2/Bax was down-regulated and c-Myc sensitized tumor cells to oxygen deprivation and led cell death; *c* Tumor cells formed VM tube following the LPPCN cells disappeared, EMT occurrence and red cell got into. $*P < 0.05$, $n = 10$



VM (Fig. 7F, red arrow) formation increased following c-Myc overexpression in MUM-2C xenografted tumor and decreased upon gene silencing of c-Myc in MUM-2B xenografted tumor (Fig. 7C, $p < 0.05$).

To evaluate the correlation between c-Myc expression and the formation of LPPCN, we performed H&E staining. Notably, there were significantly more LPPCN cells in MUM-2B tumors as compared to MUM-2B-sh-c-Myc tumors. Moreover, the number of LPPCN cells in the MUM-2C-vector groups was significantly lower than that in MUM-2C-c-Myc tumors (Fig. 7D and E, $p < 0.001$).

High c-Myc expression correlates with tumor metastasis in vivo

To detect the role of c-Myc in the invasion and metastasis of tumor, we performed metastasis assay in melanoma xenograft model. When the tumor was injected into the back of the mice, only the MUM-2B-vector tumors (1/6) has lung metastases (Fig. 7G). For colonization assay, tumors in lung were observed in 83.3 % (5/6) of MUM-2B-vector xenografted tumor (VS 0/6 of MUM-2B-sh-c-Myc tumors) and 1/6 of MUM-2C-c-Myc tumors (VS 0/6 of MUM-2C-vector tumor) following tail vein injection of tumor cells. The results indicated that c-Myc may play important roles in the tumor metastasis.

High c-Myc expression promotes VM by upregulation of EMT and LPPCN related protein expression in vivo

To further clarify the role of c-Myc in the formation of VM and LPPCN in vivo, we evaluated the expression of EMT and LPPCN related proteins by immunohistochemistry (Fig. 7H). Consistent with our results, loss of E-cadherin, increased Snail expression, and VE-cadherin expression was observed in high c-Myc expressing tumors, while the converse relationship was observed upon c-Myc shRNA-mediated silencing. LPPCN cells specifically showed to be Bax positive. Additionally, HIF-1 α , Bcl2, and Bax expression was arranged in lines or in a net. We deduced that the formation of VM was related to c-Myc over-expression, and that LPPCN was induced by c-Myc-related cell death.

Discussion

It is important to note that, it is the metastasis, rather than the primary tumor, that ultimately leads to patient death, caused by metastatic growth that compromises the function of vital organs including, most notably, the lymph nodes, liver, lungs and brain.

The proto-oncogene c-Myc encodes a nuclear phosphoprotein transcription factor that plays an integral role in a variety of cellular processes [25, 26]. Interestingly, studies have now shown that c-Myc induction of programmed cell death may contribute to carcinogenesis [27]. However, the shift in the paradoxical properties of c-Myc in which c-Myc induces a pro-apoptotic effect versus EMT and results in metastasis of melanoma remains unclear.

In this study, we demonstrated that c-Myc accelerates metastasis by promoting the formation of VM via two mechanisms. First, c-Myc upregulates Snail expression to promote EMT via the TGF- β /Snail/E-cadherin signal pathway. Second, c-Myc upregulates Bax expression, resulting in a decrease in the ratio of Bcl2 and Bax under severe hypoxic conditions and leading to cell death, thereby promoting the formation of LPPCN.

Across our cohort, nearly 50 percent of melanoma cases demonstrated high c-Myc protein expression. High c-Myc expression tumors are associated with a poor prognosis and an elevated incidence of metastasis. A study has previously demonstrated that VM significantly correlates with metastasis and a poor prognosis [9]. VM was observed in almost 60 % of melanomas with high c-Myc expression. In vivo and in vitro experiments highlighted the role of c-Myc in VM channel formation. Specifically, tumors with high c-Myc protein expression formed VM channels, whereas low c-Myc-expressing tumors did not show the same effect. Our results suggest the involvement of c-Myc in the formation of VM and promotion of disease progression.

Kang et al. demonstrated that overexpression of c-Myc mediates EMT, which plays an important roles in the formation of VM, through loss of functional E-cadherin [11, 28–30]. However, the manner by which c-Myc promotes EMT remains to be investigated in melanoma. Our results showed that exogenous c-Myc overexpression or high expression leads to increased expression of Snail and Vimentin and loss expression of E-cadherin in melanoma cells. Notably, a similar signaling axis was observed in our xenograft models. TGF- β increased the protein and gene expression of c-Myc and Snail in melanoma cells. Moreover, we demonstrated that c-Myc overexpression enhanced motility and invasiveness of A375 and MUM-2C cells, whereas c-Myc knockdown had the opposite effect. As such, we hypothesize that c-Myc may promote EMT via the TGF- β /Snail/E-cadherin signaling pathway, leading to the formation of VM.

Previous studies have demonstrated that c-Myc/Max heterodimers bind to canonical E-box elements in the promoter region of BAX [31]. Substantial evidence supports the notion that c-Myc sensitizes cells to apoptosis by altering the balance of pro- and anti-apoptotic factors, priming the cells for death when such conditions are appropriate [32]. A study has

identified c-Myc up-regulated p53 to activate Bax and promotes the release of holo-cytochrome C from mitochondria [33]. Bcl-2/Bcl-xL proteins suppress holo-cytochrome C release by inhibition of c-Myc-induced apoptosis and promotion of c-Myc oncogenicity [34]. As such, the ratio of Bcl2 and Bax determines whether cells survive or undergo apoptosis. Our results demonstrate a relationship between c-Myc-induced apoptosis and the formation of LPPCN via the competition between Bcl2 and Bax.

Nearly 80 % (54/69) of melanomas with high c-Myc expression were shown to contain LPPCN. In support of these findings, we demonstrated that c-Myc suppression in murine xenograft models reduced the incidence of LPPCN. In vitro, flow cytometry data suggested that high c-Myc expression sensitized tumor cells to hypoxia and hypoxia-mimetic agent CoCl₂, and leads to apoptosis. Western blotting and RT-PCR analysis supported this hypothesis and highlighted the hypoxic-specific relationship between c-Myc and Bax expression. Our results indicate that c-Myc overexpression induced up-regulation of Bax and decreases the ratio of Bcl2/Bax when the cells was treated with elevated levels of CoCl₂. c-Myc may regulate HIF-1 α to enhance tumor angiogenesis [35]. Furthermore, HIF-1 α may inhibit DNA damage reduced by c-Myc [36]. A study has found that the HIF-1 α -c-Myc pathway promotes cell survival only in apoptosis-deficient malignant cells [37]. Another study has suggested that c-Myc sensitization to oxygen deprivation-induced cell death depends on Bax/Bak [38]. LPPCN was recently reported in highly aggressive melanoma by Zhang et al. In their study, LPPCN cells were shown to be positive for Bax by immunohistochemistry, which was verified again in this study, realing a significant relationship between Bax and LPPCN [13].

We suggest that when the blood supply of tumor is occluded and results in severe hypoxia, elevated expression of c-Myc decreases the ratio of Bcl2/Bax and activates apoptotic pathways in tumor cells. Cell death is distributed in specific patterns of lines and networks, in a LPPCN-like manner. We conclude that the c-Myc-dependent apoptotic response via Bax-dependent signaling represents important elements in the formation of LPPCN. A study has previously suggested that the formation of LPPCN provides a basis for the spatial organization of VM and EDV structures [22]. We believe that c-Myc promotes the formation of VM by regulation of LPPCN.

The center of a bulk tumor is under hypoxic or even anoxic conditions [39, 40]. In a hypoxic microenvironment, c-Myc promotes EMT in tuomor cells, thereby inducing cell survival, metastasis and the formation of VM. However, upon acute or perfusion-limited hypoxia (such as temporal fluctuations in blood perfusion) or in instances of chronic hypoxia, high expression of c-Myc induces cells death, driving the formation of LPPCN. In this manner, c-Myc drives the creation of spatial structure necessary for VM. Taken together, we suggest that c-

Myc positive cells that have undergone EMT form VM in the space created through the disappearance of LPPCN cells and resulting in tumor metastasis. All of these processes are driven by c-Myc, as shown in Fig. 7I.

The definition of LPPCN was, until now, solely a morphological description while the molecular characteristics have thus far remained unclear. We can no longer categorize LPPCN as the kind of cell death described by the Nomenclature Committee on Cell Death in 2012 [41]. Our study provides a possible mechanism of LPPCN. Additionally, LPPCN is an active gene-regulated process, that plays an important role in cancer development and progression. Therefore, greater emphasis on characterizing the molecular features of LPPCN could improve our understanding of programmed cell death.

Our study suggest that over expressed c-Myc could be regarded as an important parameter in the malignant degree and poor prognosis of melanoma and highlights the pleiotropic nature of oncogenes in the development of cancers. Considering the central role of c-Myc in the pathobiology of numerous cancers, including melanoma, our results suggest that improved understanding and characterization of this gene could lead to the development of novel, targeted therapies.

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Author contribution statement B.C.S. conceived the study, B.C.S., R.S. and X.L. designed experiments and wrote the manuscript. X.L., X.L.Z., X.M.Z., Q.G. and X.Y.D. performed experiments. D.W.Z. and D.F.Z. analyzed the data, Y.H.Z., R.S. and Y.L.L. collected the human pathological specimen of melanoma. All authors revised the manuscript.

Compliance with ethical standards

Disclosure The authors declare that they have no conflict of interests.

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