



A mouse model of disseminated mantle cell lymphoma highlights a lack of activity of estrogen receptor β agonists toward tumor burden

Journal:	<i>Leukemia and Lymphoma</i>
Manuscript ID	GLAL-2017-0783.R1
Manuscript Type:	Letter to the Editor
Date Submitted by the Author:	n/a
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Keywords:	Lymphocytes < Hematopoiesis, Cell Lines and Animal Models < Neoplasia, Immunotherapeutic approaches < Neoplasia, Lymphoma and Hodgkin disease < Neoplasia

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48 Running head : Lack of ER β agonist activity on MCL
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5 mature B cells [1]. In the bone marrow and secondary lymphoid organs, tumor cells are
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21 receptors (ER) of α and β -types. However, while activated ER α enhances cell proliferation,
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29 lymphoma [5]. In this study, the Granta519 mantle cell lymphoma (MCL) cell line was
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12 Finally, since DPN was reported to inhibit VEGF synthesis [5], we looked at the effects of DPN
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14 and ERB-041 on angiogenesis using the tube formation assay. As shown Fig 1D and Fig S5,
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43 clinical model could be due to a protective CAM-DR. Indeed, in this *in vivo* model, MCL cells
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45 are in close contact with stromal cells delivering anti-apoptotic and pro-proliferative signals
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47 likely bypassing the effect of the drugs [7]. Alternatively, the pharmacokinetics distribution
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49 of ER β agonists may be different in the model described here and the one of Yakimchuk and
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51 colleagues [5]. A low concentration of ER β agonists could be present in the physiologic
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53 niches such as the bone marrow explaining the lack of efficiency for the treatment. Finally,
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3 the anti-angiogenic properties of ER β agonists highlighted in the work of Yakimchuk and
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5 colleagues [5], may have a greater impact on the proliferation of tumor cells in the s.c.
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7 model, where the neovascularization is crucial to bring to the tumor nutrients and oxygen.
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9 By contrast, hematopoietic organs are less dependent on neovascularization.
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11 However, our *in vitro* experiments confirmed that, although ER β are well-expressed in the
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13 MCL cell lines, ER β agonists had no effect on proliferation, migration, invasion or VEGF
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15 production, emphasizing the *in vivo* results. Interestingly, we found previously that, although
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17 able to decrease multiple myeloma cell proliferation, ER β agonists are inefficient *in vivo* [8].
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19 In conclusion, our results highlight a lack of effects of ER β agonists as single agents in mice.
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21 Nonetheless, as it has been described that estrogen play an important role in BCR pathway
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23 regulation [9], consequently further investigations would be required to assess the efficacy
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25 of ER β agonists in association with B-cell receptor inhibitors, like ibrutinib.
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34 **Acknowledgements**

35
36 The work has been financially supported by grants from ITMO *Biologie cellulaire,*
37
38 *développement et évolution* (to BS), *Ligue contre le Cancer - Comité de l'Orne,* and *Fondo de*
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40 *Investigación Sanitaria* PI12/01847 and PI15/00102, European Regional Development Fund
41
42 (ERDF) "*Una manera de hacer Europa*" (to GR). SiBo obtained a mobility grant from
43
44 *Cancéropôle Nord-Ouest.* AE-A and CR-Z were recipients of predoctoral fellowships from
45
46 *Ministerio de Ciencia e Innovación and Instituto de Salud Carlos III* (grant PIE13/00033),
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56 **Conflict of interest**

The authors declare no conflicts of interest.

Authors contribution

SB, AE-A, and CR-Z performed the research and analyzed the data. XT provided essential support. GR and BS designed and supervised the study. SB and BS wrote the paper. All authors read the final version of the manuscript and agree with its content and submission.

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9 **Figure 1. ER β are present on MCL cells but ER β agonists do not affect**
10 **migration, invasion, nor angiogenesis**

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14 **A.** Proteins were purified from cultured MCL cell lines including GFP/mCherry/Luc-expressing
15 cells with the M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific),
16 quantified, separated by SDS-PAGE, blotted onto nitrocellulose mambrane and incubated
17 with antibodies against ER β (sc-8974, Santa Cruz Biotech.) or β -actin (sc-47778) as a control
18 for gel loading and transfer. Our procedure has been described previously [10]. A band at 55
19 kDa, specific for ER β , is arrowed; * refers to an unspecific band at 75 kDa. **B.** *In vivo*
20 experiments were conducted in accordance with the recommendations of EEC (86/609/CEE)
21 and with the approval of the Animal Experimental Ethics Committee of our institution (UB-
22 CEEA agreement 251/15). Z138-GFP-Luc, REC1-GFP-Luc, JeKo1-mCherry-Luc, and
23 Granta519-GFP cell lines were engineered using lentivirus or retrovirus infections. Male NSG
24 mice, n = 3 per group (age 6-8 weeks), were injected i.v. with 10^7 cells in a final volume of
25 100 μ l. The same day, mice were separated into three groups receiving: vehicle (0.01%
26 DMSO), DPN or ERB-041 both at the concentration of 1 mg/kg. Drugs and vehicle were
27 injected i.p. 5 days a week. Tumor engraftment was determined weekly by bioluminescence
28 imaging (BLI). For this aim, mice were injected i.p. with 75 mg/kg of D-luciferine and then
29 analyzed on an Aequoria Luxiflux bioluminescent device equipped with an ORCA-ER camera
30 (Hamamatsu). Mice were euthanized at day 31 (Z138-Luc) or day 41 (REC1-GFP-Luc).
31 Endpoint BLI signal is shown for each treatment group. **C.** MCL cells were treated *in vitro*
32 with DPN or ERB-041 (100 nM or 1 μ M for 48 h or 72 h) or vehicle and assayed for migration.
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3 Cells (5×10^5 cells per insert) were suspended in RPMI 1640 medium containing 0.5% bovine
4 serum albumin (BSA). They were seeded in the top chamber of transwell inserts (Millicell®
5 Hanging Cell Culture Inserts 5 μ m PET, Millipore). Filters were transferred to wells containing
6 RPMI 1640 medium with recombinant human stromal cell-derived factor 1 (SDF1, 200 ng/ml,
7 R&D Systems) as chemoattractant. Plates were then incubated for 4 h at 37°C, and the
8 number of cells migrated in the lower chambers was counted by flow cytometry. As an
9 example, the results obtained for the Z138 cell line treated with 0.1 or 1 μ M ERB-041 or DPN
10 for 48 h are shown. The percentage of migrating cells (treated vs. vehicle) for each culture
11 condition is plotted on the graph. **D.** HUVECs were plated onto a layer of Matrigel (BD
12 Bioscience) at a density of 4×10^4 cells/well. Cells were treated with supernatants of ERB-
13 041-treated (100 nM or 1 μ M for 4 h) or vehicle-treated cells as a control. After 24 h,
14 tubular structures were photographed under a microscope (Olympus IX2-SL). The number of
15 formed capillary tubes was counted in 11 independent fields (Fig S5A) of each well from
16 each culture. The experiment has been done twice with triplicate samples. The number of
17 tubes are plotted in the histograms. **E.** The concentration of VEGF was measured with an
18 ELISA assay (Quantikine ELISA Human VEGF, R&D systems) using the supernatants of
19 Granta519 and JeKo-1 cells treated by ERB-041 (1 μ M for 48 h) according to the
20 manufacturer's recommendations. Samples were assayed in triplicate. The concentration of
21 synthesized VEGF in each culture condition was deduced from the standard curve shown in
22 Fig S5B. ns, non significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ with the Student's *t*-test.
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Table 1. Flow cytometry analysis of infiltrated organs in engrafted NSG mice

Cell line	Mouse	Spleen	Blood	Bone marrow	Liver	Lung	Kidney	Testis
JeKo1-mCherry-Luc*	J1	5	no	44	no	nd	nd	no
	J2	15	no	59	no	nd	nd	no
	J3	7	7	45	no	nd	nd	no
Z138-Luc**	Z3	22	no	68	no	nd	nd	nd
	Z4	24	no	75	no	nd	nd	nd
	Z5	38	no	82	no	nd	nd	nd
REC1-GFP-Luc***	R4	no	no	4	nd	nd	nd	nd
	R5	no	no	3	no	nd	nd	nd
	R6	no	no	3	nd	nd	nd	nd
Granta519-GFP†	G1	23	1	57	45	7	9	no
	G4	37	2	21	47	3	10	< 1

*, in the JeKo1-mCherry-Luc series, three mice were sacrificed at day 23. **, in the Z138-Luc series, three mice were sacrificed at day 30. ***, in the REC1-GFP-Luc series, three mice were sacrificed at day 33. †, in the Granta-GFP series, three mice died unexpectedly at day 17, two mice were sacrificed at the same day. At the time of euthanasia, indicated organs were collected; single-cell suspensions were prepared, then stained with IOTest® CD20-PE (phycoerythrin, IM1451, FL2) or the corresponding isotype control and analyzed on an Attune acoustic focusing cytometer (Thermo Fisher Scientific). The percentage of positive cells in each organ is indicated in the table. no, no signal; nd, not done.

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3 the anti-angiogenic properties of ER β agonists highlighted in the work of Yakimchuk and
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5 colleagues [5], may have a greater impact on the proliferation of tumor cells in the s.c.
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7 model, where the neovascularization is crucial to bring to the tumor nutrients and oxygen.
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9 By contrast, hematopoietic organs are less dependent on neovascularization.
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11 However, our *in vitro* experiments confirmed that, although ER β are well-expressed in the
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13 MCL cell lines, ER β agonists had no effect on proliferation, migration, invasion or VEGF
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15 production, emphasizing the *in vivo* results. Interestingly, we found previously that, although
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17 able to decrease multiple myeloma cell proliferation, ER β agonists are inefficient *in vivo* [8].
18
19 In conclusion, our results highlight a lack of effects of ER β agonists as single agents in mice.
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21 Nonetheless, as it has been described that estrogen play an important role in BCR pathway
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23 regulation [9], consequently further investigations would be required to assess the efficacy
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25 of ER β agonists in association with B-cell receptor inhibitors, like ibrutinib.
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34 **Acknowledgements**

35
36 The work has been financially supported by grants from ITMO *Biologie cellulaire,*
37
38 *développement et évolution* (to BS), *Ligue contre le Cancer - Comité de l'Orne,* and *Fondo de*
39
40 *Investigación Sanitaria* PI12/01847 and PI15/00102, European Regional Development Fund
41
42 (ERDF) "*Una manera de hacer Europa*" (to GR). SiBo obtained a mobility grant from
43
44 *Cancéropôle Nord-Ouest.* AE-A and CR-Z were recipients of predoctoral fellowships from
45
46 *Ministerio de Ciencia e Innovación and Instituto de Salud Carlos III* (grant PIE13/00033),
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51 respectively.
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56 **Conflict of interest**

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The authors declare no conflicts of interest.

Authors contribution

SB, AE-A, and CR-Z performed the research and analyzed the data. XT provided essential support. GR and BS designed and supervised the study. SB and BS wrote the paper. All authors read the final version of the manuscript and agree with its content and submission.

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Figure 1. ER β are present on MCL cells but ER β agonists do not affect migration, invasion, nor angiogenesis

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A. Proteins were purified from cultured MCL cell lines including GFP/mCherry/Luc-expressing cells with the M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific), quantified, separated by SDS-PAGE, blotted onto nitrocellulose membrane and incubated with antibodies against ER β (sc-8974, Santa Cruz Biotech.) or β -actin (sc-47778) as a control for gel loading and transfer. Our procedure has been described previously [10]. A band at 55 kDa, specific for ER β , is arrowed; * refers to an unspecific band at 75 kDa. **B.** *In vivo* experiments were conducted in accordance with the recommendations of EEC (86/609/CEE) and with the approval of the Animal Experimental Ethics Committee of our institution (UB-CEEA agreement 251/15). Z138-GFP-Luc, REC1-GFP-Luc, JeKo1-mCherry-Luc, and Granta519-GFP cell lines were engineered using lentivirus or retrovirus infections. Male NSG mice, n = 3 per group (age 6-8 weeks), were injected i.v. with 10^7 cells in a final volume of 100 μ l. The same day, mice were separated into three groups receiving: vehicle (0.01% DMSO), DPN or ERB-041 both at the concentration of 1 mg/kg. Drugs and vehicle were injected i.p. 5 days a week. Tumor engraftment was determined weekly by bioluminescence imaging (BLI). For this aim, mice were injected i.p. with 75 mg/kg of D-luciferine and then analyzed on an Aequoria Luxiflux bioluminescent device equipped with an ORCA-ER camera (Hamamatsu). Mice were euthanized at day 31 (Z138-Luc) or day 41 (REC1-GFP-Luc). Endpoint BLI signal is shown for each treatment group. **C.** MCL cells were treated *in vitro* with DPN or ERB-041 (100 nM or 1 μ M for 48 h or 72 h) or vehicle and assayed for migration.

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3 Cells (5×10^5 cells per insert) were suspended in RPMI 1640 medium containing 0.5% bovine
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5 serum albumin (BSA). They were seeded in the top chamber of transwell inserts (Millicell®
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7 Hanging Cell Culture Inserts 5 μ m PET, Millipore). Filters were transferred to wells containing
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9 RPMI 1640 medium with recombinant human stromal cell-derived factor 1 (SDF1, 200 ng/ml,
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11 R&D Systems) as chemoattractant. Plates were then incubated for 4 h at 37°C, and the
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13 number of cells migrated in the lower chambers was counted by flow cytometry. As an
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15 example, the results obtained for the Z138 cell line treated with 0.1 or 1 μ M ERB-041 or DPN
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17 for 48 h are shown. The percentage of migrating cells (treated vs. vehicle) for each culture
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19 condition is plotted on the graph. **D.** HUVECs were plated onto a layer of Matrigel (BD
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21 Bioscience) at a density of 4×10^4 cells/well. Cells were treated with supernatants of ERB-
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23 041-treated (100 nM or 1 μ M for 4 h) or vehicle-treated cells as a control. After 24 h,
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25 tubular structures were photographed under a microscope (Olympus IX2-SL). The number of
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27 formed capillary tubes was counted in 11 independent fields (Fig S5A) of each well from
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29 each culture. The experiment has been done twice with triplicate samples. The number of
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31 tubes are plotted in the histograms. **E.** The concentration of VEGF was measured with an
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33 ELISA assay (Quantikine ELISA Human VEGF, R&D systems) using the supernatants of
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35 Granta519 and JeKo-1 cells treated by ERB-041 (1 μ M for 48 h) according to the
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37 manufacturer's recommendations. Samples were assayed in triplicate. The concentration of
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39 synthesized VEGF in each culture condition was deduced from the standard curve shown in
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47 Fig S5B . ns, non significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ with the Student's *t*-test.
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Table 1. Flow cytometry analysis of infiltrated organs in engrafted NSG mice

Cell line	Mouse	Spleen	Blood	Bone marrow	Liver	Lung	Kidney	Testis
JeKo1-mCherry-Luc*	J1	5	no	44	no	nd	nd	no
	J2	15	no	59	no	nd	nd	no
	J3	7	7	45	no	nd	nd	no
Z138-Luc**	Z3	22	no	68	no	nd	nd	nd
	Z4	24	no	75	no	nd	nd	nd
	Z5	38	no	82	no	nd	nd	nd
REC1-GFP-Luc***	R4	no	no	4	nd	nd	nd	nd
	R5	no	no	3	no	nd	nd	nd
	R6	no	no	3	nd	nd	nd	nd
Granta519-GFP†	G1	23	1	57	45	7	9	no
	G4	37	2	21	47	3	10	< 1

*, in the JeKo1-mCherry-Luc series, three mice were sacrificed at day 23. **, in the Z138-Luc series, three mice were sacrificed at day 30. ***, in the REC1-GFP-Luc series, three mice were sacrificed at day 33. †, in the Granta-GFP series, three mice died unexpectedly at day 17, two mice were sacrificed at the same day. At the time of euthanasia, indicated organs were collected; single-cell suspensions were prepared, then stained with IOTest® CD20-PE (phycoerythrin, IM1451, FL2) or the corresponding isotype control and analyzed on an Attune acoustic focusing cytometer (Thermo Fisher Scientific). The percentage of positive cells in each organ is indicated in the table. no, no signal; nd, not done.

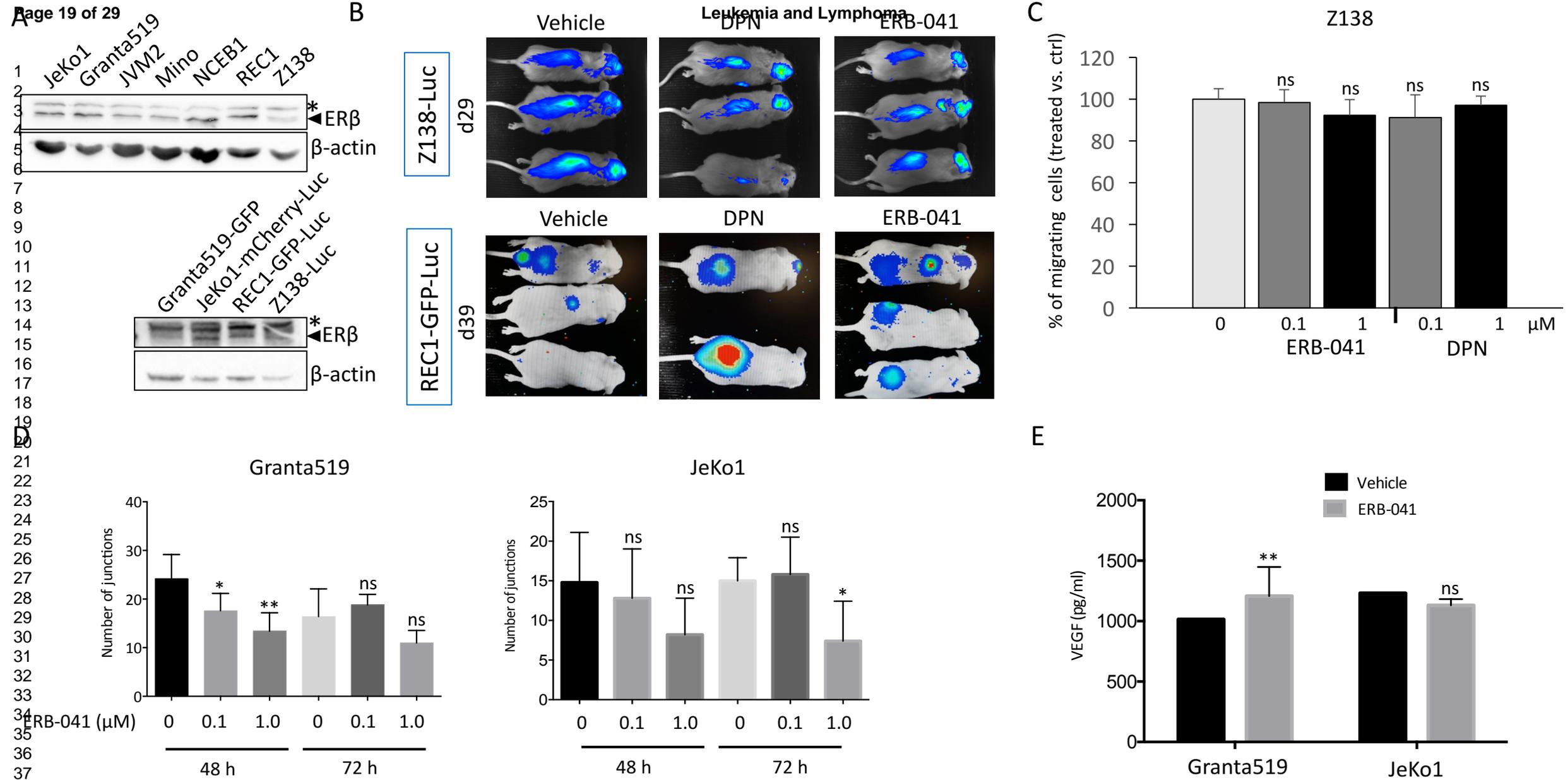


Fig 1

Additional informations

Additional methods

Cell culture and cell proliferation measurement

MCL cell lines have been described previously (Moros *et al*, 2014). Cell lines were maintained in culture in RPMI 1640 medium (Lonza) supplemented with 10-20% FCS (PAA Laboratories), 2 mM L-glutamine and antibiotics (Lonza) in a humid atmosphere at 37°C. Cell authentication was done by short tandem repeat (STR) profiling (IdentiCell, Aarhus, Denmark).

Cell proliferation was assessed by an MTS assay (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, here the CellTiter 96® AQueous One Solution 5promega) according to the supplier's instructions.

Transwell migration and invasion assays

For migration assay, 5×10^5 cells in serum-free medium plus 0.5% BSA were added to the top chamber of 24-well inserts (Costar Transwell® Permeable Support, pore size 5 μm). For invasion assay, the same number of cells were seeded in transwell inserts coated with extracellular matrix ECM obtained from Engelbrecht-Holm-Swarm mouse sarcoma (Sigma-Aldrich). Medium supplemented with SDF1 (200 ng/ml) or not (for control), was added in the bottom chambers. Plates were incubated for 4 h (migration) or 24 h (invasion) at 37°C. Migrating and invaded cells present in the lower chambers were counted by flow cytometry. The accuracy of cell count was checked by the use of fluorescent particles added in the chambers (Accucount Fluorescent particles, SpheroTech).

Tube formation assay

HUVECs were plated onto a layer of Matrigel (BD Bioscience) at a density of 4×10^4 cells/well in 24-well plates. Immediately after, cells were treated with supernatants of DPN/ERB-041-treated (100 nM or 1 μ M for 48 h) or vehicle-treated MCL cells. Cultures were observed 24 h later under an inverted microscope (Olympus IX2-SL, X 40 magnification) and the number of formed capillary tubes counted on 11 representative fields. A junction was recorded when a tube was bound to, at least, one another tube.

Western blotting

Proteins were purified from cultured MCL cell lines including GFP/mCherry/Luc-expressing cells with the M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific), quantified, separated by SDS-PAGE, blotted onto nitrocellulose paper and incubated with antibodies against ER β (sc-8974, Santa Cruz Biotech.) or β -actin (sc-47778) as a control for gel loading and transfer. Our procedure has been described previously (Moros *et al*, 2014).

MCL cells engraftment in immunocompromised mice

In vivo experiments were conducted in accordance with the recommendations of EEC (86/609/CEE). They were approved by the Animal Experimental Ethics Committee of our institution (UB-CEEA agreement 251/15 in Spain). Z138-GFP-Luc, REC1-GFP-Luc, JeKo1-mCherry-Luc, Granta519-GFP cell lines were engineered using lentiviruses or retroviruses. Male NSG (n = 6 per group) mice (age 6-8 weeks) were injected i.v. with 10^7 cells in a final volume of 100 μ l. Tumor engraftment was determined weekly by bioluminescence imaging (BLI). Mice were injected i.p. with 75 mg/kg of D-luciferine and then analyzed with a Aequoria Luxiflux bioluminescent device quipped with an ORCA-ER camera (Hamamatsu). Photon emissions were captured through the whole mouse body. At the end of the

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3 experiment mice were euthanized. Then, infiltrated organs were collected; single-cell
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5 suspensions were prepared, then stained with IOTest® CD45-PE (phycoerythrin, IM1451,
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7 FL2) or the corresponding isotype controls as recommended by the supplier.

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10 For ERβ agonists treatment, mice were either engrafted with Z138-Luc or REC1-GFP-Luc. The
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12 same day, mice were separated into three groups (n=3 in each one), one receiving DPN (1
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14 mg/kg), another ERB-041 (1mg/kg), and the last one vehicle (0.1% DMSO). Mice were
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16 treated five days a week. Mice of the Z138 group were euthanized at day 29, of the REC1
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18 group, at day 39. BLI was recorded as mentioned before.

21 22 23 24 **Additional figure legends**

25 26 **Supplemental Fig S1**

27
28 Six-week old NOD-*scid* *IL2γ*^{null} (NSG) male mice were injected in the caudal vein with 10⁷
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30 JeKo1-mCherry-Luc, Z138Luc, or REC1-GFP-Luc cells. Mice were weekly analyzed for BLI
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32 starting one week after cells injection.

33 34 35 **Supplemental Fig S2. Effect of DPN and ERB-041 treatment on MCL tumor cell infiltration** 36 37 **in vivo.**

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39 NSG mice were injected intravenously with Z138-Luc cells and received a daily dose of DPN
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41 (D, 1 mg/kg), ERB-041 (E, 1 mg/kg) or the equivalent volume of vehicle (V), for up to 14 days.
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43 Mice were then sacrificed and human CD45+ malignant B cells were recounted on a
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45 cytometer from spleen, brain, bone marrow extracts and in peripheral blood samples, as
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47 described in the methods section. Shown are the relative mean fluorescence intensity (MFI-
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49 R) values between CD45-derived fluorescence and isotypic control, calculated in the
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51 different compartments and for each treatment group (statistical significance: * $p < 0.05$).
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56 57 **Supplemental Fig S3**

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3 Granta519-GFP, JeKo1-mCherry-Luc, REC1-GFP-Luc and Z138-Luc cells were either treated
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5 with the vehicle (0.1% DMSO) or various concentrations (0.1-10⁴ nM) DPN or ERB-041 for 48
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7 h. Cell proliferation was assessed using an MTS assay (CellTiter 96[®] AQueous One Solution
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9 Cell Proliferation Assay, Promega). The experiment was performed twice with triplicate
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11 samples with similar results. The means \pm SD from one representative experiment are
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13 reported on the histograms. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant with
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15 the Student's *t*-test.
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18 19 **Supplemental Fig S4**

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21 **A. Left panel.** JeKo1 and Z138 cultured cells were seeded into the top chamber of
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23 transwell inserts. Inserts were transferred into wells containing medium with SDF1 or
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25 without SDF1 (Ctrl) and cells were allowed to migrate for 4 h. Cells present in the bottom
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27 well were then counted. Three independent inserts were seeded per experiment; the
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29 experiment was performed three times. Mean numbers of migrating cells \pm SD were plotted
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31 on the graph. *Middle and right panels.* Granta519 and JeKo1 cells were treated *in vitro* with
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33 vehicle or ERB-041 (0.1 or 1 μ M) for 48 h or 72 h. Then they were seeded and assayed for
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35 migration as described before. Three independent inserts were seeded per experiment; the
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37 experiment was performed three times. The percentage of migrating cells \pm SD was plotted
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39 on the graph. **B. Left panel.** JeKo1 cells were seeded in transwell inserts coated with
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41 extracellular matrix (ECM) proteins. Inserts were transferred into wells containing medium
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43 with SDF1 or without SDF1 (Ctrl). Cells were allowed to invade ECM for 24 h. The number of
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45 invaded cells is plotted on the graph. The experiment has been carried out twice with
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47 triplicate samples. *Middle and right panels.* JeKo1 and Z138 cells were treated with vehicle,
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49 DPN (0.1-1 μ M) or ERB-041 (0.1-1 μ M) for 48 h and seeded in ECM-coated transwell inserts.
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51 Inserts were incubated for 24 h and the number of cells present in the bottom wells
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3 counted. The experiment was repeated three times with triplicate samples. The mean ratios
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5 (treated vs. ctrl) \pm SD are plotted on the graph. ns, not significant; *, $p < 0.05$; **, $p < 0.01$;
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8 *** , $p < 0.001$ in t -tests.
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10 Supplemental Fig S5

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12 **A.** HUVECs were plated onto a layer of Matrigel (BD Bioscience) at a density of 4×10^4
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14 cells/well in the presence of supernatants from JeKo1 and Granta519 cells previously
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16 treated with ERB-041 (0.1 or 1 μ M, 4 h) or with vehicle as a control. After a 24 h-incubation
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18 period, cells were photographed under a microscope (Olympus IX2-SL). The number of
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20 branch points (circled in red) with at least two branches, corresponding to newly formed
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22 capillary tubes was counted on representative fields. The experiment has been done twice
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27 with triplicate samples.

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29 **B.** The quantification of produced VEGF was done using the Quantikine ELISA kit (R&D
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31 systems) according to the manufacturer' instructions on a Victor X4 plate-reader (Perkin
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33 Elmer). The standard curve was drawn from the OD at 450 nm of calibrated VEGF
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35 concentrations ranging from 10^3 to 15.6 pg/ml.
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40 Additional reference

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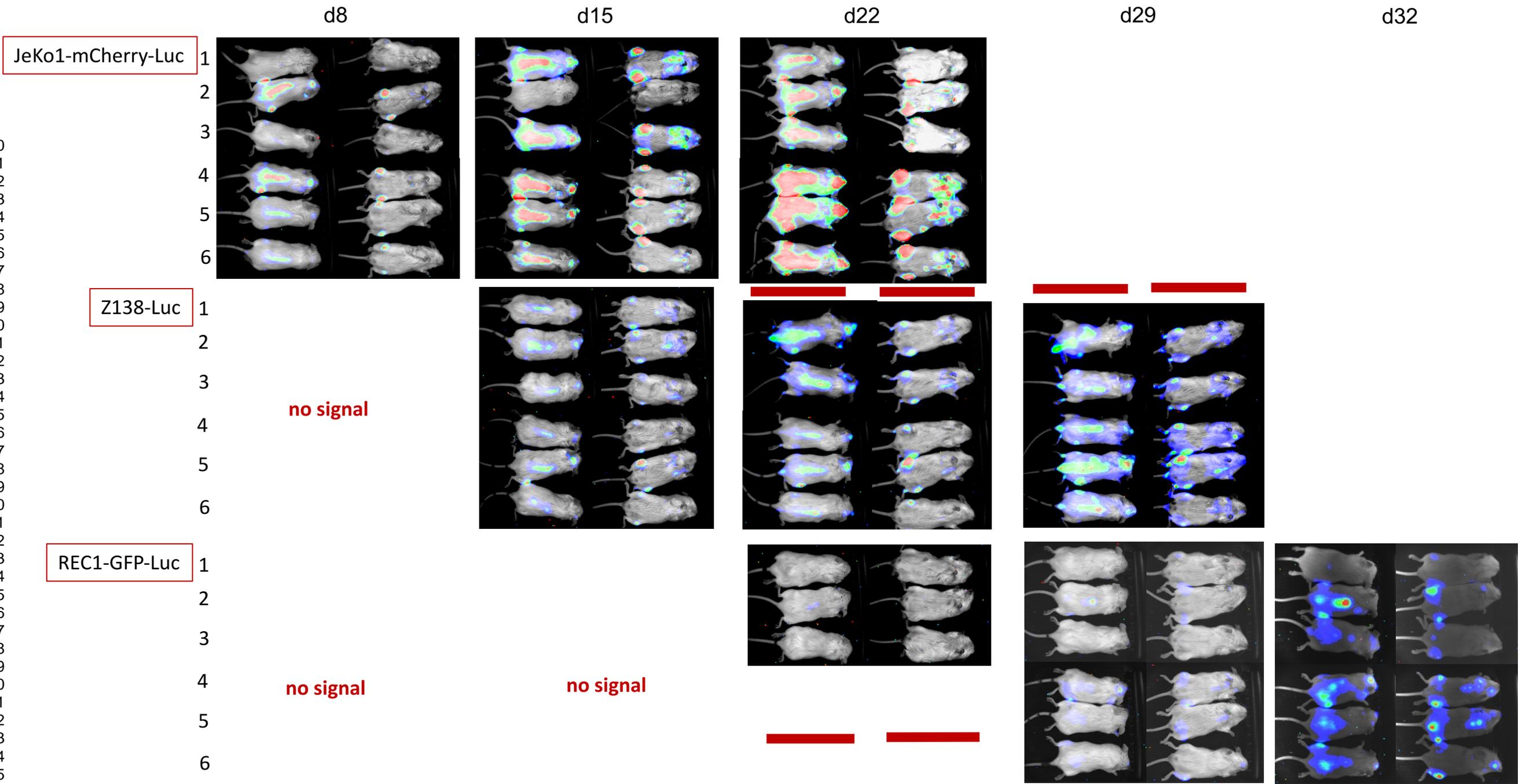


Fig S1

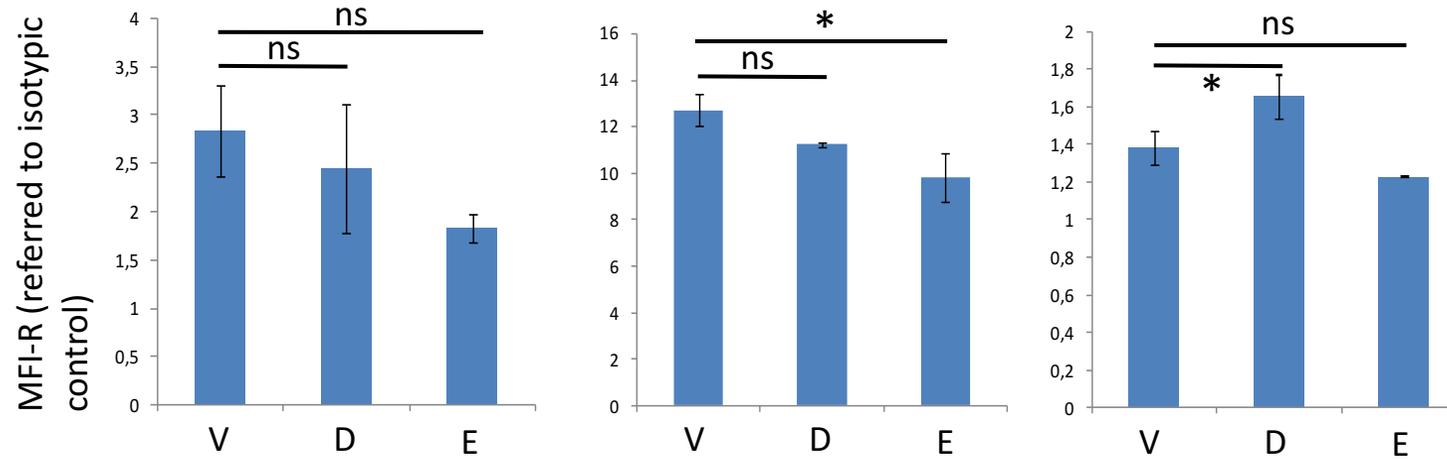


Fig S2

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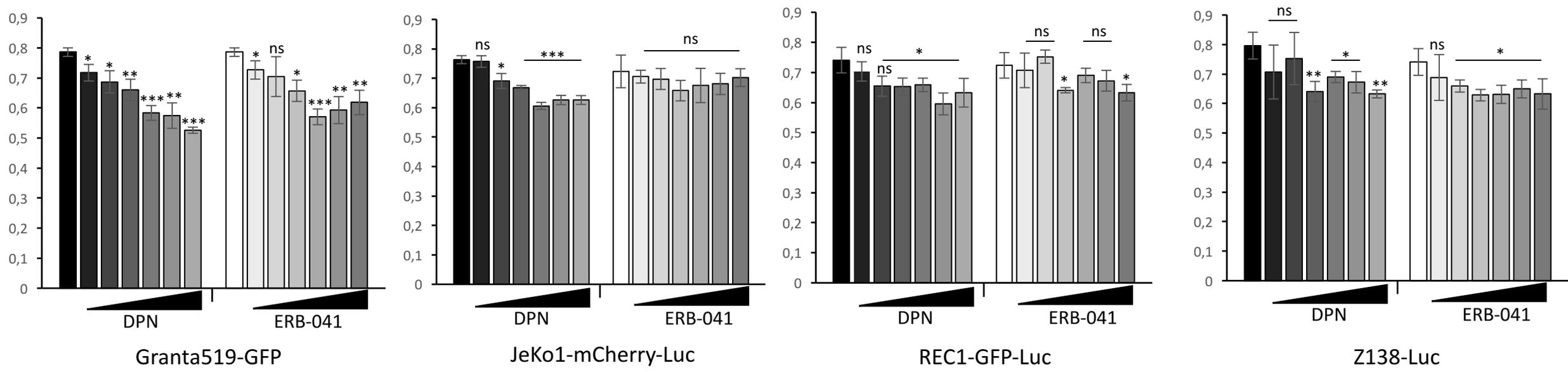
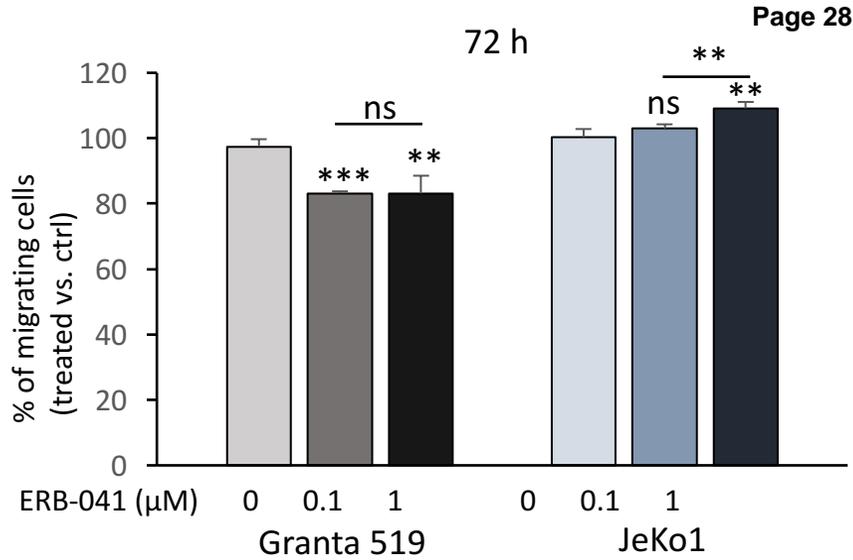
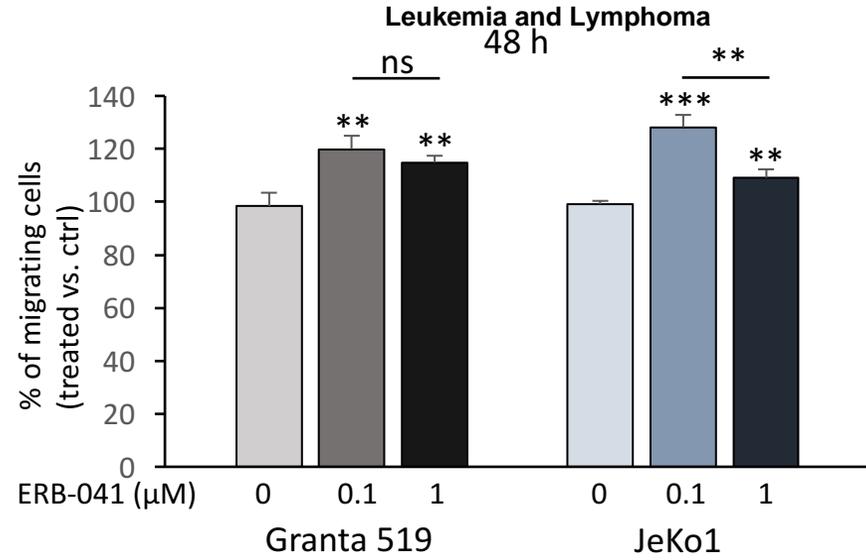
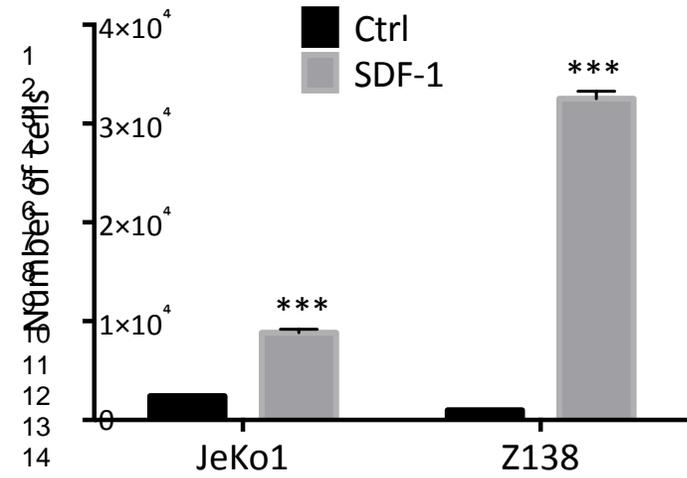
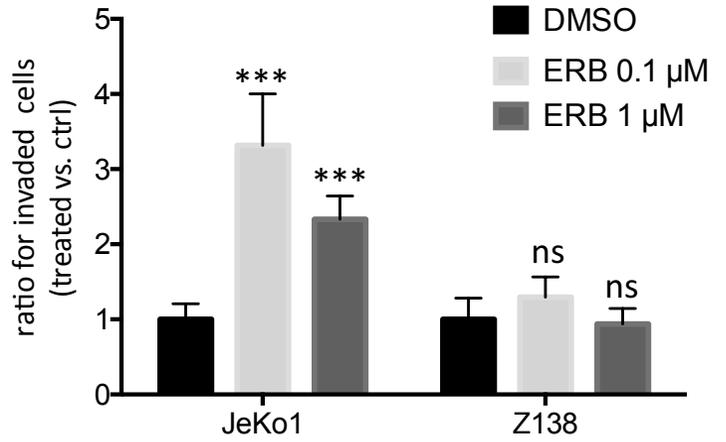
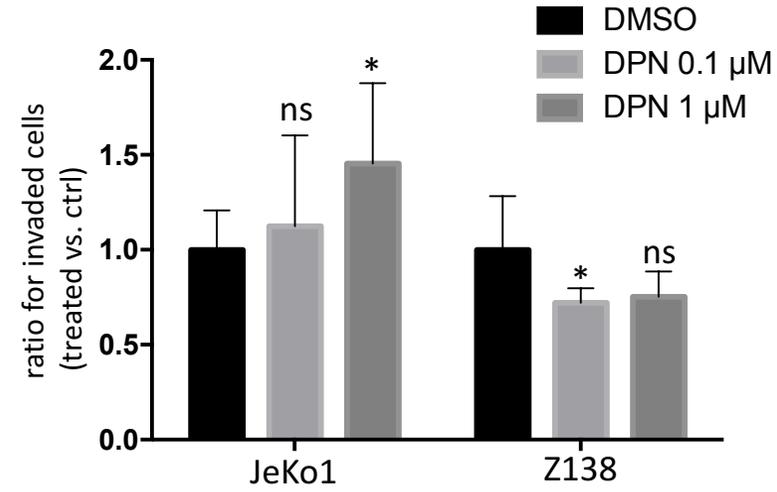
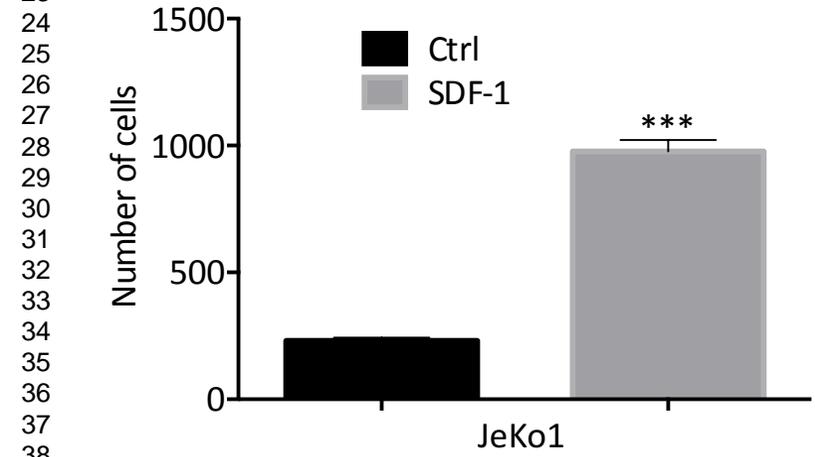


Fig S3

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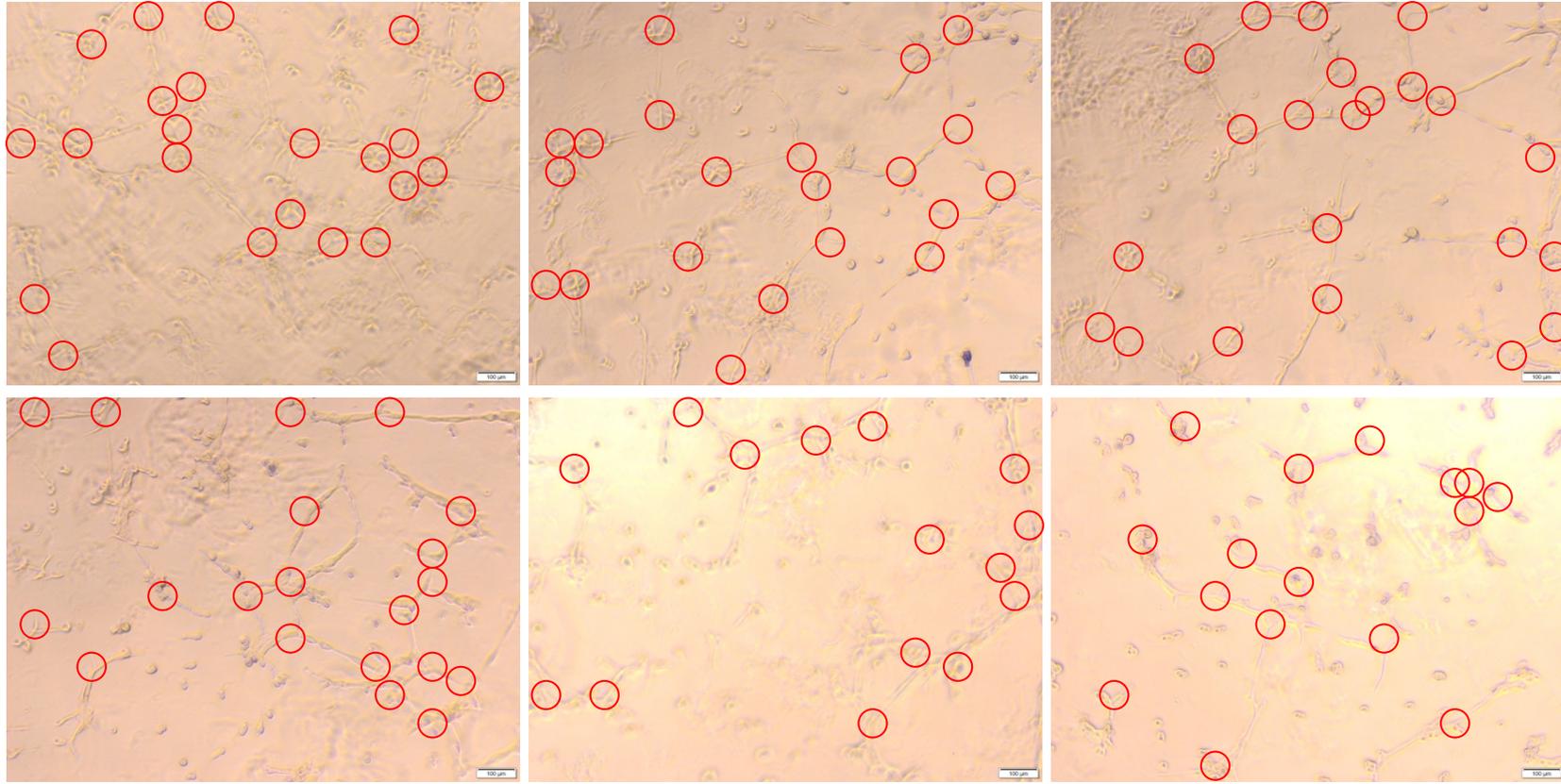


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Fig S4

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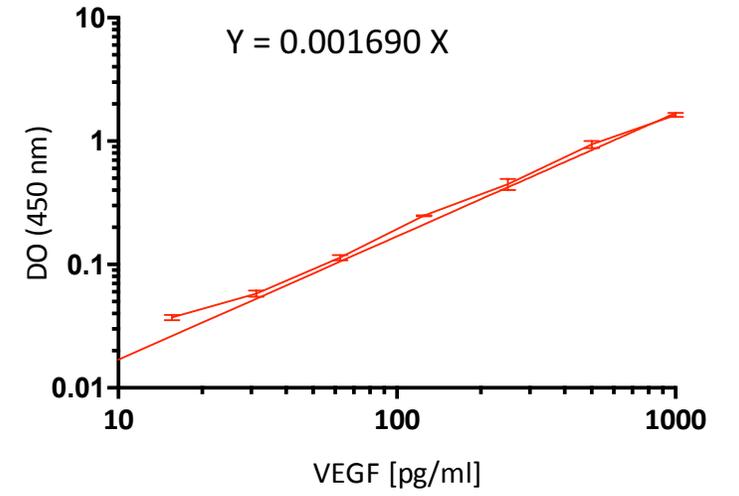


Fig S5