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Interleukin-8 is a key mediator of FKBP51-induced melanoma growth, angiogenesis and metastasis

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Background: FKBP51 is overexpressed in melanoma and impacts tumour cell properties. However, its comprehensive role in melanoma pathogenesis and underlying mechanism(s) remain elusive.

Methods: FKBP51 was stably silenced in aggressive melanoma cell lines and its effect examined *in vitro* and in mouse model. Histological/immunohistochemical analyses were performed to confirm metastasis, angiogenesis and neutrophil infiltration. Gene expression was analyzed by qRT–PCR, immunoblot and/or ELISA. NF-κB transcriptional activity and promoter binding were monitored by luciferase-based promoter-reporter and ChIP assays, respectively. Interleukin (IL)-8 inhibition was achieved by gene silencing or neutralising-antibody treatment.

Results: FKBP51 silencing reduced melanoma growth, metastasis, angiogenesis and neutrophil infiltration and led to IL-8 downregulation through NF-κB suppression in cell lines and tumour xenografts. IL-8 inhibition drastically decreased growth, migration and invasiveness of FKPB51-overexpressing cells; whereas its treatment partially restored the suppressed phenotypes of FKBP51-silenced melanoma cells. Interleukin-8 depletion in conditioned medium (CM) of FKBP51-overexpressing melanoma cells inhibited endothelial cell proliferation and capillary-like structure formation, whereas its treatment promoted these effects in endothelial cells cultured in CM of FKBP51-silenced melanoma cells.

Conclusions: FKBP51 promotes melanoma growth, metastasis and angiogenesis, and IL-8 plays a key role in these processes. Thus, targeting of FKBP51 or its upstream or downstream regulatory pathways could lead to effective therapeutic strategies against melanoma.

Melanoma is an aggressive form of skin cancer in human. It is the sixth most commonly diagnosed cancer, and one of the leading causes of skin cancer-related deaths in human in the United States (Sun *et al*, 2015). Incidence and mortality rates of melanoma have increased tremendously during the past several decades. According to an estimate, nearly 73 870 new cases of melanoma will be diagnosed and approximately 9940 patients will die from this malignancy in 2015 (Siegel *et al*, 2015). Moreover, the 5-year survival rate of melanoma patients with distant metastasis is 15% with the median survival of 6–9 months (Rodic *et al*, 2014). Together, these facts clearly highlight the need to develop effective preventive and/or therapeutic strategies against this deadly malignancy. To be able to make progress in these areas, we need to first identify molecular players involved in melanoma initiation and progression as well as characterise the mechanisms through which they confer their impact on melanoma phenotype. So far, several molecular aberrations have been recorded in malignant melanoma and have been characterised for their involvement in pathogenesis.

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FK506-binding protein 51 (FKBP51, also referred as FKBP5) belongs to a highly conserved family of immunophilins having cis-trans peptidyl-prolyl isomerase activity (Galat, 1993; Jiang *et al*, 2008). In recent years, FKBP51 has emerged as a chief regulator of immunity, hormonal physiology, drug sensitivity and biological processes involved in cell survival, protein synthesis, folding as well as in trafficking (Storer *et al*, 2011). Structurally, FKBP51 contains multiple functional domains (Storer *et al*, 2011). The N-terminal FK1 domain possesses the PPIase function, which is inhibited upon direct binding of FK506 (Bracher *et al*, 2013). The FK2 is PPIase-like domain and does not have much PPIase activity and is not inhibited by FK506 (Storer *et al*, 2011). The C-terminus of FKBP51 contains a three-unit domain of tetratricopeptide repeats that mediates its interactions with other proteins (Sinars *et al*, 2003). Recent studies have shown that FKBP51 is overexpressed in human melanoma tissues and associated with cell survival and chemoresistance (Romano *et al*, 2010, 2011). FKBP51 also plays an important role in maintenance of melanoma stemness and metastatic potential (Romano *et al*, 2013). Despite these advances, the mechanism(s) through which FKBP51 promotes melanoma progression and metastasis has remained largely unknown.

In this study, we investigated the role of FKBP51 in melanoma growth, metastasis and angiogenesis and identified interleukin 8 (IL-8), a chemokine, as a key mediator of its pathological functions. Interleukin-8, also referred as CXCL8, which is not normally produced by melanocytes, exhibits significant overexpression in melanoma along with its cognate receptors, CXCR1 and CXCR2 (Singh *et al*, 2010). Furthermore, several lines of evidence indicate its multifaceted action in the progression of human malignant melanoma (Wang *et al*, 1990; Varney *et al*, 2006; Singh *et al*, 2009a; Wu *et al*, 2012). Activation of IL-8 downstream signalling leads to altered expression of several genes involved in cell proliferation, apoptosis resistance and cytoskeletal dynamics (Waugh and Wilson, 2008; Wu *et al*, 2012). A positive correlation of IL-8 and its cognate receptors expression with melanoma aggressiveness has been demonstrated by us and others (Varney *et al*, 2006; Gabellini *et al*, 2009; Singh *et al*, 2011; Wu *et al*, 2012) and its inhibition leads to suppressive effects *in vitro* and in the preclinical studies of melanoma (Huang *et al*, 2002; Gabellini *et al*, 2009; Singh *et al*, 2011). Therefore, identification of IL-8 as a novel downstream target of FKBP51-mediated signalling is significant considering its reported aberrant expression and pathological involvement in melanoma pathogenesis.

MATERIALS AND METHODS

Reagents, plasmid constructs and antibodies. Details of all the reagents, plasmid constructs, siRNAs and antibodies used in this study are provided in Supplementary Materials.

Cell lines and culture conditions. Human melanoma cell lines, A375P and A375SM were obtained from Dr Isaiah J Fidler (University of Texas MD Anderson Cancer Center, Houston, TX, USA); FEMX-V; FEMX-1 and-FEMX-DR were generously gifted by Dr Øystein Fodstad (Oslo University Hospital, Oslo, Norway), and WM-115, WM266.4, SK-MEL-28, MEWO and human umbilical vein endothelial cells (HUVEC) were from ATCC (Manassas, VA, USA). Cell lines were maintained in DMEM medium supplemented with FBS (10%), penicillin (100 units per ml) and streptomycin ($100\,\mu g\,ml^{-1}$) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were routinely monitored for their typical morphology, and intermittently tested for mycoplasma.

Transfection. A375SM and-FEMX-1 cells were transfected with plasmids expressing shRNA against FKBP51 (pGFP-V-RS-shFKBP51) and non-targeting-scramble-sequence expressing control vector (pGFP-V-RS-NT) using FuGENE reagent. Stable pooled population were selected in puromycin ($2\,\mu g\,ml^{-1}$) containing culture medium. To dissect the role of NF-κB, cells were transiently transfected with constitutively active mutant plasmids of IκB-α and-IKKβ-SSEE along with respective control vectors using FuGENE reagent. For transient silencing, cells were transfected with 50 nM of human IL-8 specific or non-target-scrambled siRNAs.

RNA isolation and reverse transcription polymerase chain reaction (RT–PCR). Total RNA was extracted using TRIzol reagent and RT–PCR analysis was performed as previously described (Tyagi *et al*, 2014). Specific sets of primer pairs were used (Supplementary Table S1).

Western blot analysis. Cells were processed for protein extraction and western blotting as described earlier (Tyagi *et al*, 2014). Specific primary antibodies at different dilutions (1 : 50 to 1 : 1000) followed with HRP-labelled secondary antibodies (1:2000) were used. β-actin (1 : 20 000) probing was used as a loading control.

Cell motility and invasion assay. For motility (1×10^6 cells per well) and invasion (1×10^5 cells per well) assays, cells were plated on uncoated membrane (for motility assay) and on Matrigel-coated membrane (for invasion assay) chambers in serum-deprived culture medium, medium supplemented with FBS (10%) or rIL-8 ($10\,ng\,ml^{-1}$). Following 16 h incubation, migrated or invaded cells were fixed, stained and observed under microscope and counted in ten random fields of view ($\times$ 200).

***In vitro* cell growth assay.** Cells (1×10^4 cells per well) were seeded and growth was monitored after-96 h using-WST-1 assay kit (Roche Diagnostics, Mannheim, Germany) as described earlier (Bhardwaj *et al*, 2014). Separately, cells were treated with control-IgG ($200\,ng\,ml^{-1}$), IL-8-neutralising antibody ($200\,ng\,ml^{-1}$) or rIL-8 ($10\,ng\,ml^{-1}$) for 48 h. Following treatment, cells were replenished with fresh treatment media for another 48 h and growth was monitored. For HUVECs proliferation assay, cells (2×10^3 cells per well) were incubated with conditioned medium (CM) obtained from different melanoma cells (NT, FKBP51/IL-8-silenced and IL-8-neutralising antibody ($200\,ng\,ml^{-1}$) treated cells for 48 h). In addition, HUVECs cells were treated with or without-rIL-8 ($10\,ng\,ml^{-1}$) in serum-free media for 48 h, and growth was monitored.

Plating efficiency assay. Controls and FKBP51/IL-8-silenced cells (1×10^3 cells per well) were seeded in 6-well plates in complete media. Additionally, control cells were treated with control-IgG ($200\,ng\,ml^{-1}$) or with-IL-8-neutralising antibody, and FKBP51-silenced cells were treated with rIL-8 ($10\,ng\,ml^{-1}$). Every third day, media was replaced with fresh culture/treatment media. Following 2 weeks of culturing, colonies were fixed (methanol), stained (crystal violet), photographed and counted using Image analysis software (Gene Tools, Syngene, Frederick, MD, USA).

***In vitro* angiogenesis assay.** Human umbilical vein endothelial cells (1×10^4) were seeded on matrigel-coated plate in CM obtained from controls, shFKBP51/IL-8-silenced and IL-8-neutralising antibody ($200\,ng\,ml^{-1}$)/control IgG ($200\,ng\,ml^{-1}$)-treated cells. After 16 h of incubation, capillary-like structure (CLS) formation was observed and counted in ten random fields ($\times$ 100).

Enzyme-linked immunosorbent assay (ELISA). Cells (1×10^6 per well) were seeded in 6-well plates for 24 h and media replaced with serum-free media. At different intervals (24, 48 and 72 h) culture supernatants were collected and IL-8 levels were determined using human IL-8 ELISA kit.

NF-κB transcriptional activity assay. Transcriptional activity of NF-κB in controls and FKBP51-silenced cells were examined as previously described (Singh *et al*, 2012).

Nuclear and cytoplasmic fractionation. The cytoplasmic and nuclear extracts were fractioned using the nuclear extract kit as described previously (Singh *et al*, 2012).

Chromatin immunoprecipitation assay. Binding of NF-κB/p65 to the IL-8 promoter was analyzed by chromatin immunoprecipitation assay using a ChIP-IT enzymatic kit as previously described. Polymerase chain reaction was performed using specific primers sets (Supplementary Table S1), products resolved on a 2.0% agarose gel, and visualised using ethidium-bromide staining.

***In vivo* tumour growth and experimental lung metastasis.** Animal studies were performed after the approval of University of South Alabama Institutional Animal Care and Use Committee (IACUC). Athymic nude mice (Nude-Foxn1^{nu}, stock number 069; 4–6 weeks old) were purchased from Harlan Laboratories (Prattville, AL, USA) and maintained in pathogen-free conditions. A375SM-NT or A375SM-shFKBP51 cells (1×10^6 cells per 0.1 ml of HBSS; $n = 5$) were subcutaneously injected on the right rear flank of mice. Tumour growth was monitored twice a week and tumour volume was calculated using the formula: $\pi/6 \times$ (smaller diameter)$^2 \times$ (larger diameter). At the end point (45 days post implantation), mice were killed, tumours harvested, weighed, fixed and processed for immunohistochemical analysis. For experimental lung metastasis, A375SM-NT and A375SM-shFKBP51 cells (1×10^6 cells per 0.1 ml of HBSS; $n = 10$) were injected intravenously through tail vein injection. Mice were killed 8 weeks following tumour cell injection and lungs harvested and fixed in Bouin's solution.

Immunohistochemical and histological analyses. Tumour sections were deparaffinised using EZ-Dewax and rehydrated and processed for staining as described earlier (Tyagi *et al*, 2014). Mouse anti-human FKBP51 (1 : 200), mouse anti-human IL-8 (1 : 200), rat anti-mouse GR-1 (1 : 50) and biotinylated lectin GS-IB4 (1 : 50) were used for 60 min at room temperature. Subsequently, sections were incubated at room temperature with respective polymer and probe pairs as per manufacturer's instructions except for GS-IB4. Immunoreactivity was visualised using DAB substrate. Negative control tissues were incubated with all the reagents except primary antibody. The stained cells were visualised under microscope, counted in 10 random fields ($\times 200$) and photographed. Histological examination followed by haematoxylin and eosin staining was performed on lung tissue sections to examine the presence of metastatic tumour nodules. Tumour nests were visualised under microscope ($\times 200$) and photographed.

Statistical analysis. All experiments were performed at least three times and numerical data expressed as mean $\pm$ s.e.m. Wherever appropriate, the data were also subjected to unpaired two-tailed Student's t test and $P < 0.05$ was considered statistically significant.

RESULTS

FKBP51-induced growth and metastasis of melanoma cells is associated with enhanced angiogenesis and neutrophil infiltration. FKBP51 is shown to impact melanoma pathogenesis by promoting tumour cell survival, stemness and metastatic potential (Romano *et al*, 2010, 2013, 2014). Here, we set out to further explore the functional importance of FKBP51 in melanoma growth, metastasis, angiogenesis and immune cell infiltration. A375SM-shFKBP51 and A375SM-NT cells were subcutaneously implanted into the mice or delivered through tail vein injection. A375SM-shFKBP51 cells implanted subcutaneously exhibited slower tumour growth compared with A375SM-NT cells (Figure 1A). Average volume and weight of tumours developed in A375SM-NT group at the end point were 677.24 mm^3 and 1.1 g, respectively, as compared with 78.27 mm^3 and 0.14 g in A375SM-shFKBP51 group (Figure 1A and B). Furthermore, in experimental lung metastasis assay, we observed the presence of multiple metastatic tumour nodules ($\sim$10 per mice) in the lungs of all the mice from the control group, whereas no tumour cell nodule was seen in the FKBP51-knockdown group (Figure 1C and D). To further examine the effect of FKBP51 on tumour angiogenesis and neutrophil infiltration, we conducted histochemical analyses. We observed $\sim$5.5-fold decrease in the number of blood vessels in tumours developed from A375SM-shFKBP51 cells as compared with A375SM-NT cells (Figure 1E). Similarly, neutrophil infiltration was also significantly reduced ($\sim$43.0-fold) in tumours from A375SM-shFKBP51 as compared with the control group (Figure 1F). Together, these data reveal that FKBP51 promotes melanoma growth and metastasis, which is associated with increased angiogenesis and neutrophil infiltration.

Expression of FKBP51 positively correlates with IL-8 levels. Previously, we and others have shown that IL-8, a human C-X-C chemokine, plays important roles in melanoma growth, metastasis, vascularisation and recruitment of neutrophils at the tumour site (Huang *et al*, 2002; Varney *et al*, 2006; Gabellini *et al*, 2009; Singh *et al*, 2009a, b; Wu *et al*, 2012). Therefore, we examined whether FKBP51 expression impacted IL-8 production by performing immunohistochemical analysis. In tumour tissues, we observed a positive association of FKBP51 and IL-8 expression (Figure 2A). To further confirm this, we silenced FKBP51 expression in another aggressive melanoma cell line (FEMX-1) and examined changes in IL-8 expression by RT–PCR and immunoblot analyses. We also observed decreased mRNA and protein expression of IL-8 in A375SM-shFKBP51 and FEMX-1-shFKBP51 cells relative to their controls (Figure 2B). The amount of IL-8 secreted by the FKBP51-expressing and -silenced cells was also determined by ELISA. Data demonstrate the low levels of IL-8 in the culture supernatant of A375SM-shFKBP51 ($\sim$7.2-, 11.1- and 11.6-folds at 24, 48 and 72 h, respectively) and in FEMX-1-shFKBP51 ($\sim$6.1-, 9.8-, and 10.2-folds at 24, 48 and 72 h, respectively) as compared with their respective control cells (Figure 2C). Interleukin-8 expression was also examined in a panel of melanoma cell lines exhibiting variable expression of FKBP51. The data demonstrate a positive correlation between FKBP51 and IL-8 expression in melanoma cells at both mRNA and protein levels (Supplementary Figure S1). Together, these data indicate a direct association of FKBP51 and IL-8 expression, and suggest that IL-8 may serve as a mediator to regulate FKBP51-promoted melanoma pathogenesis.

FKBP51 induces IL-8 expression in melanoma cells through activation of NF-κB pathway. To further confirm the association of FKBP51 and IL-8, we investigated the mechanism underlying FKBP51-mediated regulation of IL-8. As IL-8 expression was decreased at the transcript level in FKBP51-silenced cells, we performed *in silico* analysis of $\sim$1 kb DNA region 5' upstream of their coding DNA sequence (GenBank accession number NG029889) using web-based application (ALGGEN-PROMO). We observed a putative binding site (-497 to -507) for NF-κB, a constitutively active transcription factor in melanoma, which is also shown to be regulated by FKBP51 (Romano *et al*, 2004, 2011). As expected, we observed a decreased transcriptional activity of NF-κB in A375SM-shFKBP51 and FEMX-1-shFKBP51 cells ($\sim$3.8- and 4.3-folds, respectively) as compared with their respective controls (Figure 3A). We also observed decreased nuclear accumulation with a concomitant increase in its cytoplasmic levels in FKBP51-silenced cells associated with decreased phosphorylation and enhanced level of IκB-α (inhibitor of NF-κB) (Figure 3B). Furthermore, we examined the direct binding of NF-κB to IL-8 promoter region in chromatin immunoprecipitation assay, which was decreased in A375SM-shFKBP51 and FEMX-1-shFKBP51 cells (Figure 3C). To further

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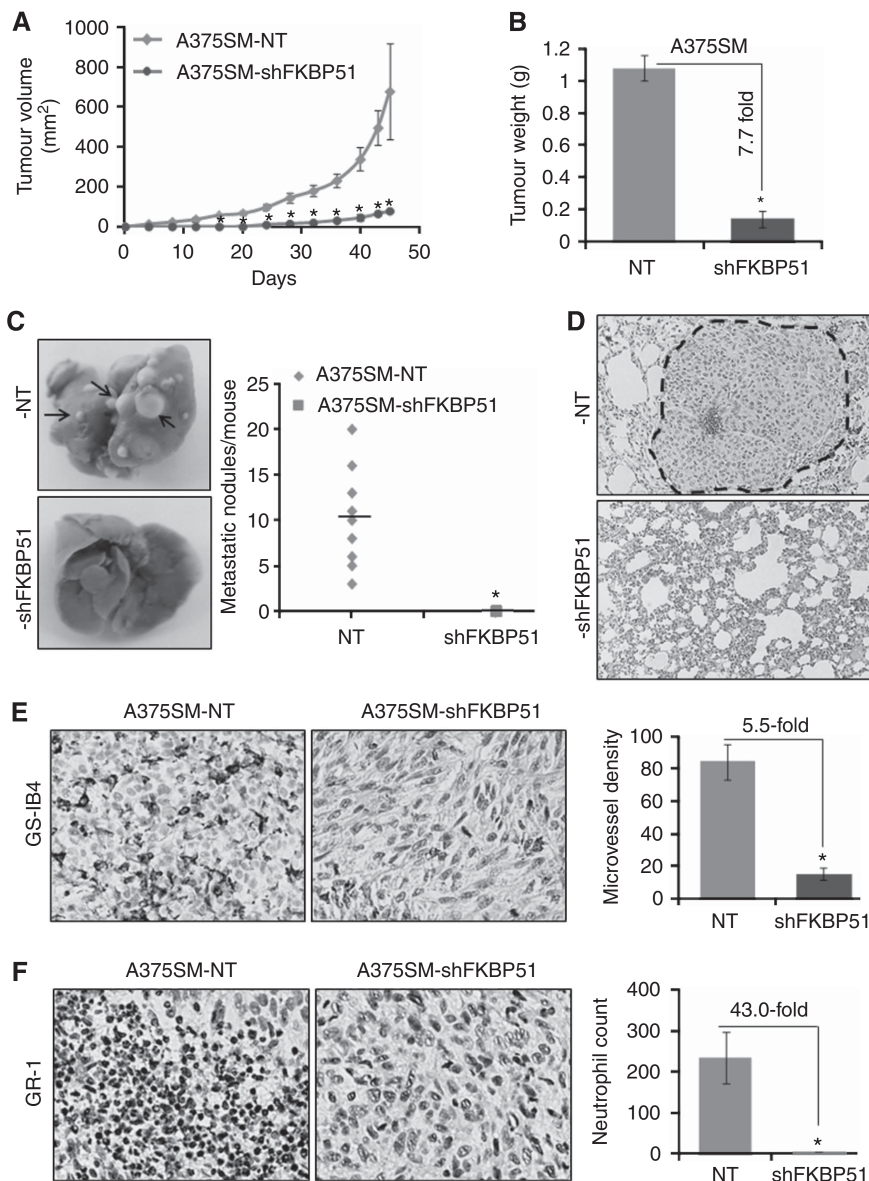

Figure 1. Knockdown of FKBP51 inhibits tumour growth, metastasis, angiogenesis and neutrophil infiltration. A375SM-shFKBP51 and A375SM-NT cells were subcutaneously injected on the right rear flank in athymic nude mice ($n=5$/group) and killed after 45 days. (**A**) Volume and (**B**) weight of the tumours from control and FKBP51-silenced group were calculated. (**C**) For experimental lung metastasis, A375SM-shFKBP51 and A375SM-NT cells were injected intravenously into the nude mice. Following 8 weeks of tumour cell injection, lungs harvested and fixed, and number of metastatic colonies per mouse were counted and plotted. Dark line indicates the median value of the group. (**D**) Haematoxylin and eosin-stained lung sections showing dense tumour nest in control group whereas FKBP51-silenced group had no gross evidence of deposits of tumour cells. (**E** and **F**) In immunostained tumour sections ($n=5$), number of microvessels and neutrophils were counted in 10 random view fields (magnification $\times 200$). All the data are representative of at least triplicate independent experiments and the data are presented as the mean ± s.e.m., $^*P<0.05$.

confirm the involvement of NF-κB in FKBP51-mediated regulation of IL-8, we transiently transfected FKBP51-expressing cells with degradation-resistant IκB-α-mutant. In parallel studies, FKBP51-silenced cells were transfected with a constitutively active mutant of IKKβ (IKKβ-SSEE), an upstream kinase of IκB-α. The data reveal that transcriptional activity of NF-κB is suppressed in FKBP51-expressing cells upon transfection of IκB-α-mutant, while the suppressive effect of FKBP51 silencing on the transcriptional activity of NF-κB is abolished following transfection of A375SM-shFKBP51 and FEMX-1-shFKBP51 cells with IKKβ mutant (Supplementary Figure S2). This is accompanied by decreased (in IκB-α MUT-transfected A375SM- and FEMX-1-NT cells) and enhanced (in IKKβ mutant-transfected A375SM- and FEMX-1 - shFKBP51 cells) nuclear accumulation of NF-κB (Figure 3D upper panel). Importantly, decreased expression of IL-8 in IκB-α MUT-transfected FKBP51-expressing cells was observed, whereas expression of IL-8 is restored in A375SM- and FEMX-1-shFKBP51 cells transfected with IKKβ mutant (Figure 3D lower panel). Altogether, these findings confirm that FKBP51 regulates IL-8 through the activation of NF-κB.

Interleukin-8 mediates FKBP51-induced growth, clonogenicity and aggressive phenotypes of melanoma cells. To explore the involvement of IL-8 in FKBP51-promoted melanoma pathogenesis, A375SM-NT and FEMX-1-NT cells were treated with IL-8-neutralising antibody or transiently transfected with IL-8-specific siRNAs, whereas A375SM-shFKBP51 and FEMX-1-shFKBP51 cells were treated with rIL-8, and effect on growth and malignant behaviour was examined. In accordance to our *in vivo* data, we observed $\sim$3.6-fold and $\sim$3.4-fold decreased

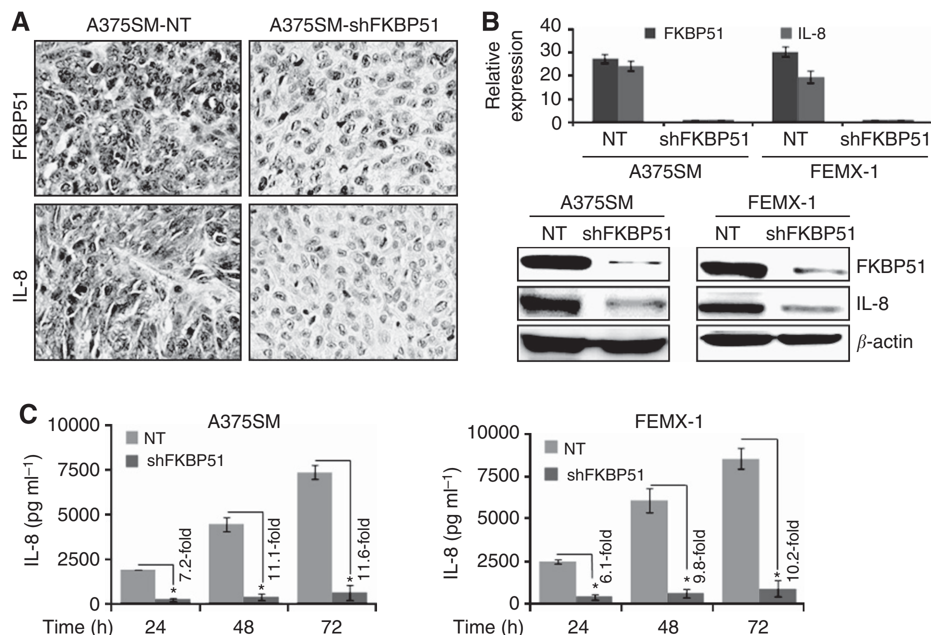

Figure 2. Expression of FKBP51 positively correlates with IL-8 levels. (**A**) Immunohistological analysis of paraffin-embedded melanoma tumours ($n = 5$) for FKBP51 expression (upper panel) and IL-8 expression (lower panel). The data are representative of at least triplicate independent experiments. (**B**) Expression of FKBP51 and IL-8 at transcript (upper panel) and protein (lower panel) levels was examined by PCR and immunoblot assays, respectively. GAPDH (for PCR) and β-actin (for immunoblot assay) served as internal controls. (**C**) Cell culture supernatants was collected after 24, 48 and 72 h and processed for ELISA. Data are presented as mean ± s.e.m., $*P < 0.05$.

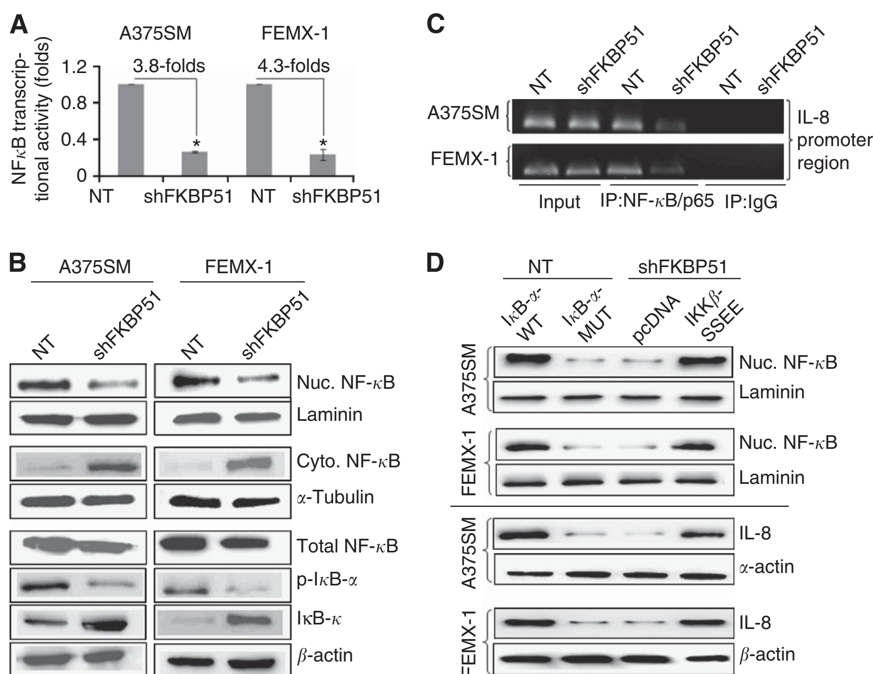

Figure 3. FKBP51-activated NF-κB regulates IL-8 in melanoma cells. (**A**) Cells were transiently co-transfected with NF-κB-responsive or control reporter plasmids. After 24 h of transfection, firefly and renilla luciferase activities were examined in the FKBP51-silenced or control cells as a measure of NF-κB transcriptional activity and transfection efficiency, respectively. (**B**) Total, nuclear and cytoplasmic extracts were prepared from FKBP51-silenced or control cells and expression of NF-κB-p65, IκB-α were determined by immunoblot analysis. Laminin, α-tubulin and β-actin were used as a loading control for nuclear, cytoplasmic and total proteins, respectively. (**C**) Chromatin immunoprecipitation assay was performed as described in materials and methods to examine the binding of NF-κB to IL-8 promoter region. (**D**) Control cells were transiently transfected with degradation-resistant IκB-α-mutant plasmid and FKBP51-silenced cells were transfected with constitutively active IKKβ-SSEE plasmid. Thereafter, nuclear and total protein lysates were prepared after 24 h and/or 48 h of transfection, and expression levels of NF-κB (in nuclear lysate after 24 h) and IL-8 (in total lysate after 48 h) were examined by immunoblot analysis. Data are presented as mean ± s.e.m., $*P < 0.05$.

growth in FKBP51-silenced A375SM and FEMX-1 cells, respectively, as compared with that in controls. Furthermore, treatment with IL-8-neutralising antibody also resulted in the significant growth inhibition of A375SM-NT (~2.2-fold) and FEMX-1-NT (~1.8-fold) cells (Figure 4A). Interestingly, treatment in A375SM- and FEMX-1-shFKBP51 cells partially abrogated the

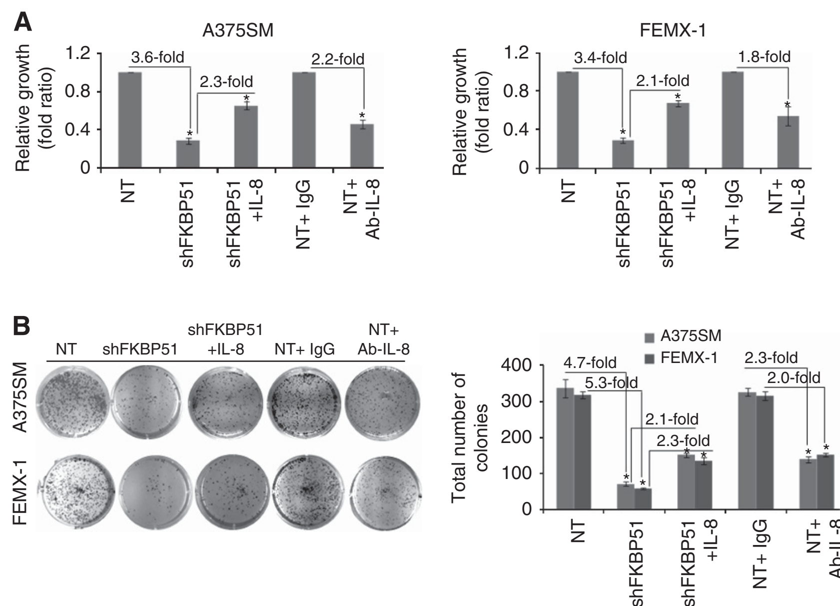

Figure 4. FKBP51 promotes melanoma growth by regulating IL-8. (A) For cell growth assay, controls and FKBP51-silenced cells were seeded (5×10^4 per well) in 96-well plate, and growth was monitored after 96 h as described in materials and methods. In parallel, NT cells seeded in 96 wells were treated with control IgG (200 ng ml^{-1}) or IL-8-neutralising antibody (200 ng ml^{-1}) and FKBP51-silenced cells were treated with rIL-8 (10 ng ml^{-1}) and effect on cell growth was determined. (B) For plating efficiency assay, cells were seeded (1×10^3cells per well) in 6-well plates in complete media and allowed to form colonies for 2 weeks. Separately, NT (1×10^3 cells per well) cells were treated with control IgG (200 ng ml^{-1}) or IL-8-neutralising antibody (200 ng ml^{-1}) and FKBP51-silenced (1×10^3 cells per well) cells were treated with rIL-8 (10 ng ml^{-1}). After 2 weeks, colonies were fixed with methanol, stained with crystal violet, photographed and counted using Image analysis software. Bars represent mean ± s.e.m., $n = 3$, *$P < 0.05$.

growth-inhibitory effects of FKBP51 silencing (Figure 4A). Next, we performed plating efficiency assay to monitor growth in the long term. Our data revealed that knockdown of FKBP51 was able to decrease the clonogenic potential of A375SM and FEMX-1 cells by ∼4.7-fold and ∼5.3-fold, respectively, as compared with control cells (Figure 4B). Additionally, inhibition of IL-8 by its neutralising antibody also decreases the clonogenic ability of A375SM-NT (∼2.3-fold) and FEMX-1-NT (∼2.0-fold) cells. Furthermore, cells (A375SM-shFKBP51 and FEMX-1-shFKBP51) treated with rIL-8 significantly enhances the colony formation (Figure 4B). In the next set of experiments, we studied the significance of IL-8 in FKBP51-induced aggressive phenotypes of melanoma cells. Our data demonstrate that FKBP51 silencing diminishes the migration (∼3.3- and ∼4.2-folds; Figure 5A) and invasion (∼6.7- and ∼5.2-folds; Figure 5B) in A375SM and FEMX-1 cells, respectively, as compared with their relevant controls. Inhibition of IL-8 by neutralising antibody (in FKBP51-expressing cells) also decreased the cell migration (∼2.7- and ∼2.3-folds) and invasion (∼2.4- and ∼2.6-folds) (Figure 5A and B). On the other hand, cells treated with rIL-8, partially neutralised the effects of FKBP51 silencing and promoted their migration (∼3.0- and ∼2.8-folds, respectively) and invasion (∼2.3- and ∼1.9-folds, respectively) (Figure 5A and B). Similar to the effects of IL-8-neutralising antibody, we also observed the inhibitory effects of IL-8 silencing on the growth and malignant behaviour of melanoma cells (Supplementary Figure S3). Altogether, our data suggest that FKBP51 regulated melanoma growth and aggressiveness is mediated, at least in part, through IL-8.

Interleukin-8 mediates the effect of FKBP51 overexpression on angiogenesis. Having observed an association of FKBP51-expressing cells-derived xenograft with enhanced angiogenesis, we next examined whether this effect is also mediated through IL-8. For this, we performed *in vitro* endothelial (HUVEC) cell proliferation and capillary-like structure formation assays in the presence of CM from FKBP51-expressing and -silenced cells. The data demonstrate a decreased proliferation of HUVEC when grown in CM from A375SM-shFKBP51 (∼5.5-fold) and FEMX-1-shFKBP51 (∼5.8-fold) cells, respectively, as compared with that from control cells (Figure 6A). Treatment of HUVECs with the CM (treated with IL-8-neutralising antibody for 24 h) obtained from A375SM-NT and FEMX-1-NT cells resulted in significant reduction of HUVECs growth (∼2.9- and ∼2.5-folds, respectively) (Figure 6A). In a separate experiment, treatment of HUVECs with rIL-8 promoted their proliferation by ∼6.3-fold (Figure 6B). These effects were further substantiated by conducting CLS formation assay. Cells seeded on Matrigel in the presence of CM (from A375SM-NT and FEMX-1-NT), exhibited extensive CLS formation (∼17–20 CLS/field), while very less CLS formation was observed when HUVECs were treated with CM from FKBP51-silenced cells (Figure 6C). In order to validate a role of IL-8 in FKBP51-induced CLS formation of HUVEC, we treated HUVEC with CM obtained from FKBP51-expressing cells that had been pre-treated with IL-8-neutralising antibody. Data demonstrate that neutralising antibody to IL-8 significantly inhibited the CLS formation (Figure 6C) of HUVECs. Similarly, we also observe significant reduction in the HUVEC proliferation as well as CLS formation upon treatment with CM of IL-8 silenced melanoma cells (Supplementary Figure S4A and B). On the contrary, higher number (∼20 CLS per field) of CLS is observed upon treatment of HUVEC with rIL-8 (Figure 6D). Thus, these data support the involvement of IL-8 in mediating the effect of FKBP51 on angiogenesis in melanoma.

DISCUSSION

In the present study, we demonstrated that inhibition of FKBP51 significantly reduced the tumourigenicity, metastatic potential, neutrophil infiltration and angiogenesis of melanoma cells.

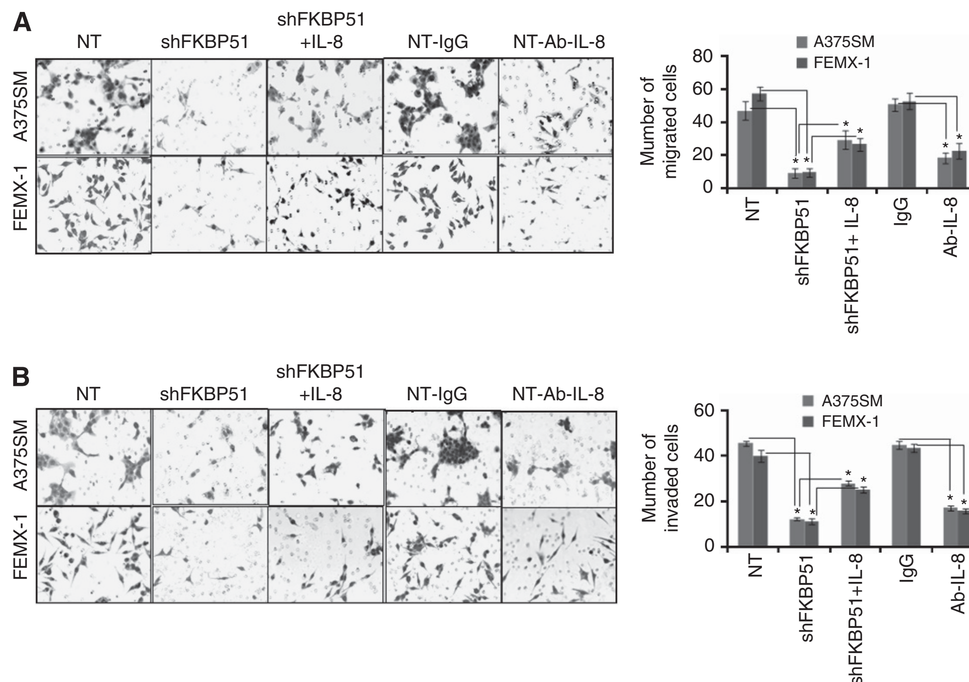

Figure 5. FKBP51-induced malignant potential is mediated through IL-8. Controls and FKBP51/IL-8-silenced cells were seeded on (**A**) non-coated (for motility assay), or (**B**) Matrigel-coated (for invasion assay) membranes. Media containing 10% FBS (for controls, FKBP51/IL-8-silenced cells) or rIL-8 (10 ng ml^{-1}, for FKBP51-silenced cells, pretreated with rIL-8) was added as a chemoattractant. Migrated (**A**) and invaded (**B**) cells were counted and presented as average number of cells per field ± s.e.m., *$P<0.05$. Photographs are representative of three experiments done in triplicate.

Moreover, we demonstrated that FKBP51 expression correlated with IL-8 level in tumour xenograft developed from FKBP51-silenced melanoma cells as well as in cell lines. Furthermore, mechanistic and functional studies revealed that FKBP51-induced IL-8 expression is mediated through NF-κB activation, and it serves as a key mediator in FKBP51-induced melanoma growth, metastasis, immune cell infiltration and angiogenesis.

A role of FKBP51 in melanoma pathogenesis has been suggested in recent studies (Romano *et al*, 2004, 2013, 2014). In the same line, this study provided direct evidence for a functional role of FKBP51 in melanoma growth and metastasis. Moreover, in additional novel findings, FKBP51 overexpression was associated with enhanced neutrophil infiltration and angiogenesis within the tumour xenograft. This is quite interesting as these phenotypes are significant for progressive growth of tumour. New blood vessel formation is required to support the increasing need of food supply to the growing tumour mass as well as to facilitate their haematogenous spread to distant organs (Li *et al*, 2003; Autiero *et al*, 2005; Sadanandam *et al*, 2010). Similarly, in the last decade, significant attention has been focused on tumour-associated neutrophils that are recruited into the tumour mass to support cancer progression. During the extravasation to the tumour site, neutrophils remodel the extracellular matrix by releasing several proteases, heparinase and various enzymes (Mollinedo *et al*, 1999). These released factors can act as growth stimulators and/or chemoattractants for the tumour and/or endothelial cells and thus assist in the metastatic processes (De Larco *et al*, 2004; Tazzyman *et al*, 2009; Spicer *et al*, 2012). Accordingly, higher level of neutrophil infiltration has been shown to be associated with poor survival, tumour-grade and aggressiveness in a variety of cancers (Fossati *et al*, 1999; Reid *et al*, 2011). In that regard, our data on the effect of FKBP51 on vessel density and neutrophil infiltration add a novel insight into its multiple pathological involvements in melanoma growth and metastasis.

For the recruitment of neutrophils to the tumour site, they must leave the general circulation and transmigrate across the vasculature (Tazzyman *et al*, 2009). It is well known that chemokines may stimulate the transmigration of neutrophils, and IL-8 is one of the chemokine, which acts as a major neutrophil chemoattractant (Huber *et al*, 1991). Moreover, it has been demonstrated that IL-8-expressing melanoma cells physically interact with neutrophils to facilitate their lung metastasis (Huh *et al*, 2010). Interleukin-8 has been demonstrated to positively influence the endothelial cells as well as tumour growth through autocrine and paracrine signalling (Li *et al*, 2003; Singh *et al*, 2011). Overexpression of IL-8 significantly induced the primary tumour growth and lung metastasis in melanoma (Wu *et al*, 2012). Moreover, its role in modulating growth and invasiveness of oestrogen receptor-negative breast cancer cells (Yao *et al*, 2007) and prostate cancer cells (Ma *et al*, 2009) has also been reported. Similarly, significant data exist to support a role of IL-8 in angiogenesis. Overexpression of IL-8 or exogenous addition of IL-8 was shown to promote endothelial cell survival, proliferation and induced capillary formation (Li *et al*, 2003; Wu *et al*, 2012). Interleukin-8 exerted these effects upon binding to CXCR1/CXCR2 receptors on endothelial cells and silencing of these receptors suppressed angiogenic phenotypes (Singh *et al*, 2011). More importantly, in our previous study, we provided direct evidence for a role of IL-8-CXCR2 in melanoma growth, metastasis and angiogenesis using a CXCR2 knockout mouse model (Singh *et al*, 2009a).

Interleukin-8 is constitutively expressed in melanoma, however, it has remained largely unknown what factors elicit its upregulation in melanoma cells. Like many other cytokines and chemokines, its expression can be regulated through a variety of mechanisms (Roebuck, 1999; Xie, 2001; Sakamoto *et al*, 2003). Here, our studies revealed that FKBP51 is one of the dysregulated inducers of IL-8 expression in melanoma cells. Moreover, we identified that IL-8 regulation by FKBP51 was mediated through NF-κB activation.

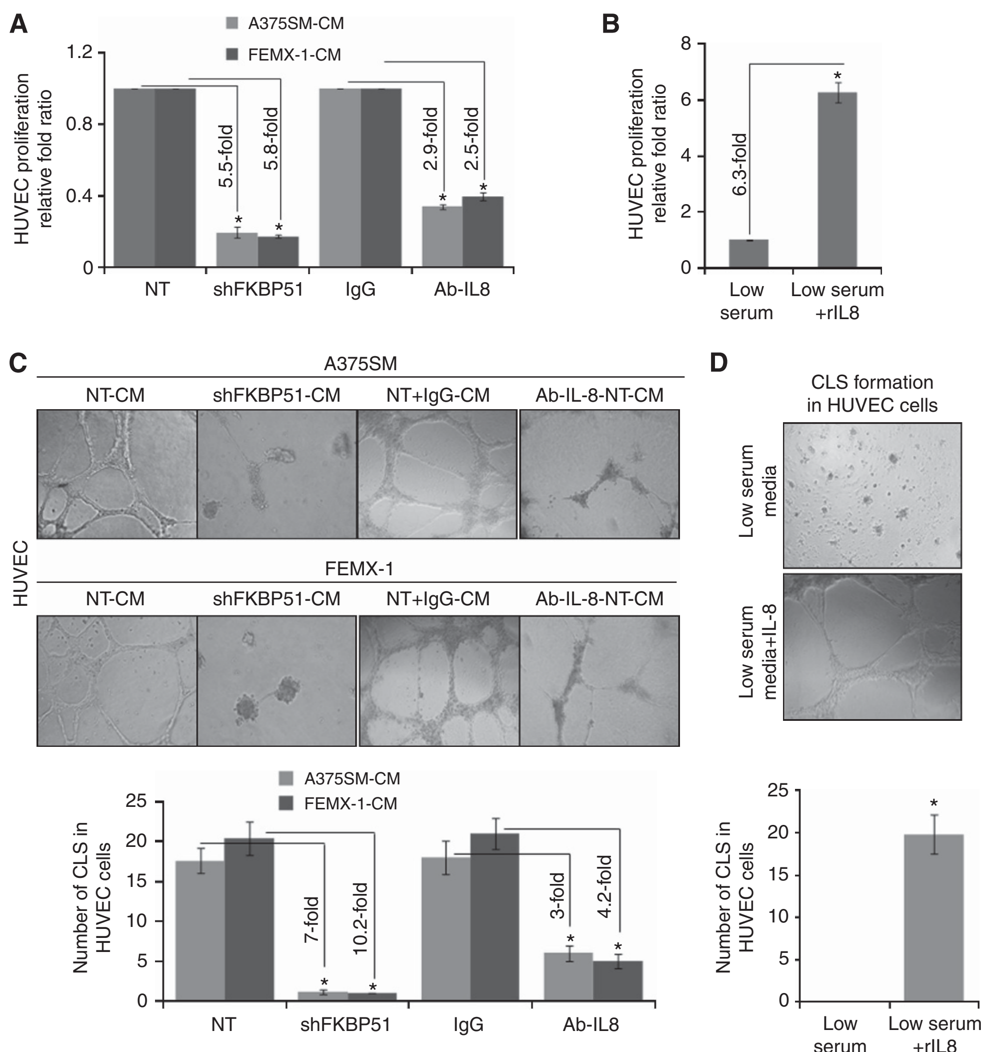

Figure 6. FKBP51 silencing decreases *in vitro* angiogenesis through inhibiting IL-8. For HUVEC proliferation assay (**A** and **B**), HUVECs were incubated with CM obtained from NT, FKBP51-silenced cells for 48 h and effect on cell proliferation was monitored using WST-1 assay kit. Separately, CM from FKBP51-NT cells was treated with IL-8-neutralising antibody (200 ng ml^{-1}) for 24 h. Subsequently, effect on HUVECs growth was examined. In a parallel study, HUVECs were cultured in low serum (2% serum) media or low serum media containing rIL-8 (10 ng ml^{-1}), and cell proliferation was examined after 48 h as described above. For *in vitro* angiogenesis assay (**C** and **D**), cells were plated on Matrigel-coated 96-well plates and incubated with CM from controls, FKBP51-silenced/IL-8-neutralising antibody (200 ng ml^{-1})-treated cells. After 16 h of incubation, CLS formation was observed and photographed. In a separate experiment, HUVECs were plated on Matrigel-coated 96-well plates in 2% serum containing media or media (with 2% serum) containing rIL-8 (10 ng ml^{-1}), and CLS formation was examined after 16 h. Data are presented as mean ± s.e.m., $^{*}P<0.05$.

NF-κB is a transcription factor that is aberrantly activated in several malignancies and there are indications that its constitutive activation may involve positive feedback mechanisms (Lin *et al*, 2010). For example, *CXCR4* is known to be a downstream target gene of NF-κB (Bist *et al*, 2011), whereas activation of CXCR4 itself is shown to promote NF-κB nuclear translocation and enhanced transcriptional activity of NF-κB-responsive promoter (Kukreja *et al*, 2005). Interleukin-8 is also reported to be one of the target genes of NF-κB (Kunsch and Rosen, 1993) and is also known to activate it in a positive feed-back mechanism (Manna and Ramesh, 2005). Therefore, FKBP51-induced enhanced production of IL-8 may elicit multiple autocrine and/or paracrine signalling pathways to promote melanoma pathogenesis.

In conclusion, our study have shown that FKBP51 regulates IL-8 through NF-κB activation, which then contributes to enhanced growth, metastatic potential and angiogenesis in melanoma. These novel findings strengthen the pathological significance of FKBP51 in melanoma and are quite significant from the perspective of molecular pathogenesis of melanoma. Together, our data shed new light into involved cancer mechanisms that could be targeted for development of effective melanoma therapy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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