HOXB7 induces STAT3-mediated transformation and lung metastasis in an immortalized mammary gland NMuMG cells

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Abbreviations

HOXB7, homeobox B7; NMuMG, normal murine mammary gland; HER2, human epidermal growth factor receptor 2; TCGA, the cancer genome atlas; TGFß, transforming growth factor beta:; EMT, epithelial to mesenchymal transition; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IRES, internal ribosome entry site; PEI, polyethylenimine; METABRIC, the molecular taxonomy of breast cancer international consortium; IVIS, *in vivo* imaging system.

Abstract:

Genes from the homeobox family are often dysregulated in cancer. Particularly, homeobox B7 (*HOXB7*) amplification and overexpression correlate with poor prognosis in various cancers, such as gastric, pancreatic, and lung cancers. Moreover, *HOXB7* is known to contribute to cancer progression by promoting epithelial to mesenchymal transition, drug resistance, and angiogenesis. In this study, we show that *HOXB7* overexpression correlates with a poor prognosis in patients with breast cancer. Coamplification of *HOXB7* with *ERBB2* enhanced the expression of *ERBB2* and the malignancy of HER2-positive breast cancer cells. Additionally, *HOXB7* overexpression in an immortalized murine mammary gland epithelial cell line, NMuMG, induced cellular transformation through an activation of the JAK-STAT signaling pathway, which resulted in lung metastasis upon orthotropic transplantation in mice. These findings reveal that *HOXB7* alone is sufficient to induce tumorigenesis and lung metastasis using *HOXB7*-expressing NMuMG cells.

Keywords: homeobox B7, oncogene, breast cancer, metastasis, ERBB2, normal murine mammary gland

1. Introduction

Oncogenic alterations involving gene amplifications, mutations, chromosomal translocation, and epigenetic dysregulation induce tumorigenesis *in vivo* [1]. Activated oncogenes also trigger typical phenotypic changes, so-called transformation, in immortalized cell lines such as NIH3T3 and normal murine mammary gland (NMuMG) cells *in vitro*. Therefore, assay systems evaluating phenotypic changes, including focus formation, soft agar colonization, and tumorigenesis, have been used for the screening of novel oncogenes in previous studies including ours [2,3].

Most oncogenes cause a dysregulation of signal transduction cascades relating to cellular proliferation, cell cycle, and survival [4]. Oncogenic signaling pathways, such as RAS-MAPK and PI3K-AKT are typically activated in various cancer cell types and hence targeted by several anti-cancer drugs. Recent studies have shown that the JAK-STAT signaling is also important for cancer initiation and progression [5]. STAT3 is activated in cancer stem cell-like populations [6] and regulates stemness via fatty metabolism in breast cancer [7]. In addition, aberrant activation of STAT3 within IL6-enriched tumor microenvironments is associated with an immunosuppression of tumor-infiltrating immune cells [8]. JAK-STAT signaling further coactivates and supports cancer growth and progression via the activation of the RAS-MAPK and PI3K-AKT pathways [9].

Homeobox (HOX) genes encode transcriptional factors controlling development and organ homeostasis. Homeobox genes of the HOX superfamily (HOXA, HOXB, HOXC and HOXD clusters) are often dysregulated in breast, prostate, colon, and lung cancers. The differential dysregulation of HOX family genes in various solid tumors provides an opportunity to better understand cancer development [10,11]. The HOXB clusters are composed of *HOXB1*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXB6*, *HOXB7*, *HOXB8*, *HOXB9*, and *HOXB13*, which are located at 17q23.32. HOXB cluster genes are co-amplified by gene amplification in several types of cancer. Notably, an overexpression of *HOXB7* contributes to cancer progression in breast, hepatocellular, oral, and gastric cancer cell lines [12-14]. *HOXB7* induces an activation of TGFß signaling, epithelial to mesenchymal transition (EMT), and migration in breast cancer cells [15,16]. In addition, the breast cancer cell line MCF7 of the luminal subtype acquires tamoxifen resistance via *HOXB7* expression [17]. Acting as a cofactor

of estrogen receptor (ER), *HOXB7* induces cancer progression through the expression of downstream genes [18]. In this study, we reveal that HOXB7 activates the JAK-STAT pathway and results in cellular transformation *in vitro*. We also found that HOXB7 is sufficient to induce tumorigenesis and metastasis in the immortalized NMuMG cell line.

2. Materials and methods

2.1. Cell culture

NMuMG and NMuMG-luc cells [19] were cultured in Dulbecco's modified Eagle's medium (DMEM; Fujifilm Wako, Osaka Japan) supplemented with 10% fetal bovine serum (FBS; NICHIREI BIOSCIENCES INC., Tokyo, Japan), 100 U/mL penicillin G (Meiji-Seika Pharma Co., Ltd., Tokyo, Japan), 100 µg/mL streptomycin (Meiji-Seika Pharma Co., Ltd.), 10 µg/mL insulin (Fujifilm Wako), and 4.5% glucose (Fujifilm Wako) at 37°C with 5% CO_2. Plat-E cells for retroviral packaging were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin G, and 100 µg/mL streptomycin at 37°C with 5% CO_2. The cells were treated with 0.1–1 µM Ruxolitinib (LC Laboratories, MA, USA).

2.2. Retroviral packaging and infection

Plat-E cells were seeded in 6-well plates and transfected with pMXs-Venus-IRES-Puromycin^R and pMXs-*HOXB7*-IRES-Puromycin^R plasmid DNAs using 6 µg polyethylenimine (PEI; Polysciences, Inc., PA, USA). The NMuMG-Venus and HOXB7-expressing NMuMG (NMuMG-HOXB7) cell lines were established by retroviral infection using the cell supernatants and 800 µg/mL polybrene (SIGMA ALDRICH Co., MO, USA), as previously described [2]. To select the *Venus* or *HOXB7* expressing cells, 1 µg/mL of puromycin (Fujifilm Wako) was added to the culture medium for 2 days.

2.3. Western blotting

The infected cell lines were lysed with SDS sampling buffer (3.5% SDS, 12% glycerol, 0.05M

Tris-HCl [pH 6.8], 0.003% bromo phenol blue, and 5% 2-mercaptoethanol) for Western blotting. Western blotting was performed as previously described [2] using the antibodies listed in the Supplementary Table S1.

2.4. Cell proliferation assay

A total of 5.0×10^5 NMuMG cells were seeded into a 12-well plate. Cells were harvested with 0.25% trypsin (Thermo Fisher Scientific, MA, USA) and counted at each time point (24, 48, and 72 h) examined [20].

2.5. Focus formation assay

To perform a focus formation assay, 7.0×10^4 parental NMuMG cells and 3.5×10^4 NMuMG-HOXB7 cells were mixed and seeded into 12-well plates. Mixed cells were cultured for over 10 days. Cells were stained with 0.01% crystal violet in 70% methanol to detect the cellular foci [2].

2.6. Tumorigenesis assay

A total of 1.0×10^6 NMuMG cells were transplanted into 6–8-weeks-old *Rag2*$^{-/-}$ female mice with 100 µL 50% Matrigel (BD Bioscience, CA, USA) as previously described [19]. All the animal experiments were approved by the Animal Committee of Waseda University (WD19-058, 2019-A068).

2.7. Orthotopic transplantation and bioluminescence imaging

A total of 1.0 × 106 NMuMG-HOXB7 cells were transplanted into the fourth fat pads of 6-week-old BALB/cSlc-nu/nu female mice as previously described [21]. After injection, the transplantation sites were palpated for every 2–3 days to calculate tumor formation time and the mice were weekly monitored for primary tumor formation and metastasis formation using an *in vivo* imaging system (IVIS Lumina Xr3; Perkin-elmer, Waltham, MA, USA). Mice were intraperitonially injected with 200 µL of D-Luciferin (15 mg/mL; Gold Biotechnology, Inc., St. Luis, MO, USA), and luminescence was taken under anesthesia using IVIS. Sixteen weeks after injection, all mice were sacrificed, and excised

lungs. Lungs were transferred to a 12-well plate with 900 μl of D-PBS (-) (Wako Pure Chemical Industries) and 100 μL of D-Luciferin (15 mg/ml), and then luminescence was imaged.

2.7. Quantitative real-time PCR

Total RNA was extracted using an ISOGEN kit (NIPPON GENE Co., LTD, Tokyo, Japan), then cDNA was synthesized using the Superscript III first-strand synthesis system (Thermo Fisher Scientific). Quantitative RT-PCR was performed using a THUNDER BIRD SYBR qPCR mix (TOYOBO, Osaka, Japan) with 18S rRNA serving as a relative control. The primers used are listed in the Supplementary Table S2.

2.8. Clinical database analysis

The genomic profiles and RNA-seq datasets of The Cancer Genome Atlas (TCGA) breast cancer (BRCA) cohorts were subjected to OncoPrint visualization and expression analyses. OncoPrint visualization of *HOXB7* and *ERBB2* was conducted using the cBioPortal [22]. The Kaplan-Meier method was then used to perform survival analyses of molecular taxonomy according to the breast cancer international consortium (METABRIC) [23], using the GSE1456 and GSE9893 datasets, as previously described [24].

2.9. Statistical analysis

Statistical analyses using the Welch's t-test, Fisher's exact test and log-rank test were performed using R 3.4.1 (https://www.r-project.org/).

3. Results

3.1. *HOXB7* expression correlates to a poor prognosis in patients with HER2-positive breast cancer

To elucidate the clinical significance of *HOXB7* in breast cancer, we first analyzed RNA-seq data of TCGA BRCA cohorts for *HOXB7* expression. To further examine the genomic profiles of *HOXB7* in TCGA BRCA cohorts, the mutational status of *ERBB2* and *HOXB7* was subjected to an OncoPrint visualization using the cBioPortal (Figure 1A). *HOXB7* was significantly co-amplified with *ERBB2* in 30 out of 48 patients with *HOXB7* amplification (Figure 1B). Gene expression analysis based on breast cancer subtype revealed a significantly higher expression of *HOXB7* in the HER2-positive subtype compared to other subtypes and normal tissue (Figure 1C). These results suggest that *HOXB7* was preferentially amplified and expressed in patients with HER2-positive breast cancer.

Next, to determine the relationship between *HOXB7* expression and progression in breast cancer, we performed a survival analysis using several public clinical datasets. In the METABRIC cohorts, a high expression of *HOXB7* was correlated with a poor prognosis in all the patients with breast cancer (Figure 1D left) including those of the HER2-high subtype (Figure 1D right). The correlation of poor prognosis with *HOXB7* expression was further confirmed by analyzing other public breast cancer cohorts comprising all subtypes (Figure 1E).

3.2. *HOXB7* induces oncogenic focus formation and activation of JAK-STAT3 signaling

To elucidate the oncogenic function of *HOXB7*, we first expressed *HOXB7* in an immortalized murine breast epithelial cell line, NMuMG, which has been used for both the *in vitro* and *in vivo* evaluation of oncogenes [2,19]. Expressing HOXB7 changed the morphology of NMuMG cells from epithelial-like to spindle-like shapes (Figure 2A). Furthermore, the NMuMG-HOXB7 cells formed foci (Figure 2B).

Next, to identify oncogenic signaling pathways downstream of *HOXB7*, we performed Western blotting using antibodies against major cancer-related signaling proteins. We found that *HOXB7* overexpression increased the protein levels of vimentin (a mesenchymal marker gene) (Figure 2C) without changing those of E-cadherin and N-cadherin, suggesting that *HOXB7* induces a partial epithelial-mesenchymal transition. In addition, *HOXB7* overexpression significantly increased Stat3 phosphorylation and the protein levels of Jak1, Jak2, Stat3 and Erbb2 (Figure 2D). Major proliferative signals, such as Erk and Akt, were unchanged by HOXB7 expression. However, Stat3

and Erbb2 were increased at the transcriptional level, while Jak2 was increased at the protein level (Figure 2E). These results indicate that *HOXB7* induces focus formation and activates JAK-STAT signal.

3.3. JAK-STAT signaling regulates *HOXB7*-mediated focus formation in NMuMG cells

The phosphorylation of Stat3 induced by *HOXB7* expression was completely inhibited in a dose-dependent manner by a JAK1/2 inhibitor, Ruxolitinib, confirming JAK1/2 involvement in Stat3 phosphorylation (Figures 3A and 3B). Lapatinib, which inhibits the activation of Erbb2, decreased p-Erk and partially reduced p-Stat3 in HOXB7-expressing cells (Figure 3C), suggesting its partial involvement in the phosphorylation of Stat3. The protein levels of Stat3, Jak2, and Erbb2 were unchanged by Ruxolitinib treatment, indicating that the transcriptional regulation of Stat3 and Erbb2 is not taking place downstream of Stat3 signaling. To elucidate the contribution of the oncogenic activity of p-Stat3, we next performed proliferation and focus formation *in vitro* assays following Ruxolitinib treatment. *HOXB7* decreased the proliferative activity of NMuMG cells, but Ruxolitinib treatment did not affect the proliferative activity of NMuMG-HOXB7 cells (Figure 3D). Nevertheless, Ruxolitinib inhibited their focus-forming activity (Figure 3E). These results suggest that activation of JAK-STAT signaling by *HOXB7* induces cellular transformation.

3.4. Overexpression of *HOXB7* induces tumorigenesis and metastasis

To assess oncogenic activities *in vivo*, we performed a subcutaneous tumorigenesis assay and a metastasis assay by orthotopic transplantation. We found that NMuMG-HOXB7 cells formed tumors when subcutaneously injected into immunodeficient mice (Figure 4A). Tumor formation was similarly observed upon orthotopic transplantation. Primary tumors of fat pads were formed in half the mice within one week and eleven out of twelve mice within ten days (Figure 4B). They continued to grow until sixteen weeks after transplantation (Figure 4C), confirming that *HOXB7* induces oncogenic activities *in vivo*. We next examined the metastatic activity of NMuMG-HOXB7 cells *in vivo* and *ex vivo*. Using *in vivo* imaging, bioluminescence from the lung was detected in one mouse out of twelve,

sixteen weeks after transplantation (Figure 4D). To detect lung metastasis with more sensitivity, we excised the lungs and performed *ex vivo* bioluminescence imaging. As a result, lung metastasis was detected in four out of twelve mice (Figure 4E). Our results indicate that *HOXB7* induces not only *in vivo* tumor formation but also lung metastasis in an orthotropic transplantation model.

4. Discussion

In this study, we demonstrated that *HOXB7* induces cellular transformation by activating the JAK-STAT pathway, which suggests its involvement in determining the poor prognosis of HER2-positive patients. Additionally, overexpression of *HOXB7* in NMuMG cells transplanted into mouse models induced the transition from tumorigenesis to metastasis.

HOX family genes regulate development and homeostasis as transcriptional factors [10,11,25]. Several HOX genes contribute to cancer malignancy [10,11]. Overexpression and knockdown experiments together support the idea that *HOXB7* is acting as an oncogene in breast, prostate, hepatocellular and gastric cancer cell lines [12-14]. HOXB7 also induces EMT and migration/invasion activity in MCF10A normal human mammary epithelial cells [15]. *In vivo*, *HOXB7* was also shown to promotes malignant progression through the activation of TGFß signaling in a MMTV-Hoxb7/Her2 transgenic mouse model [16]. In addition, *HOXB7* overexpression in MCF7 cells (luminal A subtype) induces acquired resistance to tamoxifen via the activation of receptor tyrosine kinases and ERα signaling [17,18]. Previous analysis of microarray data also showed that high levels of HOXB7 are associated with a poor prognosis in HER-2–positive, but not HER-2–negative breast cancers [26]. Consistently, our analysis with recent genomic and RNA-seq data confirmed their study and further demonstrated that *HOXB7* is significantly co-amplified with *ERBB2*. Another study showed that *HOXB7* expression induces upregulation of *ERBB2* in MCF7 cells [18], a finding which was also confirmed by our experiments using different cell lines. Taken together, these results indicate that coamplification of *HOXB7* with *ERBB2* may enhance the expression of *ERBB2* to

enhance the malignancy of HER2-positive breast cancer cells.

Using an immortalized mammary gland epithelial cell line, NMuMG, *HOXB7* was shown to induce cellular transformation *in vitro* and tumor formation *in vivo*. In this cellular context, *HOXB7* increased the mRNAs of *STAT3* and protein levels of JAK2 and STAT3 as well as phosphorylated STAT3. Intriguingly, treatment with Ruxolitinib, a JAK1/2 inhibitor, abolished cellular transformation with inhibition of Stat3 phosphorylation, suggesting involvement of the JAK-STAT pathway in this HOXB7-mediated transformation. As observed previously [27], Jak2 phosphorylation was found to be upregulated by Ruxolitinib treatment. This phenomenon was also observed following treatment with Pacritinib, another JAK2 inhibitor [28], but the molecular mechanism underlying the upregulation of phosphorylated Jak2 remains unclear. The JAK-STAT pathway is activated in several cancers and is considered to be involved in tumorigenesis [5]. Kinase inhibitors against JAK including Ruxolitinib were thus developed. Even though *HOXB7* cannot be directly targeted, the downstream JAK-STAT pathway could be targeted in cancers associated with *HOXB7* alterations [8].

A previous study revealed that *HOXB7* is not sufficient to induce tumors in *MMTV-Hoxb7* transgenic mice [26]. By contrast, we showed that *HOXB7* alone triggers tumorigenesis and lung metastasis using *HOXB7*-expressing NMuMG cells. While this discrepancy remains unclear, the immortalization status of NMuMG cells may be involved. In any case, our observation that *HOXB7* enhances lung metastasis in an orthotropic mouse model underpins the importance of *HOXB7* in lung metastasis, consistently with a previous study of M*MTV-Hoxb7/Erbb2* transgenic mice [26]. The transplantation models using NMuMG-HOXB7 cells are expected to be useful in discovering the role of HOXB7 in cancer initiation and metastasis. Furthermore, a combined therapy against HOXB7 or downstream JAK-STAT together with ERBB2 may lead to efficient therapeutic outcomes in the "*ERBB2-* and *HOXB7*-amplified" subtype of breast cancer.

Acknowledgments

We thank Dr. Yusuke Yamamoto (National Cancer Center Research Institute) and Dr. Jiro Fujimoto (Japan Biological Informatics Consortium; JBic) for their meaningful comments and discussion, Prof. K. Miyazawa (University of Yamanashi, Yamanashi, Japan) for kindly providing the NMuMG cell line, and Prof. T. Kitamura (Institute of Medical Science, The University of Tokyo, Tokyo, Japan) for kindly providing the Plat-E cell line. This study was conducted in part through the Fukushima Translational Research Project program.

Funding

This work was supported by JSPS KAKENHI [grant no. 23241064, Grant-in-Aid for Scientific Research (A) to K.S.; grant no. 18K16269, Grant-in-Aid for Early-Career Scientist to J.N.; grant no. 20J01794, Grant-in-Aid for JSPS Fellows to J.N.] and the JBic.

Authors' contributions

J.N. and K.S. conceived and designed the study. K.A, M.S., S.K., J.N., and A.M. performed the experiments. K.A., M.S. and J.N. analyzed the data. N.G., S.W. and K.S. interpreted the data. J.N. wrote the manuscript. All the authors reviewed and edited the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

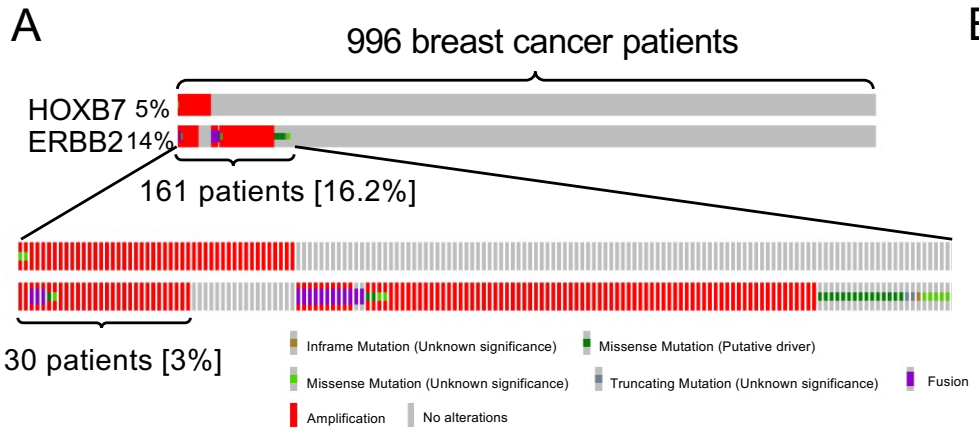
Figure 1. High expression of HOXB7 is correlated to a poor prognosis in breast cancer patients. (A) OncoPrint visualization of *ERBB2* and *HOXB7* genomic status in TCGA. (B) Altered genetic profiles of ERBB2 and HOXB7. Fisher's exact test ($p < 0.05$). (C) Comparative expression analysis of *HOXB7* in breast cancer subtypes of TCGA. Luminal A (n = 499), luminal B (n = 197), HER2-positive (n = 78), basal-like (n = 171), and normal-like (n = 36) subtypes. Welch's t-test ($p < 0.005$). (D left) Survival analysis of all METABRIC cohorts with *HOXB7* expression and the overall survival status shown in a Kaplan-Meier plot. Shading along the curves shows 95% confidence interval. Log-rank test ($p = 0.0056$) (D right) METABRIC cohorts with high HER2 expression (476 patients; Top 25% of all METABRIC cohorts) Log-rank test ($p = 0.0088$) (E) Survival analysis of other breast cancer cohorts (GSE1456; disease specific survival; GSE9893; overall survival). GSE1456, log-rank test ($p = 0.00022$); GSE9893, log-rank test ($p < 0.0001$).

Figure 2. HOXB7 expression induces transformation and activation of the JAK-STAT and ERBB2 signaling pathways. (A) Brightfield imaging of HOXB7-expressing cells. (B) Focus formation assay in HOXB7-expressing NMuMG cells. Left: crystal violet staining. Right: brightfield focus. (C) Western blotting analysis of HOXB7-expressing NMuMG cells. Venus-expressing NMuMG cells were used as a negative control. (D) Western blotting analysis of HOXB7-expressing cells. (E) qRT-PCR of *Stat3*, *Erbb2*, and *Jak2*. Welch's t-test (* $p < 0.001$, n = 3).

Figure 3. Focus formation by HOXB7 is regulated by Stat3 activation. (A) Western blotting of p-Stat3 analysis following Ruxolitinib treatment. (B) Signal analysis following Ruxolitinib treatment of HOXB7-expressing NMuMG cells. (C) Signal analysis following Lapatinib treatment of HOXB7-expressing NMuMG cells. (D) *In vitro* growth curve of HOXB7-expressing NMuMG cells treated with Ruxolitinib. (n.s.: Not significant, n = 3) (E) Focus formation assay of HOXB7-expressing NMuMG cells treated with Ruxolitinib. Foci were stained with crystal violet.

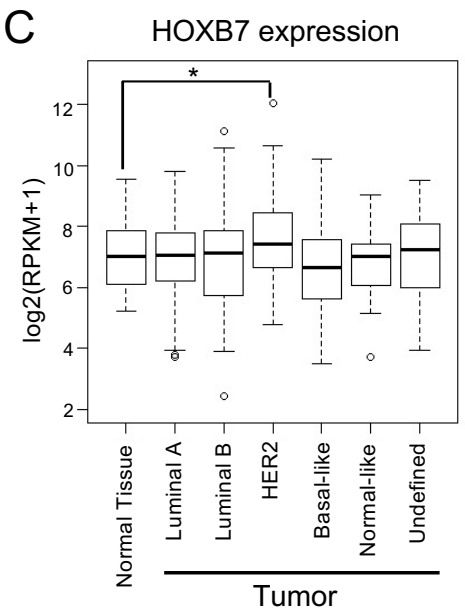
Figure 4. Overexpression of HOXB7 induces tumorigenesis and lung metastasis. (A) Tumorigenesis assay of HOXB7-expressing NMuMG cells. Left: Tumor growth curve with volume. HOXB7-expressing NMuMG cells formed tumors following subcutaneous transplantation in *Rag2*$^{-/-}$ mice (n = 10). Right: Image of HOXB7-positve tumor. (B) Primary tumor formation curve for orthotopic transplantation. HOXB7-expressiong NMuMG cells were transplanted to BALB/cSlc-nu/nu mice (n = 12). (C) Representative *in vivo* luminescence images of primary tumor in orthotopic transplantation. (D) Representative *in vivo* luminescence image of lung metastasis in orthotopic transplantation. (E) *Ex vivo* luminescence images of lungs excised from orthotopically transplanted mice. Left: Lungs with no metastasis. Right: Lungs with metastasis.

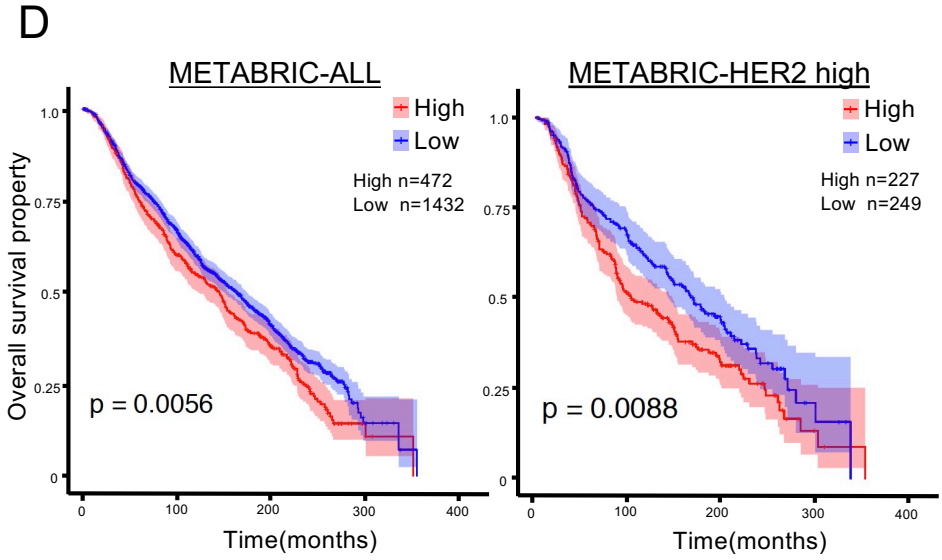
Figure 1



B

		ERBB2 amplification		
		Yes	No	SUM
HOXB7 amplification	Yes	30	18	48
	No	70	878	948
	SUM	100	896	996





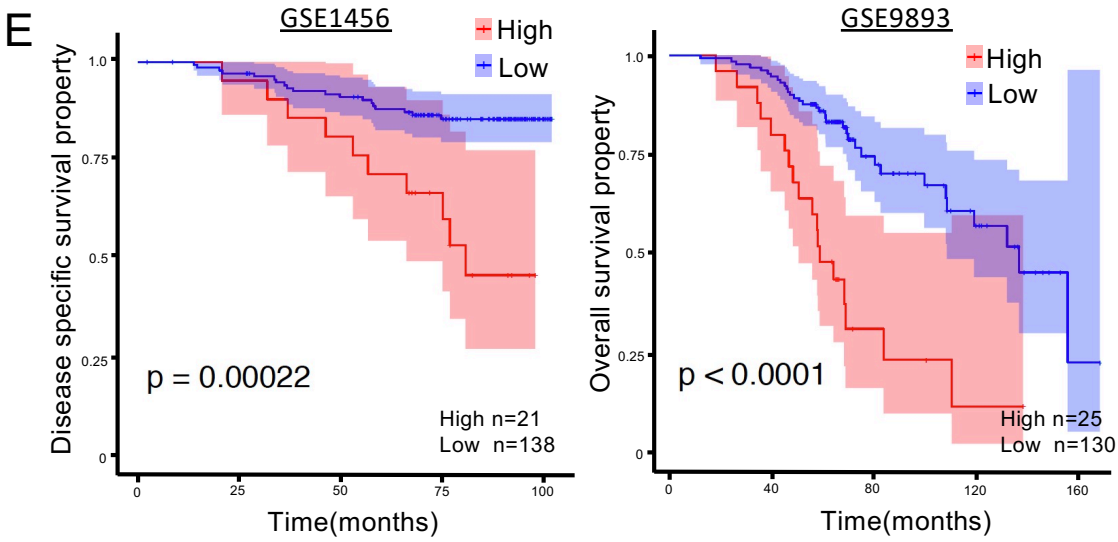


Figure 2

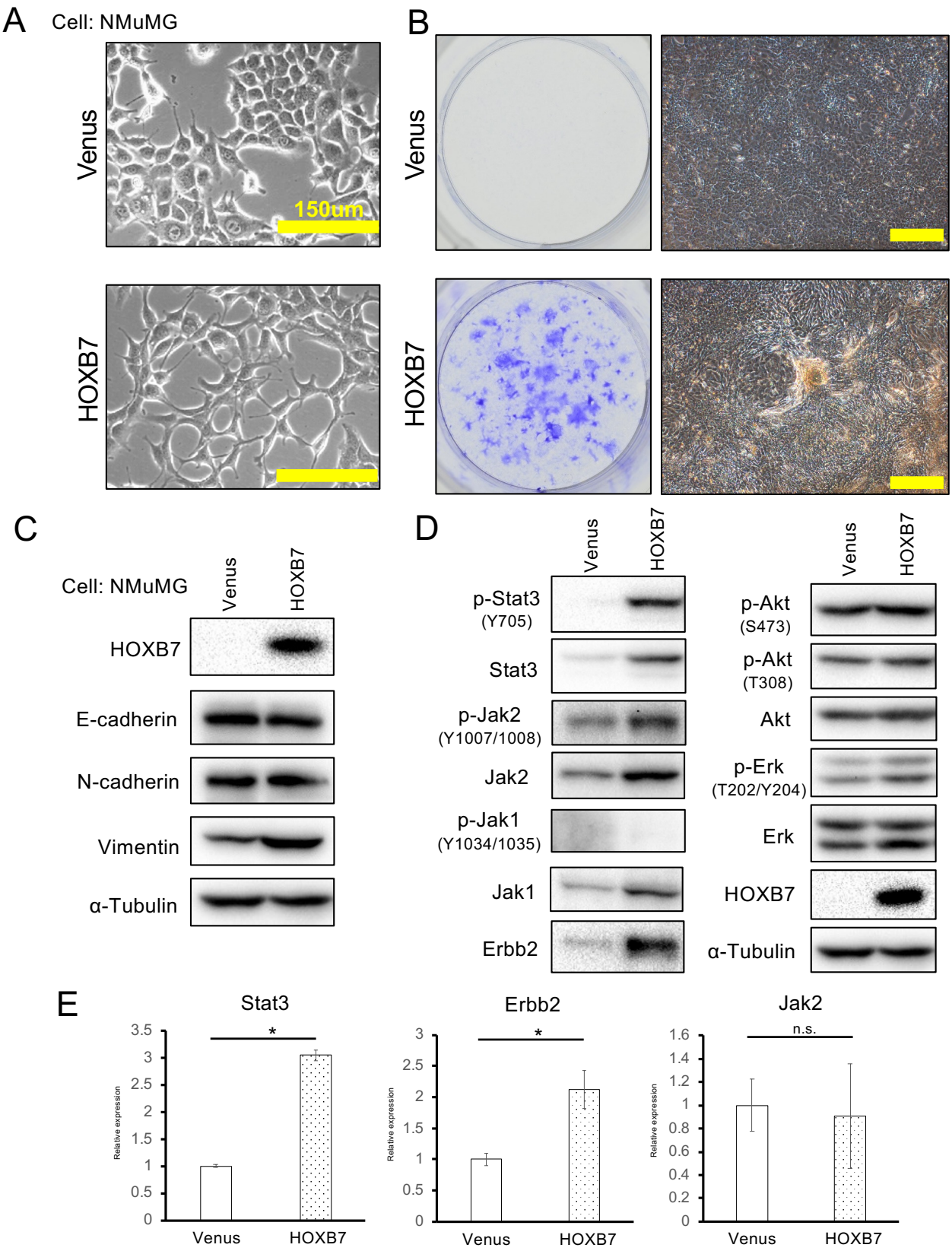

A
Cell: NMuMG
Venus
HOXB7
150um
B
Venus
HOXB7
C
Cell: NMuMG
Venus
HOXB7
HOXB7
E-cadherin
N-cadherin
Vimentin
α-Tubulin
D
Venus
HOXB7
p-Stat3 (Y705)
Stat3
p-Jak2 (Y1007/1008)
Jak2
p-Jak1 (Y1034/1035)
Jak1
Erbb2
p-Akt (S473)
p-Akt (T308)
Akt
p-Erk (T202/Y204)
Erk
HOXB7
α-Tubulin
E
Stat3
Erbb2
Jak2
Relative expression
*
n.s.
Venus
HOXB7

Figure 3

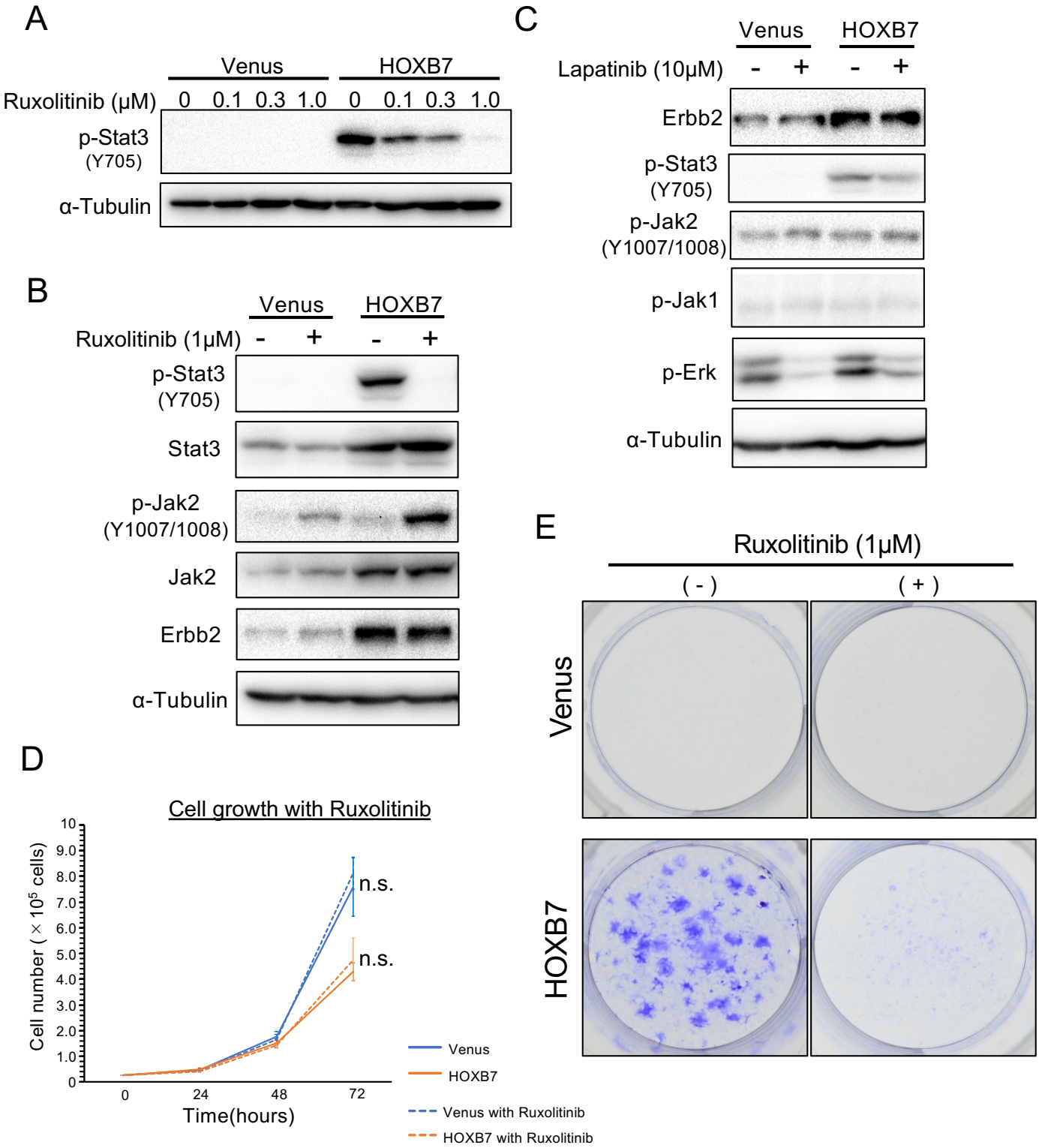
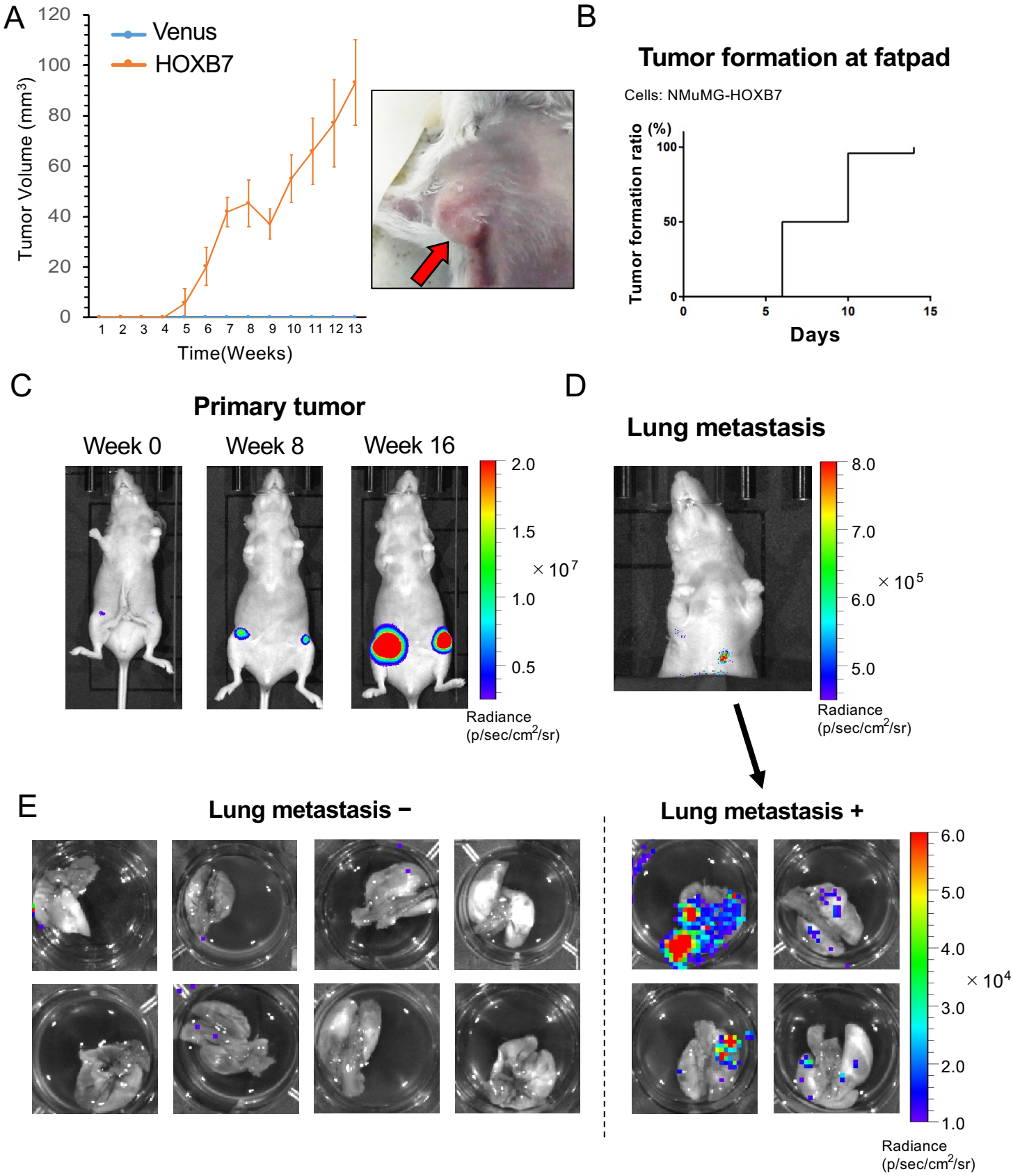

A
Venus
HOXB7
Ruxolitinib (μM)
0 0.1 0.3 1.0 0 0.1 0.3 1.0
p-Stat3 (Y705)
α-Tubulin
B
Venus
HOXB7
Ruxolitinib (1μM)
- + - +
p-Stat3 (Y705)
Stat3
p-Jak2 (Y1007/1008)
Jak2
Erbb2
α-Tubulin
C
Venus
HOXB7
Lapatinib (10μM)
- + - +
Erbb2
p-Stat3 (Y705)
p-Jak2 (Y1007/1008)
p-Jak1
p-Erk
α-Tubulin
D
Cell growth with Ruxolitinib
Cell number (× 10^5 cells)
10
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0
24
48
72
Time(hours)
n.s.
n.s.
Venus
HOXB7
Venus with Ruxolitinib
HOXB7 with Ruxolitinib
E
Ruxolitinib (1μM)
(-)
(+)
Venus
HOXB7

Figure 4


A
Venus
HOXB7
Tumor Volume (mm3)
Time(Weeks)
B
Tumor formation at fatpad
Cells: NMuMG-HOXB7
Tumor formation ratio (%)
Days
C
Primary tumor
Week 0
Week 8
Week 16
Radiance (p/sec/cm2/sr)
D
Lung metastasis
Radiance (p/sec/cm2/sr)
E
Lung metastasis −
Lung metastasis +
Radiance (p/sec/cm2/sr)