IJC
International Journal of Cancer

Interleukin 8 mediates bcl-xL-induced enhancement of human melanoma cell dissemination and angiogenesis in a zebrafish xenograft model

Chiara Gabellini[1,2a], Elena Gómez-Abenza[1,2], Sofia Ibáñez-Molero[1,2], Maria Grazia Tupone[3], Ana B. Pérez-Oliva[1,2], Sofia de Oliveira[1,2,4b], Donatella Del Bufalo[3] and Victoriano Mulero[1,2]

[1] Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, Spain
[2] Instituto Murciano de Investigación Biosanitaria (IMIB)-Arrixaca, Murcia, Spain
[3] Preclinical Models and New Therapeutic Agents Unit, Regina Elena National Cancer Institute, Rome, Italy
[4] Microvascular Biology and Inflammation Unit, Molecular Medicine Institute, Biochemistry Institute, Faculty of Medicine, University of Lisbon, Lisbon, Portugal

The protein bcl-xL is able to enhance the secretion of the proinflammatory chemokine interleukin 8 (CXCL8) in human melanoma lines. In this study, we investigate whether the bcl-xL/CXCL8 axis is important for promoting melanoma angiogenesis and aggressiveness *in vivo*, using angiogenesis and xenotransplantation assays in zebrafish embryos. When injected into *wild-type* embryos, bcl-xL-overexpressing melanoma cells showed enhanced dissemination and angiogenic activity compared with control cells. Human CXCL8 protein elicited a strong proangiogenic activity in zebrafish embryos and zebrafish Cxcr2 receptor was identified as the mediator of CXCL8 proangiogenic activity using a morpholino-mediated gene knockdown. However, human CXCL8 failed to induce neutrophil recruitment in contrast to its zebrafish homolog. Interestingly, the greater aggressiveness of bcl-xL-overexpressing melanoma cells was mediated by an autocrine effect of CXCL8 on its CXCR2 receptor, as confirmed by an shRNA approach. Finally, correlation studies of gene expression and survival analyses using microarray and RNA-seq public databases of human melanoma biopsies revealed that bcl-xL expression significantly correlated with the expression of CXCL8 and other markers of melanoma progression. More importantly, a high level of co-expression of bcl-xL and CXCL8 was associated with poor prognosis in melanoma patients. In conclusion, these data demonstrate the existence of an autocrine CXCL8/CXCR2 signaling pathway in the bcl-xL-induced melanoma aggressiveness, encouraging the development of novel therapeutic approaches for high bcl-xL-expressing melanoma.

Molecular Cancer Biology

Key words: bcl-xL, CXCL8, melanoma, angiogenesis, zebrafish

Abbreviations: CXCL8: interleukin 8; GPF: green fluorescent protein; NF-κB: nuclear factor κB; hFGF: human fibroblast growth factor; VEGF: vascular endothelial growth factor; SOX10: SRY-related HMG box-containing factor 10; IκB: inhibitor of NF-κB; Mo: morpholino; MITF: microphthalmia-associated transcription factor; hr: hours; hpi: hours postinjection; dpi: days postinjection; std: standard; sh: short hairpin; SIVs: subintestinal veins

Additional Supporting Information may be found in the online version of this article.

Conflict of Interest: No potential conflicts of interest were disclosed.

Grant sponsor: UMU Incoming Mobility Programme ACTion (U-IMPACT) under European Union Seventh Framework Programme-Marie Curie COFUND (FP7/2007–2013); **Grant number:** 267143; **Grant sponsor:** Spanish Ministry of Economy and Competiveness; **Grant number:** BIO2011-23400, BIO2014-52655-R; **Grant sponsor:** Fondos Europeos de Desarrollo Regional/European Regional Development Funds; **Grant sponsor:** Italian Association for Cancer Research; **Grant number:** 18560; **Grant sponsor:** Italian Foundation for Cancer Research (MG.T. received a fellowship); **Grant sponsor:** Fundação para a Ciência e a Tecnologia (S.d.O. received a Ph.D. Fellowship); **Grant number:** SFRH/BD/62674/2009

DOI: 10.1002/ijc.31075

History: Received 13 Dec 2016; Accepted 19 Sep 2017; Online 26 Sep 2017

Correspondence to: Victoriano Mulero or Chiara Gabellini, Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, IMIB-Arrixaca, Campus Espinardo, Murcia, Spain, Tel.: +34-868-887-581, Fax: +34-868-883-963,
E-mail: vmulero@um.es or chiara.gabellini@um.es

[a]Chiara Gabellini's current address is: Unit of Cell and Developmental Biology, Department of Biology, University of Pisa, S.S. 12 Abetone e Brennero 4, Pisa, Italy

[b]Sofia de Oliveira's current address is: Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, Wisconsin 53706, USA

What's new?

The protein bcl-xL is able to enhance the secretion of proinflammatory chemokine interleukin 8 (CXCL8) in human melanoma lines. Using the zebrafish model, here the authors demonstrate that bcl-xL enhances melanoma *in vivo* angiogenesis and invasion through the CXCL8/CXCR2 axis. In human biopsies, melanoma bcl-xL expression correlates with CXCL8, and high bcl-xL/CXCL8 levels significantly correlate with the poor prognosis of melanoma patients. These findings point to novel markers of melanoma aggressiveness associated with the activated bcl-xL/CXCL8 axis and support the use of the zebrafish model to study the efficacy of therapeutic compounds inhibiting the CXCL8/CXCR2 axis to counteract tumor progression.

Defects in apoptosis signaling pathways are common in cancer cells. Such defects may play an important role not only in tumor initiation but also in tumor progression, promoting metastasis by enabling tumor cells to survive the transit in the bloodstream and to grow in ectopic tissue sites lacking the otherwise required survival factors. Furthermore, cancer metastasis depends closely on the continuous crosstalk between cancer cells and the surrounding cellular and extracellular microenvironment.

Melanoma is a very aggressive form of skin cancer, arising from epidermal melanocytes, and its high lethality is associated with metastasis.[1]

bcl-xL is an antiapoptotic protein belonging to bcl-2 family and in addition to promoting cell survival, has been found to regulate different cellular processes, including tumor metastasization.[2] In fact, increased bcl-xL protein levels are associated with melanoma progression.[3] In this context, we have previously demonstrated that bcl-xL overexpression in melanoma and glioblastoma cell lines increases tumor angiogenesis, enhancing the secretion of the proinflammatory chemokine interleukin 8 (CXCL8)[4] through the involvement of the nuclear factor κB (NF-κB) pathway.[5]

CXCL8 acts as an autocrine/paracrine growth invasive and angiogenic factor, and influences the process of melanoma progression by activating CXCR1 and CXCR2 receptors.[6,7] The expression of CXCL8 protein correlates with the Clark level[8] and poor prognosis[9] in malignant melanoma. Moreover serum concentrations of CXCL8 in malignant melanoma patients correlate with tumor progression, survival and response to treatment.[10,11] However, a possible correlation between bcl-xL/CXCL8 co-expression and melanoma patient outcome has not been described. Besides, it is unknown whether the bcl-xL/CXCL8 axis is important in promoting tumor angiogenesis and aggressiveness *in vivo*.

In recent years, the teleost zebrafish (*Danio rerio*) has emerged as a promising vertebrate system for modeling cancer,[12] and for studying tumor biology and the mechanisms associated with disease progression. Benign and malignant tumor development in virtually all zebrafish organs occurs after exposure to mutagens and transgenesis of oncogenic proteins, resembling the histological and biochemical changes observed in human tumors,[13] including melanoma.[14] Moreover when xenografted in zebrafish embryos, human melanoma cells are not rejected, exhibit motility and invasive behavior[15–18] and induce neovascularization.[19] Such angiogenic responses are triggered by proangiogenic factors, such as fibroblast growth factor 2 (FGF2), which is produced by mammalian tumor cells.[18,20] Importantly, proinflammatory signaling pathways are conserved in zebrafish,[21–23] but the conservation of the proangiogenic role of CXCL8 in zebrafish has not been investigated in depth.[24]

The aim of the present work was to define the relationship between the bcl-xL/CXCL8 axis and cancer microenvironment, with particular regard to tissue invasion and angiogenesis in human melanoma using the unique strengths of the zebrafish as an experimental model. Our analysis extended to microarray and RNA-Seq from TCGA datasets of human melanoma specimens, in the search for a possible correlation between the expression of bcl-xL and CXCL8 and their impact on melanoma patient prognosis.

Material and Methods

Zebrafish husbandry

All experiments with live animals were performed using protocols prepared according to the European Union Council Guidelines (86/609/EU) and approved by the Bioethical Committee of the University of Murcia (approval no. #537/2011, #75/2014 and #216/2014). Zebrafish fertilized eggs were obtained from natural spawning of *wild-type* and transgenic fish held at our facilities following standard husbandry practices. Animals were maintained in a 12 hr light/dark cycle at 28.5°C. Transgenic zebrafish lines included the *Tg(flia:EGFP)*^{y1} (Zebrafish International Resource Center),[25] expressing green fluorescent protein (GFP) under the endothelial cell-specific promoter *fli1a*, the *Tg(mpx:EGFP)*^{i114} (Dr SA Renshaw)[26] expressing GFP under the neutrophil-specific promoter of myeloperoxidase and the transparent *roy*$^{a9/a9}$; *nacre*$^{w2/w2}$ (casper)[27] in which pigment cell production is inhibited.

Reagents

Human recombinant CXCL8 was purchased from R&D Systems (Minneapolis, MN). Zebrafish recombinant Cxcl8-L2 was produced in *Escherichia coli* as an N-terminal 6× His fusion protein, obtained from inclusion bodies with 6 M guanidine hydrochloride, and purified by a Ni-HiTrap column.[23] To anesthetize zebrafish larvae, a solution of 0.16 mg/ml tricaine (Sigma, St. Louis, MI) in embryo medium was used. To inhibit melanogenesis, zebrafish larvae were exposed to 0.3% phenylthiourea (PTU,

Sigma). The specific bcl-xL inhibitor WEHI-539 hydrochloride and cisplatin (PRONTO PLATAMINE infusion solution) were purchased from MCE MedChem Express Europe (Sollentuna, Sweden) and Pfizer (New York, NY), respectively.

Cell lines, transfection and viral infection

The human bcl-xL overexpressing clone MXL90 and Mneo control clone, derived from stable transfection of M14 line with a bcl-xL expression vector (pcDNA3-bcl-xL) and empty vector (pcDNA3),[4] respectively, were grown at 37°C, in a 5% CO_2/95% air atmosphere, in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamin, antibiotics and 800 μg/ml geneticin (Sigma, Saint Louis, MO). The human bcl-xL-overexpressing clone JXL8 and Jneo control clone, derived from stable transfection of JR8 line with a bcl-xL expression vector (pcDNA3-Flag-bcl-xL) and empty vector (pcDNA3), respectively, were grown in the same conditions as described above. 29-mer shRNA constructs in retroviral vector against human CXCL8 and CXCR2 genes (OriGene, Rockville, MD) were transfected into Phoenix amphotropic packaging line using LyoVec™ (InvivoGen, San Diego, CA). The following shRNA sequences were used: 5′-GCCAAGGA GTGCTAAAGAACTTAGATGTC-3′ (shIL8-B), 5′-GTATTA GCCACCATCTTACCTCACAGTGA-3′ (shIL8-D), 5′-TGG TCTCACTCCTGAAGGAAGTCAACTTC-3′ (shCXCR2). A shRNA construct containing the noneffective sequence 5′-GCACTACCAGAGCTAACTCAGATAGTACT-3′ was used as control. Transfected Phoenix cells were incubated for 48 hr for virus production, and then virus-containing medium was collected, filtered and used to infect Mneo and MXL90 cells in the presence of 8 μg/ml of polybrene (Sigma). Mixed cell populations were cultured in the presence of 1 μg/ml puromycin (Sigma).

In vivo invasion assay

Human melanoma cells were labelled with 1,1′-di-octa-decyl-3,3,3′,3′-tetra-methyl-indo-carbo-cya-nine perchlorate (DiI, Molecular Probes, Invitrogen, Waltham, MA), resuspended in a buffer containing 5% FBS in PBS. Two hundred cells/embryo were then injected in the yolk sac of *wild-type* or Casper zebrafish larvae 48 hr postfertilization (hpf) and after 5 days at 35°C, larvae were analyzed for human melanoma cells dissemination by fluorescence microscopy.[28] Melanoma cell invasion score was calculated as the percentage of human melanoma cell-invaded larvae over the total number of larvae analyzed.

Zebrafish yolk membrane (ZFYM) angiogenesis assay

Dechorionated embryos at 48 hpf were anaesthetized with tricaine and injected into the perivitelline space of *Tg(fli1a:GFP)*^{y1} larvae in the proximity of developing subintestinal veins (SIVs) with different amounts of human recombinant CXCL8, and zebrafish recombinant cxcl8-L2 or PBS, as control. As positive control, larvae were injected with 4 ng/embryo of recombinant human hFGF (Peprotech, Rocky Hill, NJ).[20] When indicated, DiI-labeled cells were injected, and 5% FBS buffer in PBS was used as control. The angiogenic response was evaluated 24 hr postinjection (hpi), based on the appearance of ectopic vessels sprouting from the SIVs and/or intervessel sprouting.[17,20] Data are expressed as the percentage of positive embryos/total injected embryos.

Otic injection

Otic injection was performed as previously described.[23] Briefly, anesthetized *Tg(mpx:GFP)*^{i114} 72 hpf larvae were mounted in 1% low melting point agarose (Sigma), and human recombinant CXCL8, zebrafish recombinant cxcl8-L2 or PBS, as control, was injected to the otic vesicle of the larvae. Images of neutrophil recruitment to the ear were taken 90 min after injection.

Morpholino knockdown

150 μM cxcr2 morpholino targeting the ATG region (ACTCTGTAGTAGCAGTTTCCATGTT) and 75 μM cxcr1 morpholino targeting the exon 1–intron 1 boundary (TGTCAGGATACTAAACTTACCAGTC),[29] purchased from Gene Tools (Philomath, OR), were microinjected in 0.5× Tango buffer and 0.05% phenol red solution into one-cell stage embryos using a microinjector (Narishige, Japan) (1 nl/embryo). Standard morpholino (GeneTools) was used as control.

ELISA and Western blot analysis

CXCL8 protein secretion in conditioned medium (CM) from the different human melanoma lines, exposed to serum-free medium for 24 hr, was evaluated by ELISA assay (R&D Systems). The level of secreted CXCL8 in CM was normalized to the number of adherent cells counted at the time of collection. The sensitivity of the ELISA assay was 31.2 pg/ml. Protein expression in cell extracts was evaluated as previously described,[5] using antibodies directed to bcl-xL (#sc-634, Santa Cruz Biotechnology, CA), Flag epitope (#F1804, Sigma), cleaved PARP (#AB3565, Millipore, Billerica, MA) and β-actin (#A1978, Sigma).

Real-time qPCR

Total RNA was extracted from melanoma cell lines using TRIzol® reagent (Thermo Fisher Scientific, Carlsbad, CA) in conjunction with PureLink® RNA Mini Kit (Thermo Fisher Scientific). cDNA was reverse transcribed using SuperScript III First-Strand synthesis system (Thermo Fisher Scientific). The quantitative PCR reaction was performed in triplicate with Applied Biosystems 7500 Fast Real-Time PCR System using SYBR® Select Master Mix (Thermo Fisher Scientific). Relative mRNA expression of each gene was normalized to β-actin (ACTB) levels. The primers used were as follows: human CXCR2 forward 5′-ATTCTGGGCATCCTTCACAG-3′, human CXCR2 reverse 5′-TGCACTTAGGCAGGAGGTCT-3′; human SOX10 forward 5′-AAGACACTAGAATCCTGACC-3′, human SOX10 reverse 5′-CTGCAGAACAGGAAAATAGG-3′; human MITF forward 5′-CAGTACCTTTCTACCACTTTAG-3′, human MITF reverse 5′-CCTCTTTTTCACAGTTGGAG-3′;

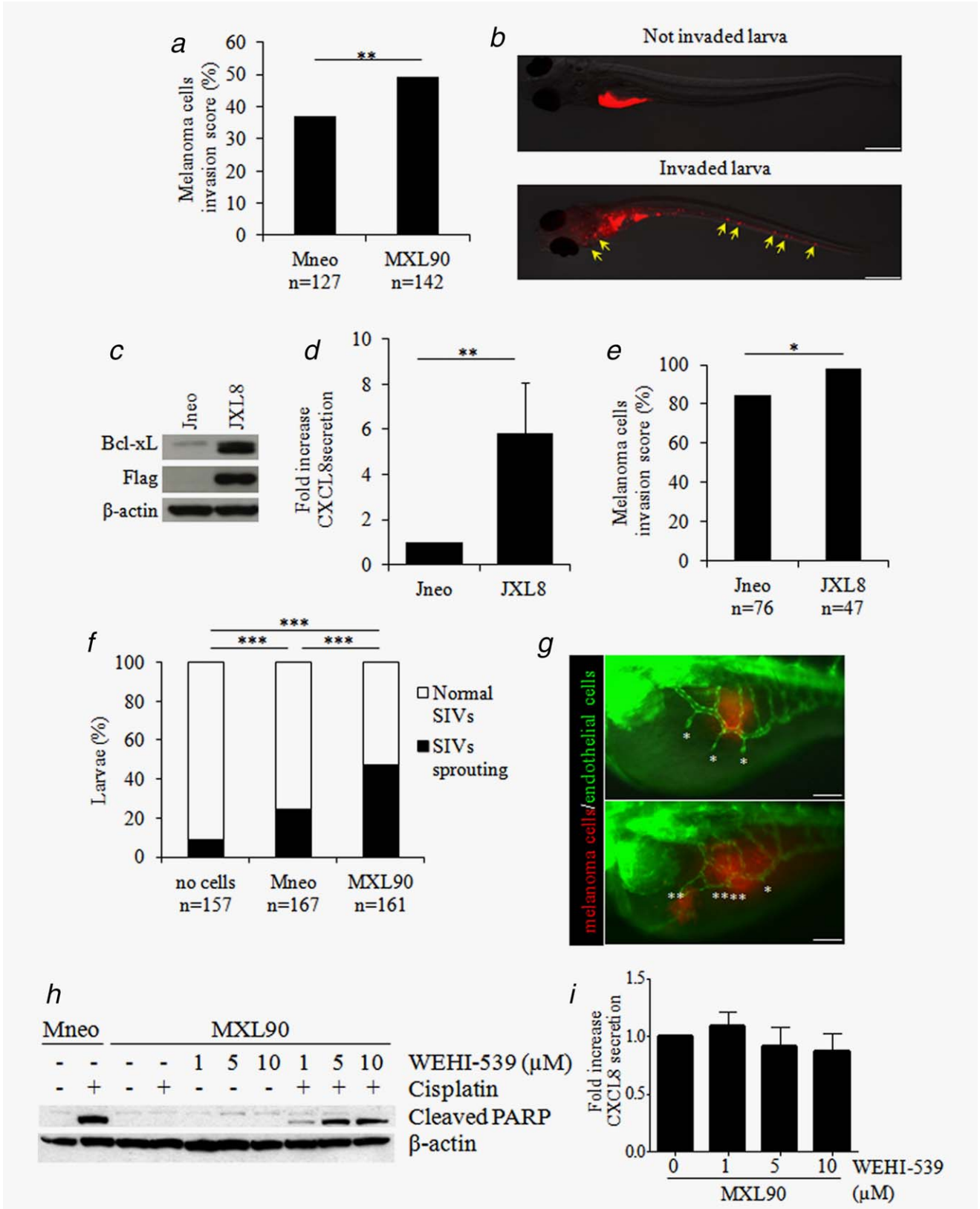
a
Melanoma cells invasion score (%)
60
50
40
30
20
10
0
**
Mneo n=127
MXL90 n=142
b
Not invaded larva
Invaded larva
c
Jneo
JXL8
Bcl-xL
Flag
β-actin
d
Fold increase CXCL8secretion
10
8
6
4
2
0
**
Jneo
JXL8
e
Melanoma cells invasion score (%)
100
80
60
40
20
0
*
Jneo n=76
JXL8 n=47
f
Larvae (%)
100
80
60
40
20
0
***
***
***
Normal SIVs
SIVs sprouting
no cells n=157
Mneo n=167
MXL90 n=161
g
melanoma cells/endothelial cells
h
Mneo
MXL90
- - - - 1 5 10 1 5 10 WEHI-539 (μM)
- + - + - - - + + + Cisplatin
Cleaved PARP
β-actin
i
Fold increase CXCL8 secretion
1.5
1.0
0.5
0.0
0 1 5 10 WEHI-539 (μM)
MXL90

Figure 1.

human ACTB forward 5′-GGCACCACACCTTCTACAATG-3′, human ACTB reverse 5′-GTGGTGGTGAAGCTGTAGCC-3′.

Image acquisition and processing

Images were taken using a Leica MZ16F fluorescence stereo microscope and processed using ImageJ software (http://rsb.info.nih.gov/ij/) and Photoshop CS.

Human microarray and RNA-seq dataset analysis

Data from the microarray datasets GDS1375 and GDS3966 were downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) website. Kaplan–Meier overall survival curves for the GSE19234 microarray of metastatic melanoma patient samples were obtained from PROGgeneV2 Pan cancer Prognostic database website (http://www.compbio.iupui.edu/proggene). The probes analyzed were 212312_at (BCL2L1/BCL-XL), 202859_x_at (CXCL8), 209843_s_at (SOX10) and 207233_s_at (MITF) and the values of each gene expression level are reported as counts.

Normalized gene expression and patient survival data were downloaded from Skin Cutaneous Melanoma repository of The Cancer Genome Atlas (TCGA) (https://gdc.cancer.gov) and analyzed in R programming language (http://www.r-project.org) using the R environment Rstudio (http://www.rstudio.com) and TCGAbiolinks packages.[30] Gene expression plots and regression curves for correlation studies were obtained using GraphPad Prism 5.03 (GraphPad Software, Inc., La Jolla, CA).

Statistical analysis

Statistical significance was determined by χ^2 test, t test, a one-way ANOVA analysis of variance followed by a Newman–Keuls or Tukey post-test, Pearson's and Spearman's correlation coefficients using GraphPad Prism 5.03 and Statistic Calculator software (StatPac Inc., Bloomington, MN).

Results

bcl-xL overexpression enhances melanoma invasion and angiogenesis in zebrafish larvae

To investigate the ability of bcl-xL protein to enhance human melanoma invasion *in vivo*, cells derived from human M14 melanoma line stably overexpressing bcl-xL protein[4] were implanted in the yolk sac of zebrafish larvae 48 hpf and melanoma cell invasion was evaluated 5 days postinjection (dpi). As shown in Figures 1*a* and 1*b*, injection of bcl-xL transfectant MXL90 resulted in a significant higher dissemination from the primary site of injection and colonization into distal parts of the larvae compared with the control clone Mneo. The same result was obtained for the stable overexpression of bcl-xL protein in another human melanoma line, JR8 (Fig. 1*c*): bcl-xL overxepressing clone JXL8 showed increased secretion levels of CXCL8 (Fig. 1*d*), associated with a significant higher dissemination in zebrafish larvae compared to the control line Jneo (Fig. 1*e*).

As our group previously demonstrated an increase of proangiogenic activity in the MXL90 cell line compared to control clone Mneo in Matrigel assays in mice,[4] it was decided to evaluate whether the enhanced invasiveness shown by bcl-xL overexpressing cells in zebrafish larvae might be associated with the increase of melanoma angiogenesis. For this, a zebrafish-based angiogenesis test[20] was performed by engrafting bcl-xL overexpressing melanoma cells and the control line in the proximity of the developing SIVs plexus of 2 dpf *Tg(fli1a:EGFP)*^{y1} zebrafish embryos, in which endothelial cells express GFP under the control of an endothelial-specific promoter.[31] The ability of melanoma cells to induce SIVs sprouting and/or the formation of collateral vessels inside the plexus was assessed. Both melanoma lines increased the percentage of zebrafish larvae positive for SIVs sprouting 24 hpi compared to buffer only. However, MXL90 cells overexpressing bcl-xL enhanced the percentage of zebrafish embryos positive for SIV sprouting compared to control clone Mneo (Figs. 1*f* and 1*g*).

We previously demonstrated that bcl-2, a bcl-xL homolog, enhances the proangiogenic hypoxia-inducible factor 1/vascular endothelial growth factor (VEGF) axis independently of its antiapoptotic activity.[32] Analogously, we found that doses of the specific inhibitor WEHI-539[33] able to counteract the antiapoptotic effect of bcl-xL (Fig. 1*h*) did not reduce CXCL8 secretion in bcl-xL overexpressing clone MXL90 (Fig. 1*i*).

Human CXCL8 induces angiogenesis in zebrafish larvae through Cxcr2 receptor

As melanoma cells overexpressing bcl-xL are characterized by enhanced CXCL8 protein secretion,[4] we tested the potential

Figure 1. bcl-xL protein overexpression enhances human melanoma cell dissemination and angiogenesis in zebrafish larvae. (*a*) Evaluation of invasion score of control clone Mneo and bcl-xL overexpressing clone MXL90 5 days after injection in the yolk sac of 2 dpf *wild-type* zebrafish larvae. (*b*) Representative images of larvae invaded or not by red-fluorescent human melanoma cells are shown. The magnification bar is 500 μm. (*c*) Evaluation of bcl-xL protein and Flag expression by Western blot analysis, (*d*) fold increase of CXCL8 secretion by ELISA assay in control clone Jneo and Flag-Bcl-xL overexpressing clone JXL8. (*e*) Evaluation of invasion score of control clone Jneo and Flag-Bcl-xL overexpressing clone JXL8 5 days after injection in the yolk sac of 2 dpf *wild-type* zebrafish larvae. (*f*) Evaluation of angiogenic activity of Mneo and MXL90 cells after injection in the yolk sac of *Tg(fli1a:GFP)*^{y1} zebrafish embryos, expressing GFP in endothelial cells. Larvae were analyzed 24 hpi for subintestinal veins (SIVs) sprouting by fluorescence microscopy. (*g*) Representative images of SIVs sprouting after transplantation of red-fluorescent melanoma cells are shown. Single sprouting (*) and fused sprouts (**) are indicated. The magnification bar is 100 μm. (*h*) Evaluation of PARP protein cleavage in Mneo and MXL90 cells after treatment for 24 hr with 20 μg/ml Cisplatin, in presence of increasing doses of the specific Bcl-xL inhibitor WEHI-539. (*i*) Evaluation of fold increase of CXCL8 secretion in MXL90 cells exposed for 24 hr to increasing doses of WEHI-539. (*a, e, f*) The results presented are a pool from three different experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ χ^2 test. (*d*) The results are presented as mean ± SD of two different experiments performed in duplicate. **$p < 0.01$ *t*-student test. (*i*) The results are presented as mean ± SD of two different experiments, performed in duplicate. (*a*, *e*, *f*) n = number of larvae.

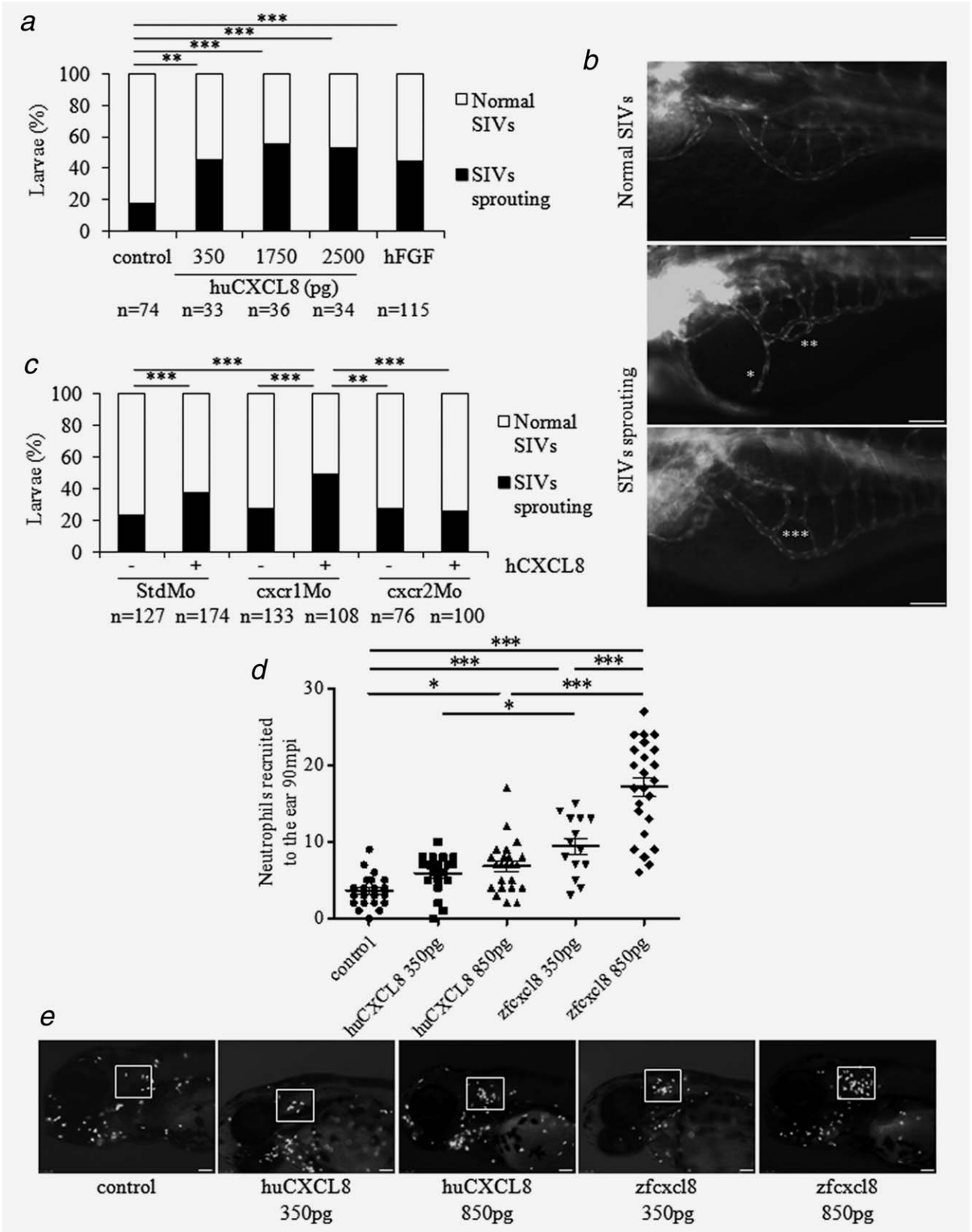
a
***
***
***
**
100
80
60
40
20
0
Larvae (%)
Normal SIVs
SIVs sprouting
control
350
1750
2500
hFGF
huCXCL8 (pg)
n=74 n=33 n=36 n=34 n=115
b
Normal SIVs
SIVs sprouting
**
*
***
c
***
***
***
***
**
Larvae (%)
Normal SIVs
SIVs sprouting
-
+
-
+
-
+
hCXCL8
StdMo
cxcr1Mo
cxcr2Mo
n=127 n=174 n=133 n=108 n=76 n=100
d
***
***
***
*
***
*
30
20
10
0
Neutrophils recruited to the ear 90mpi
control1
huCXCL8 350pg
huCXCL8 850pg
zfcxcl8 350pg
zfcxcl8 850pg
e
control
huCXCL8 350pg
huCXCL8 850pg
zfcxcl8 350pg
zfcxcl8 850pg

Figure 2.

of human CXCL8 protein to induce angiogenesis in zebrafish larvae. We found that increasing amounts of human recombinant CXCL8 increased the percentage of larvae positive for SIV sprouting compared to the larvae injected with control buffer (Figs. 2*a* and 2*b*), although a dose-dependent effect of the recombinant CXCL8 protein was not observed. Given that Cxcl8 signaling is conserved in zebrafish and is mediated through cell-surface Cxcr1 and Cxcr2 receptors,[29] we downregulated the expression of these receptors through morpholinos to identify the mediator of human CXCL8-induced angiogenesis in zebrafish. As shown in Figure 2*c*, while the human recombinant CXCL8 still exerted its proangiogenic activity in Cxcr1 morphants, the dowregulation of Cxcr2 expression impaired the capability of the protein to induce SIV sprouting.

Next, it was evaluated whether human CXCL8 was able to induce a proinflammatory response in zebrafish larvae, as this activity is conserved between humans and zebrafish.[21,23,29] To this end, an *in vivo* neutrophil recruitment assay was performed by injecting this chemokine into the otic vesicle of *Tg(mpx:gfp)*^{i114} zebrafish larvae, which express GFP under the neutrophil-specific promoter of mieloperoxidase (mpx). As shown in Figures 2*d* and 2*e*, only the higher dose of human recombinant CXCL8 was able to induce a slight but significant proinflammatory response 90 min post-injection. On the contrary, and as expected,[23] the zebrafish recombinant homolog cxcl8-L2 stimulated a significant and dose-dependent proinflammatory reaction, even at the lower dose.[4] Then we tested zebrafish cxcl8-L2 proangiogenic activity in zebrafish by injecting this recombinant protein into *Tg(fli1a:GFP)*^{y1} zebrafish embryos. It was observed that zebrafish Cxcl8-L2 was able to induce SIV sprouting at 24 hpi (Supporting Information, Fig. 1), but to a lower extent than human CXCL8 (Figs. 2*a* and 2*b*).

CXCL8 mediates bcl-xL-induced enhancement of human melanoma cell invasion and angiogenesis in zebrafish larvae

To analyze the possible role of CXCL8 as the mediator of angiogenesis and cell invasion enhanced by bcl-xL overexpression, we inhibited the secretion of this chemokine in MXL90 melanoma cells by means of a retrovirus-mediated shRNA approach. Two different shRNA sequences were used, shIL8-B and shIL8-D, and a reduced secretion of CXCL8 of about 50% and 30%, respectively, was observed compared to cells expressing a control shRNA sequence (shCTRL) (Fig. 3*a*). Importantly, such a reduction in CXCL8 secretion impaired melanoma cells overexpressing bcl-xL angiogenic (Fig. 3*b*) and invasive potential (Figs. 3*c* and 3*d*) in zebrafish larvae.

The autocrine CXCL8/CXCR2 axis mediates bcl-xL-induced enhancement of human melanoma cell invasion in zebrafish larvae

Given that the biological effects of CXCL8 in terms of melanoma angiogenesis and cell invasion are mainly mediated through cell-surface CXCR2 receptor,[7] the contribution of the autocrine loop involving the binding of secreted CXCL8 chemokine by melanoma cells to their own receptor was investigated. The expression of CXCR2 receptor was reduced by infecting MXL90 melanoma clone overexpressing bcl-xL and control clone Mneo with retrovirus carrying a shRNA targeting CXCR2, as confirmed by qPCR analysis (Fig. 4*a*). The reduced expression in CXCR2, about 25%, impaired the capability of bcl-xL overexpression to enhance melanoma cell invasion compared to the Mneo cells infected with a control shRNA (Figs. 4*b* and 4*c*) in our xenotransplantation model. Notably, Figures 4*b* and 4*c* also show that the 60% reduction of CXCR2 expression did not modify the invasion score of Mneo control cells, which release CXCL8 at low levels.[4] Besides, any involvement of CXCL8 paracrine signaling in bcl-xL-induced enhancement of melanoma cells invasion could be excluded as, when implanted in Cxcr1- and Cxcr2-low-expressing zebrafish larvae, neither melanoma cells overexpressing bcl-xL nor the control line significantly modified their dissemination ability (Supporting Information, Fig. 2).

bcl-xL expression correlates with CXCL8 expression in both primary and metastatic melanoma biopsies

To confirm the existence of a bcl-xL/CXCL8 axis in human melanoma, the expression of these two genes and their possible correlation was evaluated in microarray data profiles and RNA-seq database from human melanoma biopsies. Data from the GDS1375 microarray dataset indicated that bcl-xL was expressed in human normal skin and its basal expression was not altered in benign nevi (Supporting Information, Fig. 3*a*). Moreover, the progression to malignant primary

Figure 2. Human CXCL8 induces angiogenesis in zebrafish larvae through Cxcr2 receptor. (*a*) Evaluation of angiogenic activity of increasing amounts of human recombinant CXCL8 (huCXCL8) 24 hr after injection into the yolk sac of 2 dpf *Tg(fli1a:GFP)*^{y1} zebrafish embryos, expressing GFP in endothelial cells. Larvae were analyzed for subintestinal veins (SIVs) sprouting by fluorescence microscopy. Four nanograms of embryo recombinant human fibroblast growth factor (hFGF) was used as positive control. BSA was injected as negative control. (*b*) Representative images of normal SIVs and SIVs sprouting in *Tg(fli1a:GFP)*^{y1} zebrafish embryos 24 hpi of human CXCL8. Single sprouting (*), fused sprouts (**), and intervessel sprouting (***) are indicated. The magnification bar is 100 μm. (*c*) Evaluation of angiogenic activity of 1750 pg huCXCL8 after injection in the yolk sac of 2 dpf *Tg(fli1a:GFP)*^{y1} zebrafish embryos injected with morpholinos against Cxcr1 (cxcr1Mo) or Cxcr2 (cxcr2Mo) receptor at one-cell stage. Control morphants were injected with Standard Morpholino (StdMo). (*a*,*c*) n = number of larvae. (*d*) Quantification of neutrophils recruited after injection of increasing amounts of huCXCL8 or zfcxcl8 in the ear of 72 hpf *Tg(mpx:GFP)*^{i114} zebrafish embryos. Larvae were analyzed 90 min after injection by fluorescence microscopy. PBS was injected as control. The results presented are a pool of three different experiments. (*e*) Representative images of green neutrophils recruited to the ear are shown. The magnification bar is 100 μm. (*a*,*c*,*d*) The results presented are a pool of three different experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (*a*,*c*) χ^2 test and (*d*) one-way ANOVA analysis of variance test followed by Newman–Keuls post-test.

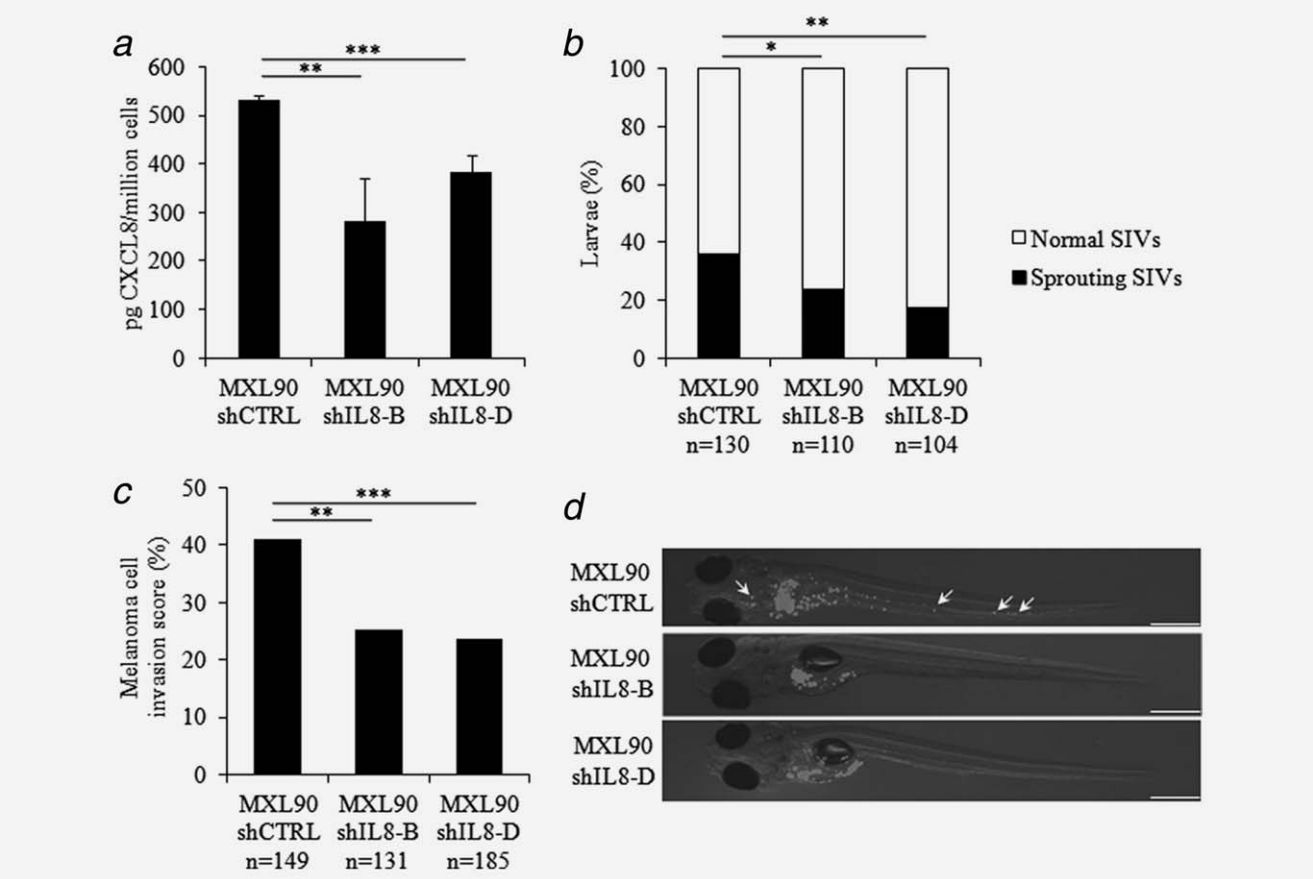

Figure 3. CXCL8 protein is the mediator of bcl-xL-induced enhancement of human M14 melanoma cell invasion and angiogenesis in zebrafish larvae. (*a*) Expression of CXCL8 protein in the conditioned media of bcl-xL overexpressing clone MXL90 stably expressing two different short hairpin RNA sequences to downregulate the expression of CXCL8 (shIL8-B and shIL8-D) or a scrambled short hairpin RNA sequence (shCTRL). CXCL8 protein content was analyzed by ELISA and normalized to the adherent cell number 24 hr after medium change. The results presented are mean ± SD of two different experiments evaluated in duplicate. (*b*) Evaluation of angiogenic activity of MXL90 shCTRL, shIL8-B and shIL8-D cells 24 hr after injection into the yolk sac of *Tg(fli1a:GFP)*^{y1} zebrafish embryos, expressing GFP in endothelial cells. Larvae were analyzed 24 hpi for SIV sprouting by fluorescence microscopy. (*c*) Evaluation of invasion score and (*d*) representative images of MXL90 shCTRL, shIL8-B and shIL8-D cells 5 days after injection in the yolk sac of 2 dpf *wild-type* zebrafish larvae. (*b*,*c*) The results presented are a pool of two different experiments. (*a*–*c*) $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (*a*) one-way ANOVA and (*b*,*c*) χ^2 test. (*b*,*c*) n = number of larvae. (*d*) The magnification bar is 500 μm.

melanoma was characterized by a significant increase in bcl-xL expression. As observed for bcl-xL, CXCL8 transcript levels were not modified in the biopsies of benign nevi when compared to normal skin tissues, whereas there was tendency for CXCL8 expression to increase in malignant primary melanoma samples, but not to a statistically significant extent. By contrast, the comparison between the expression level of bcl-xL and CXCL8 in primary and metastatic melanoma from the GDS3966 microarray dataset showed that there was no significant difference in the case of bcl-xL; however, there was a significant increase of CXCL8 expression in metastatic melanoma compared to primary melanoma (Supporting Information, Fig. 3*b*). When we calculated the correlation index of gene expression from the GDS3966 microarray dataset, bcl-xL expression was positively correlated with CXCL8 levels in both primary and metastatic melanoma (Fig. 5*a*).

To evaluate the impact of the expression of both bcl-xL and CXCL8 on melanoma patient prognosis, the ProgGeneV2 survival analysis tool was used to conduct analyses on the GSE19234 microarray, which besides data on gene expression, also includes information about melanoma patient survival. In Figure 5*b*, Kaplan–Meier overall survival curves for the metastatic melanoma patient cohort, divided into high and low combined bcl-xL and CXCL8 gene expression, showed that high bcl-xL/CXCL8 expression significantly correlates with a poor prognosis of patients. Indeed, analyzing the gene expression of bcl-xL and CXCL8 in tumor samples from the same GSE19234 microarray set, a significant positive correlation was again found between bcl-xL and CXCL8 expression (Fig. 5*c*), confirming the data obtained from the analysis of GDS3966 microarray set (Fig. 5*a*).

Our investigation was extended to a larger cohort of skin melanoma samples analyzed by RNA-seq, derived from the TGCA repository. While co-expression of bcl-xL and CXCL8 is not statistically significant in primary melanoma, we found that bcl-xL expression was positively correlated with the level of

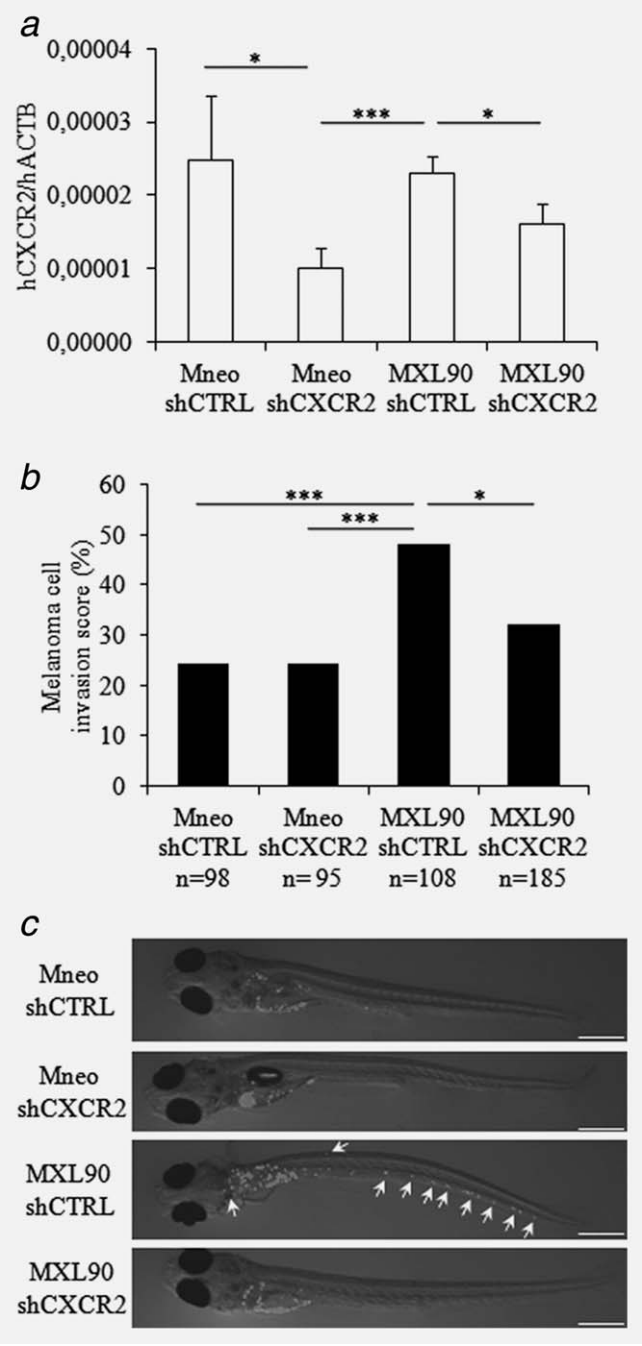

Figure 4. Cell surface CXCR2 mediates bcl-xL-induced enhancement of human M14 melanoma cell invasion in zebrafish larvae. (*a*) Quantification of CXCR2 expression by RT-qPCR in control clone Mneo and bcl-xL overexpressing clone MXL90 stably expressing a short hairpin RNA sequence to downregulate the expression of CXCL8 receptor CXCR2 or the scrambled sequence shCTRL. The results presented are mean ± SD of two different experiments evaluated in triplicate. (*b*) Evaluation of invasion score and (*c*) representative images of Mneo and MXL90 cells stably expressing a short hairpin RNA sequence to downregulate the expression of CXCL8 receptor CXCR2 or the scrambled sequence shCTRL 5 days after injection in the yolk sac of 2 dpf *wild-type* zebrafish larvae. The results presented are a pool of two different experiments. (*a*,*b*) $*p < 0.05$, $***p < 0.001$ (*a*) one-way ANOVA test and (*b*) χ^2 test. (*b*) n = number of larvae. (*c*) The magnification bar is 500 μm.

CXCL8 in metastatic melanoma samples (Fig. 5*d*) and its receptor CXCR2 (Supporting Information, Fig. 4). Moreover high bcl-xL/CXCL8 co-expression analyzed by RNA-seq significantly correlates with a poor prognosis of melanoma patients (Fig. 5*e*), confirming the results obtained from survival data associated to the GDS3966 microarray set (Fig. 5*b*).

Next, we analyzed the microarray data profiles from human primary and metastatic melanoma biopsies from GDS3966 microarray and TCGA RNA-seq databases to extend the correlation studies to molecules involved in melanoma development. Among all the genes analyzed, the expression of well-characterized melanoma marker SRY-related HMG box-containing factor 10 (SOX10)[34] was positively correlated with bcl-xL expression, in both primary and melanoma samples analyzed by microarray (Fig. 6*a*) and RNA-seq (Fig. 6*b*). Also in the case of another player in melanocyte development, microphthalmia-associated transcription factor (MITF), which is actively involved in the dynamics of melanoma development,[35] there was a positive correlation with bcl-xL expression in both primary and metastatic melanoma in the microarray dataset (Fig. 6*a*), that was confirmed in both primary and metastatic melanoma cases analyzed by RNA-seq (Fig. 6*b*).

To confirm whether the enhanced expression of SOX10 and MITF in melanoma depends on bcl-xL protein levels, and to demonstrate that CXCL8 is the mediator of this modulation, RT-qPCR analysis was performed to analyze the levels of these genes in Mneo control cells and in MXL90 cells and after downregulating CXCL8 by shRNA. As shown in Figure 6*c*, the enhanced expression of bcl-xL protein significantly increases the expression of all these genes in the MXL90 cell line. Strikingly, the reduction of CXCL8 expression rescued the upregulation of SOX10 and MITF expression induced by bcl-xL.

Discussion

Overexpression of bcl-xL protein is able to enhance melanoma cell angiogenesis through increasing chemokine CXCL8 secretion.[4] In this study, using zebrafish xenotransplantation models, we demonstrate that this feature is associated with the increased ability of bcl-xL overexpressing cells to enhance invasion *in vivo*. This experimental model emerged as a powerful tool in the cancer research field to monitor, by means of *in vivo* live imaging, cancer-associated features such as cell invasion,[15,36] tumor angiogenesis[19] and the interaction with immune cells in tumor microenvironment.[37] Using the rapid ZFYM angiogenesis assay and transgenic larvae expressing GFP in endothelial cells,[17] the capability of melanoma cells overexpressing bcl-xL protein to induce the formation of new blood vessels was confirmed, by quantifying SIV sprouting positive zebrafish larvae, as observed previously in a mouse-based matrigel assay.[4] Here we study the potential of human CXCL8 as a mediator of bcl-xL-induced angiogenesis, performing the ZFYM angiogenesis assay using increasing amounts of recombinant human CXCL8. It was demonstrated for the first time that human CXCL8 exerts angiogenic activity in zebrafish larvae, as previously shown for other human proangiogenic

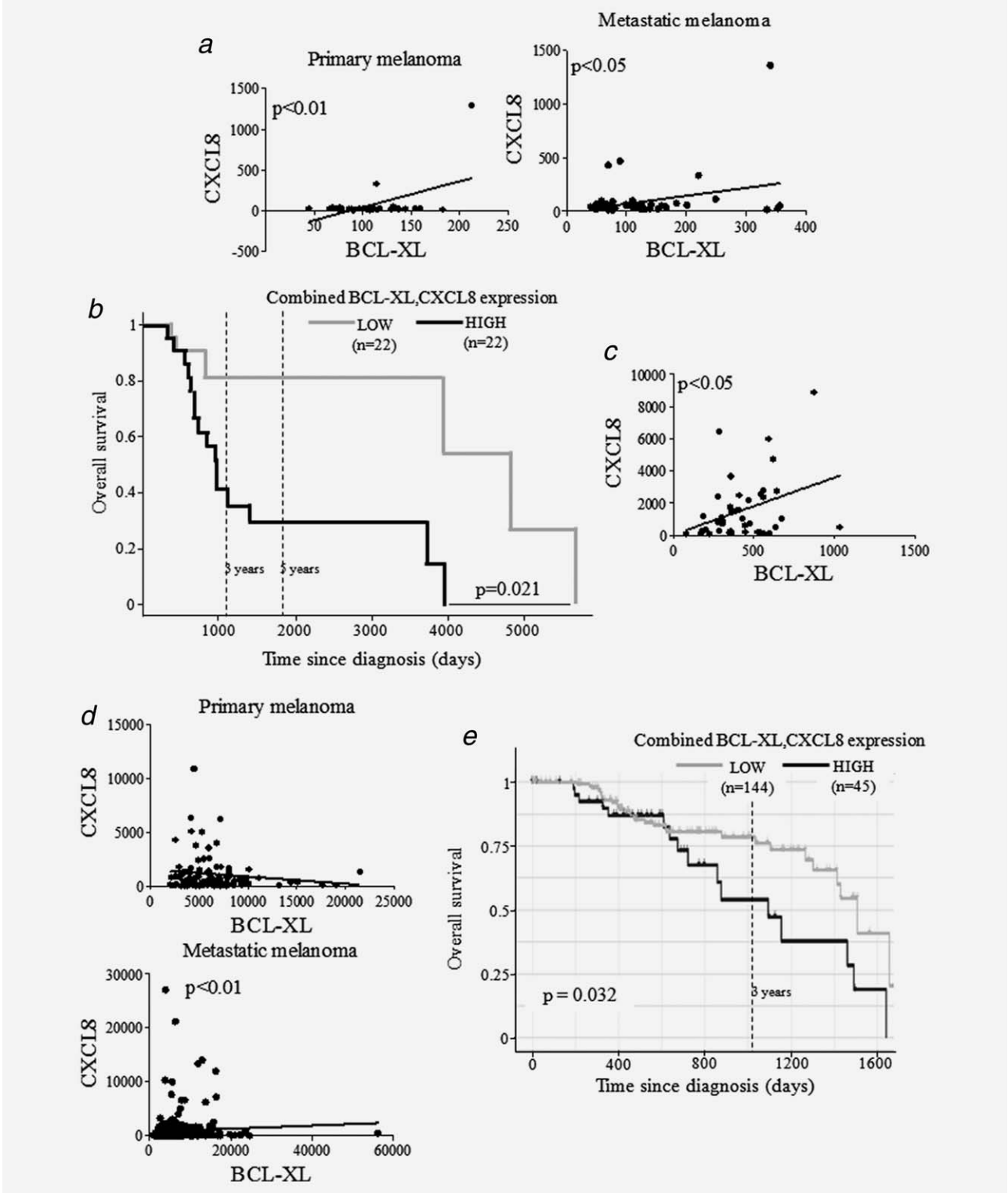

Figure 5. bcl-xL expression correlates with CXCL8 expression in both primary and metastatic melanoma biopsies and BCL-XL/CXCL8 co-expression is associated with poor prognosis in human melanoma patients. (*a*) Correlation of BCL-XL and CXCL8 gene expression in human primary ($n = 31$) and metastatic ($n = 52$) melanoma biopsies from microarray expression data set GDS3966. (*b*) Kaplan–Meier overall survival curves for the GSE19234 microarray metastatic melanoma patient cohort divided into high and low combined BCL-XL and CXCL8 gene expression. (*c*) Correlation of BCL-XL and CXCL8 gene expression in metastatic melanoma biopsies ($n = 44$) from microarray data set GSE19234. (*d*) Correlation of BCL-XL and CXCL8 gene expression in human primary ($n = 103$) and metastatic ($n = 368$) melanoma biopsies from TCGA RNA-seq database. (*e*) Kaplan–Meier overall survival curves for TCGA RNA-seq database melanoma patient cohort ($n = 189$) divided into high and low combined BCL-XL and CXCL8 gene expression. (*a*, *c*, *d*) The statistical significance of the correlation was determined using (*a*, *c*) Pearson's correlation coefficient and (*d*) Spearman's correlation coefficient. A linear regression-fitting curve is shown.

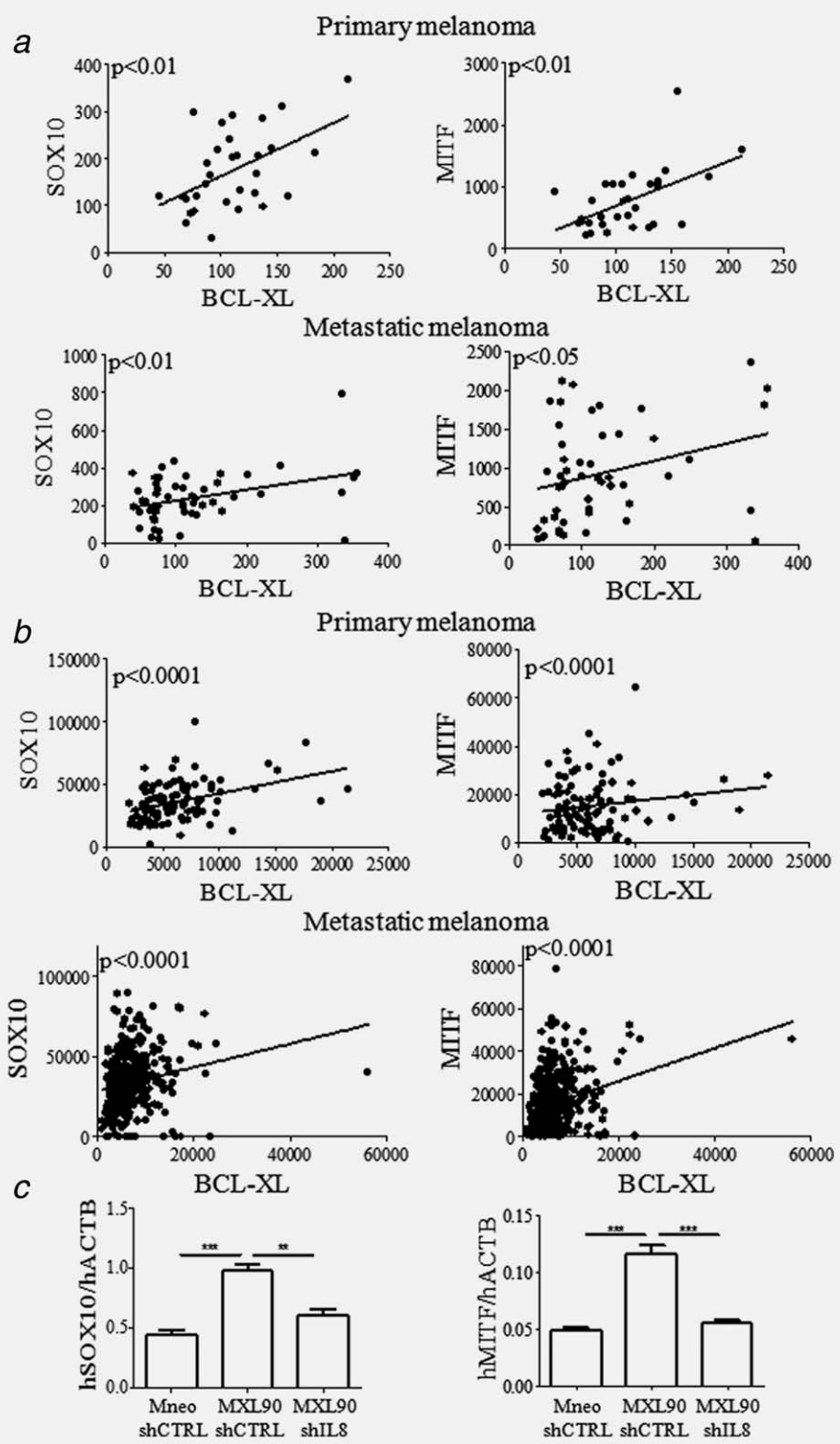

Figure 6. bcl-xL expression correlates with expression of aggressiveness markers expression in primary and metastatic melanoma biopsies. (*a*) Correlation of BCL-XL gene expression with SOX10 and MITF in human primary ($n = 31$) and metastatic ($n = 52$) melanoma biopsies from microarray expression data set GDS3966. (*b*) Correlation of BCL-XL gene expression with SOX10 and MITF in human primary ($n = 103$) and metastatic ($n = 368$) melanoma biopsies from TCGA RNA-seq database. (*a*, *b*) The statistical significance of the correlation was determined using (*a*) Pearson's correlation coefficient and (*b*) Spearman's correlation coefficient. A linear regression-fitting curve is shown. (*c*) Quantification of SOX10 and MITF expression by RT-qPCR in control clone Mneo and bcl-xL overexpressing clone MXL90 stably expressing a short hairpin RNA sequence to downregulate CXCL8 expression (shIL8) or the scrambled sequence shCTRL. The results presented are mean ± SD of two different experiments evaluated in triplicate. $^{**}p < 0.01$, $^{***}p < 0.001$ one-way ANOVA test.

factors, such as VEGF[38] and FGF-2. However FGF-2 does not elicit a dose-dependent proangiogenic effect in zebrafish at high doses,[20] as we observed for human CXCL8. Moreover, we demonstrated that this proangiogenic activity of CXCL8 is mediated by Cxcr2, the zebrafish homologue of the human protein CXCR2, which displays a key receptor role in mediating CXCL8-induced angiogenesis.[39] The definition of this homology of function opens up the possibility of using zebrafish larvae to set up high-throughput screenings to test the efficacy of novel inhibitors of the CXCL8 proangiogenic pathway with potential antitumoral activity, as previously demonstrated for compounds targeting VEGF signaling.[40–43] We were also able to show that recombinant zebrafish Cxcl8-L2 induces SIV sprouting, although to a lesser extent than human CXCL8. Moreover, as established for humans, the role of Cxcl8 as a proinflammatory protein has been extensively demonstrated in zebrafish.[23,29] In our study, we also addressed human recombinant CXCL8 role in neutrophil recruitment, demonstrating it has a weak proinflammatory activity in zebrafish larvae compared to zebrafish Cxcl8-L2.

Next, by dowregulating CXCL8 expression by shRNA, it was demonstrated that this chemokine is the mediator of proangiogenic activity in bcl-xL overexpressing cells in xenotransplanted zebrafish embryos. In addition, the decrease in CXCL8 expression significantly counteracted the invasive profile of melanoma cells induced by bcl-xL overexpression. Since the downregulation of Cxcl8 receptor expression did not counteract the capability of bcl-xL overexpressing cells to invade zebrafish larvae, we focused our attention on the possible role of the autocrine CXCL8 pathway in the ability of bcl-xL protein to enhance melanoma cell invasion in zebrafish embryos through CXCL8, demonstrating that the cell-surface receptor CXCR2 is the mediator of the increased cell invasion induced by the bcl-xL/CXCL8 axis. Importantly, bcl-xL protein has recently been described as increasing invasiveness in a murine model of pancreatic neuroendocrine tumor and in breast cancer cell lines in mice, through enhancing epithelial-to-mesenchimal transition.[44] Interestingly, in this model, bcl-xL protein might exert an epigenetic activity in the nucleus, leading to increased expression of transforming growth factor β, which is the mediator of bcl-xL-induced cell invasion. In our model, bcl-xL protein overexpression increases CXCL8 gene transcription enhancing NF-κB signaling by increasing the degradation of cytoplasmic inhibitor of NF-κB (IκB), as we previously demonstrated,[5] although we do not exclude a possible role for nuclear bcl-xL in enhancing cell invasion through CXCL8-dependent or -independent mechanisms. The same authors demonstrated that bcl-xL enhances tumor aggressiveness through a mechanism independent of its antiapoptotic activity and in line with this we found that even though the specific inhibitor WEHI-539[33] counteracted the antiapoptotic effect of bcl-xL, it did not reduce CXCL8 secretion in bcl-xL overexpressing clone MXL90.

Finally, using public microarray and RNA-seq databases of human melanoma patient specimens, we found that there is a positive correlation between bcl-xL and CXCL8 expression, confirming the evidence obtained *in vitro* with human melanoma cell lines.[4] Indeed, in melanoma specimens, bcl-xL expression correlated with markers of aggressiveness: MITF, a survival factor in melanocytes and melanoma,[45] frequently amplified in this kind of tumor[46] and recently described as being regulated by bcl-xL homolog bcl-2,[47] and SOX10, which promotes primary melanoma and its metastatization[48] and recently proposed as a promising new serum marker for detection of early stage melanoma.[49] Interestingly, it was confirmed that bcl-xL overexpression is able to enhance MITF and SOX10 expression in human melanoma cells and that this increase is counteracted by CXCL8 secretion inhibition. This finding suggests that CXCL8 is required for bcl-xL-induced enhancement of MITF and SOX10 expression and suggests novel mechanisms of melanoma aggressiveness induced by the bcl-xL/CXCL8 axis, should be investigated in depth. Furthermore, using the publicly available Kaplan–Meier Plotter database to explore the clinical significance of our *in vitro* and *in vivo* findings, we found that a poor prognosis of melanoma patients is associated with higher combined expression of both bcl-xL and CXCL8 in tumor samples, completing the previous evidence and demonstrating the negative impact of single high bcl-xL[50] and CXCL8[9] expression on melanoma patient survival.

In summary, our data throw light on the involvement of CXCL8 signaling in bcl-xL-induced melanoma progression, supporting the development of new therapeutic approaches for tumors characterized by high bcl-xL expression. Moreover, the conservation of the proangiogenic activity of the CXCL8 molecule in zebrafish establishes the possible use of this interesting model to study the efficacy of novel compounds inhibiting the CXCL8 axis to counteract tumor progression.

Acknowledgements

The authors thank Inma Fuentes and Pedro J. Martínez for their excellent technical support, Dr Stephen Renshaw for providing the *Tg(mpx:eGFP)*^{i114}, and Dr Sergio Candel for helpful suggestions about GEO microarray database data analysis.

References

1. World Health Organization, World Cancer Report, 2014.
2. Martin SS, Ridgeway AG, Pinkas J, et al. A cytoskeleton-based functional genetic screen identifies Bcl-xL as an enhancer of metastasis, but not primary tumor growth. *Oncogene* 2004;23:4641–5.
3. Bush JA, Li G. The role of Bcl-2 family members in the progression of cutaneous melanoma. *Clin Exp Metastasis* 2003;20:531–9.
4. Giorgini S, Trisciuoglio D, Gabellini C, et al. Modulation of bcl-xL in tumor cells regulates angiogenesis through CXCL8 expression. *Mol Cancer Res* 2007;5:761–71.
5. Gabellini C, Castellini L, Trisciuoglio D, et al. Involvement of nuclear factor-kappa B in bcl-xL-induced interleukin 8 expression in glioblastoma. *J Neurochem* 2008;107:871–82.
6. Singh S, Singh AP, Sharma B, et al. CXCL8 and its cognate receptors in melanoma progression and metastasis. *Future Oncol* 2010;6:111–6.

7. Gabellini C, Trisciuoglio D, Desideri M, et al. Functional activity of CXCL8 receptors, CXCR1 and CXCR2, on human malignant melanoma progression. *Eur J Cancer* 2009;45:2618–27.
8. Varney ML, Johansson SL, Singh RK. Distinct expression of CXCL8 and its receptors CXCR1 and CXCR2 and their association with vessel density and aggressiveness in malignant melanoma. *Am J Clin Pathol* 2006;125:209–16.
9. Nürnberg W, Tobias D, Otto F, et al. Expression of interleukin-8 detected by in situ hybridization correlates with worse prognosis in primary cutaneous melanoma. *J Pathol* 1999;189:546–51.
10. Ugurel S, Rappl G, Tilgen W, et al. Increased serum concentration of angiogenic factors in malignant melanoma patients correlates with tumor progression and survival. *J Clin Oncol* 2001;19:577–83.
11. Sanmamed MF, Carranza-Rua O, Alfaro C, et al. Serum interleukin-8 reflects tumor burden and treatment response across malignancies of multiple tissue origins. *Clin Cancer Res* 2014;20:5697–707.
12. Barriuso J, Nagaraju R, Hurlstone A. Zebrafish: a new companion for translational research in oncology. *Clin Cancer Res* 2015;21:969–75.
13. Hsu CH, Wen ZH, Lin CS, et al. The zebrafish model: use in studying cellular mechanisms for a spectrum of clinical disease entities. *Curr Neurovasc Res* 2007;4:111–20.
14. Meierjohann S, Schartl M. From Mendelian to molecular genetics: the Xiphophorus melanoma model. *Trends Genet* 2006;22:654–61.
15. Taylor AM, Zon LI. Zebrafish tumor assays: the state of transplantation. *Zebrafish* 2009;6:339–46.
16. Weinstein BM. Plumbing the mysteries of vascular development using the zebrafish. *Semin Cell Dev Biol* 2002;13:515–22.
17. Nicoli S, Presta M. The zebrafish/tumor xenograft angiogenesis assay. *Nat Protoc* 2007;2:2918–23.
18. Jensen LD, Cao R, Cao Y. In vivo angiogenesis and lymphangiogenesis models. *Curr Mol Med* 2009;9:982–91.
19. Nicoli S, Ribatti D, Cotelli F, et al. Mammalian tumor xenografts induce neovascularization in zebrafish embryos. *Cancer Res* 2007;67:2927–31.
20. Nicoli S, De Sena G, Presta M. Fibroblast growth factor 2-induced angiogenesis in zebrafish: the zebrafish yolk membrane (ZFYM) angiogenesis assay. *J Cell Mol Med* 2009;13:2061–8.
21. Oehlers SH, Flores MV, Hall CJ, et al. Expression of zebrafish cxcl8 (interleukin-8) and its receptors during development and in response to immune stimulation. *Dev Comp Immunol* 2010;34:352–9.
22. van der Aa LM, Chadzinska M, Tijhaar E, et al. CXCL8 chemokines in teleost fish: two lineages with distinct expression profiles during early phases of inflammation. *PLoS One* 2010;5:e12384.
23. de Oliveira S, Reyes-Aldasoro CC, Candel S, et al. Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response. *J Immunol* 2013;190:4349–59.
24. Stoll SJ, Bartsch S, Augustin HG, et al. The transcription factor HOXC9 regulates endothelial cell quiescence and vascular morphogenesis in zebrafish via inhibition of interleukin 8. *Circ Res* 2011;108:1367–77.
25. Lawson ND, Weinstein BM. In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev Biol* 2002;248:307–18.
26. Renshaw SA, Loynes CA, Trushell DM, et al. A transgenic zebrafish model of neutrophilic inflammation. *Blood* 2006;108:3976–8.
27. White RM, Sessa A, Burke C, et al. Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell* 2008;2:183–9.
28. Marques IJ, Weiss FU, Vlecken DH, et al. Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model. *BMC Cancer* 2009;9:128.
29. Deng Q, Sarris M, Bennin DA, et al. Localized bacterial infection induces systemic activation of neutrophils through Cxcr2 signaling in zebrafish. *J Leukoc Biol* 2013;93:761–9.
30. Colaprico A, Silva TC, Olsen C, et al. TCGAbiolinks: an R/Bioconductor package for integrative analysis of TCGA data. *Nucleic Acids Res* 2016;44:e71.
31. Baldessari D, Mione M. How to create the vascular tree? (Latest) help from the zebrafish. *Pharmacol Ther* 2008;118:206–30.
32. Trisciuoglio D, Gabellini C, Desideri M, et al. Involvement of BH4 domain of bcl-2 in the regulation of HIF-1-mediated VEGF expression in hypoxic tumor cells. *Cell Death Differ* 2011;18:1024–35.
33. Lessene G, Czabotar PE, Sleebs BE, et al. Structure-guided design of a selective BCL-X(L) inhibitor. *Nat Chem Biol* 2013;9:390–7.
34. Shakhova O, Zingg D, Schaefer SM, et al. Sox10 promotes the formation and maintenance of giant congenital naevi and melanoma. *Nat Cell Biol* 2012;14:882–90.
35. Koludrovic D, Davidson I. MITF, the Janus transcription factor of melanoma. *Future Oncol* 2013;9:235–44.
36. White R, Rose K, Zon L. Zebrafish cancer: the state of the art and the path forward. *Nat Rev Cancer* 2013;13:624–36.
37. Feng Y, Martin P. Imaging innate immune responses at tumour initiation: new insights from fish and flies. *Nat Rev Cancer* 2015;15:556–62.
38. Yang X, Zhang Y, Hosaka K, et al. VEGF-B promotes cancer metastasis through a VEGF-A-independent mechanism and serves as a marker of poor prognosis for cancer patients. *Proc Natl Acad Sci USA* 2015;112:E2900–9.
39. Addison CL, Daniel TO, Burdick MD, et al. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. *J Immunol* 2000;165:5269–77.
40. Zhou Z, Zhao C, Wang L, et al. A VEGFR1 antagonistic peptide inhibits tumor growth and metastasis through VEGFR1-PI3K-AKT signaling pathway inhibition. *Am J Cancer Res* 2015;5:3149–61.
41. Paauwe M, Heijkants RC, Oudt CH, et al. Endoglin targeting inhibits tumor angiogenesis and metastatic spread in breast cancer. *Oncogene* 2016;35:4069–79.
42. Baek YY, Lee DK, So JH, et al. The tetrapeptide Arg-Leu-Tyr-Glu inhibits VEGF-induced angiogenesis. *Biochem Biophys Res Commun* 2015;463:532–7.
43. Zhang S, Cao Z, Tian H, et al. SKLB1002, a novel potent inhibitor of VEGF receptor 2 signaling, inhibits angiogenesis and tumor growth in vivo. *Clin Cancer Res* 2011;17:4439–50.
44. Choi S, Chen Z, Tang LH, et al. Bcl-xL promotes metastasis independent of its anti-apoptotic activity. *Nat Commun* 2016;7:10384.
45. Levy C, Khaled M, Fisher DE. MITF: master regulator of melanocyte development and melanoma oncogene. *Trends Mol Med* 2006;12:406–14.
46. Garraway LA, Widlund HR, Rubin MA, et al. Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. *Nature* 2005;436:117–22.
47. De Luca T, Pelosi A, Trisciuoglio D, et al. miR-211 and MITF modulation by Bcl-2 protein in melanoma cells. *Mol Carcinog* 2016;55:2304–12.
48. Cronin JC, Wunderlich J, Loftus SK, et al. Frequent mutations in the MITF pathway in melanoma. *Pigment Cell Melanoma Res* 2009;22:435–44.
49. Blokzijl A, Chen LE, Gustafsdottir SM, et al. Elevated levels of SOX10 in serum from vitiligo and melanoma patients, analyzed by proximity ligation assay. *PLoS One* 2016;11:e0154214.
50. Zhuang L, Lee CS, Scolyer RA, et al. Mcl-1, Bcl-XL and Stat3 expression are associated with progression of melanoma whereas Bcl-2, AP-2 and MITF levels decrease during progression of melanoma. *Mod Pathol* 2007;20:416–26.