

Everolimus prolonged survival in transgenic mice with EGFR-driven lung tumors

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Abstract

Everolimus is an orally administered mTOR inhibitor. The effect, and mechanism of action, of everolimus on lung cancers with an epidermal growth factor receptor (EGFR) mutation remain unclear. Four gefitinib-sensitive and -resistant cell lines were used in the present work. Growth inhibition was determined using the MTT assay. Transgenic mice carrying the EGFR L858R mutation were treated with everolimus (10 mg/kg/day), or vehicle alone, from 5 to 20 weeks of age, and were then sacrificed. To evaluate the efficacy of everolimus in prolonging survival, everolimus (10 mg/kg/day) or vehicle was administered from 5 weeks of age. The four cell lines were similarly sensitive to everolimus. Expression of phosphorylated (p) mTOR and pS6 were suppressed upon treatment with everolimus *in vitro*, whereas the pAKT level increased. The numbers of lung tumors with a long axis exceeding 1 mm in the everolimus-treated and control groups were 1.9 ± 0.9 and 9.4 ± 3.2 (t-test, $p < 0.001$), respectively. pS6 was suppressed during everolimus treatment. Although apoptosis and autophagy were not induced in everolimus-treated EGFR transgenic mice, angiogenesis was suppressed. The median survival time in the everolimus-treated group (58.0 weeks) was significantly longer than that in the control group (31.2 weeks) (logrank test, $p < 0.001$). These findings suggest that everolimus had an indirect effect on tumor formation by inhibiting angiogenesis

and might be effective to treat lung tumors induced by an activating EGFR gene mutation.

Highlights

- Everolimus was similarly efficacious, *in vitro*, on cells harboring various EGFR mutations.
- Everolimus suppressed lung tumors in transgenic mice expressing the L858R mutation of EGFR and prolonged the overall survival of these mice.
- Everolimus might be effective for EGFR-mutated lung cancer by the inhibiting tumor angiogenesis.

Abbreviations: EGFR, epidermal growth factor receptor; eIF4E, eukaryotic translation initiation factor complex 4E; 4E-BP, 4E-binding protein; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NSCLC, non-small cell lung cancer; pS6, phospho-S6 ribosomal protein; TKI, tyrosine kinase inhibitor.

Keywords: Non-small cell lung cancer; adenocarcinoma; everolimus; mTOR; EGFR

1. Introduction

Somatic mutants of the epidermal growth factor receptor (EGFR), HER2, KRAS, BRAF, PIK3CA, and EML4-ALK of non-small cell lung cancers (NSCLCs) are viewed as targets of anti-cancer agents [1]. Of tumors in 52 East Asian never-smokers with lung adenocarcinomas, 78.8% harbored EGFR mutations, 5.8% (three) EML4-ALK fusions, 3.8% (two) HER2 mutations, and 1.9% (one) a KRAS mutation. Only 9.6% (five of 52) tumors did not harbor any of these known oncogenic driver mutations [2]. Never-smokers with NSCLC could be divided into four distinct genotypic groups based on genetic profiling of three major oncogenes (EGFR, EML4-ALK, and KRAS), yielding unique and non-overlapping subsets of patients with lung cancer who exhibited different therapeutic responses and survival outcomes [3]. The “Biomarker-integrated approaches of targeted therapy for lung cancer elimination” (BATTLE) program of personalized medicine has been recently developed [4]. Adaptive randomization was successfully used to assign NSCLC patients to the treatment offering the greatest potential benefit, based on the markers of prospectively biopsied tumors. Moreover, EGFR mutations and EML4-ALK gene rearrangements in NSCLC have been established as real molecular targets from the clinical studies [5]. Thus, it is important to treat patients with reference to their genetic profiles to determine whether patients can

benefit from specifically targeted therapies.

EGFR mutations are more frequently observed in females, non-smokers, and adenocarcinoma patients, especially in Asian populations, wherein the mutation rate is 30–50% [6]. The EGFR tyrosine kinase inhibitors (TKIs), erlotinib and gefitinib, improved progression-free survival in patients with NSCLC harboring EGFR mutations [7-11]. The majority of EGFR mutant lung cancers initially sensitive to EGFR-TKI become resistant to these agents within 1 year. Some possible mechanisms for acquisition of resistance have been identified, the most common being development of an EGFR T790M “gatekeeper” mutation in about 50% of cases [12][13]. Although clinical work seeking to overcome resistance acquired via T790M has been conducted, no generally accepted therapy has been established [14].

The mammalian target of rapamycin (mTOR) is located downstream of EGFR and plays a major role in regulation of protein translation, cell growth, and metabolism [15-18]. Alterations in the mTOR signaling pathway are common in cancer patients and mTOR is being actively explored as a therapeutic target [17]. Inhibition of mTOR by targeting agents causes G1 cell-cycle arrest mediated by inactivation of phospho-S6 ribosomal protein (pS6) and hypophosphorylation of 4E-BP1 [16]. Everolimus is an orally administered rapamycin analog showing anticancer effects on renal cell

carcinoma and pancreatic neuroendocrine tumors [17]. Furthermore, in preclinical studies, everolimus showed anticancer effects in various cancer cell lines and xenograft models, including lung cancer [16]. However, the effect of mTOR inhibitors on NSCLC harboring EGFR mutations remains unclear. In our present study, we investigated the preclinical efficacy of everolimus in NSCLC harboring EGFR mutations. Our findings suggest that everolimus might be effective for NSCLC by the inhibiting tumor angiogenesis.

2. Materials and Methods

2.1. Cell lines and mouse model

PC-9 is a lung adenocarcinoma cell line that has an in-frame deletion mutation at exon 19 of EGFR and amplification of the EGFR gene. We used RPC-9 cells with the T790M mutation of EGFR that were 400-fold more resistant to gefitinib than parental PC-9 cells [19]. The RPC-9 cell line was established in our laboratory by continuously exposing the PC-9 cell line to gefitinib. H3255 is a lung adenocarcinoma cell line that has a L858R point mutation at exon 21 of EGFR and amplification of the EGFR gene. A549 cells harbor wild-type EGFR. The cell lines were cultured in RPMI-1640 with 10% (v/v) fetal bovine serum and 1% (w/v) penicillin/streptomycin in a tissue culture

incubator at 37°C under 5% (v/v) CO₂.

We generated transgenic mice expressing the L858R mutation of EGFR driven by the SP-C promoter [20]. In the absence of treatment, these transgenic mice developed atypical adenomatous hyperplasia at 4 weeks of age and adenocarcinoma at 6 to 7 weeks of age, and died from tumor progression at 25 to 40 weeks. The transgenic mice were randomly assigned to two groups that received either vehicle alone or everolimus (10 mg/kg/day) from 5 to 20 weeks of age. Vehicle and everolimus were administered once a day, five times a week, by gavage. Body weights were determined weekly. All mice were killed at 20 weeks of age and the number of superficial left lung tumors per mouse (with long axes exceeding 1 mm) was counted. The right lungs were used for protein extracts. To evaluate the efficacy of everolimus in terms of survival, mice were treated with oral everolimus (10 mg/kg/day) or vehicle alone from 5 weeks of age until death. All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research.

2.2 Sensitivity test

Everolimus was the kind gift of Novartis. Drug sensitivity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [21]. The

cells were added to wells of 96-well plates at a density of 2,000 cells per well and exposed continuously to the drug for 96 h. The reason for which MTT assay was done with 96 h of drug incubation was that our previous study showed the cell viability assay with 96 h drug exposure could replace standard human clonogenic assay in evaluating the effects of anticancer agents [22]. The 50% inhibitory concentration (IC₅₀) of everolimus was determined by plotting the logarithm of drug concentration versus the proportion of survivors. Each assay was performed in triplicate or quadruplicate.

2.3. Immunoblotting

Protein extracts were prepared from crushed tissue samples that had been suspended in lysis buffer (1% [v/v] Triton X-100, 0.1% [w/v] SDS, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerol phosphate, 10 mM NaF, and 1 mM Na-orthovanadate) containing protease inhibitors (Roche Diagnostics, Basel, Switzerland) and centrifuged at 15,000 rpm for 20 min at 4°C. After quantification using a Bio-Rad (Bio-Rad, Hercules, CA) protein assay, ~50 μg of protein were subjected to 5–15% SDS-PAGE (Bio-Rad) and then transferred to nitrocellulose membranes. Specific proteins were detected by the enhanced chemiluminescence assay (GE Healthcare, Buckinghamshire, UK) using antibodies (from Cell Signaling unless otherwise indicated) against EGFR (1:1,000 dilution), mTOR (1:1,000 dilution), AKT

(1:1,000 dilution), MAPK (1:1,000 dilution), PI3K (1:5,000 dilution; Upstate), S6, 4E-binding protein (4E-BP) (1:1,000 dilution), pEGFR (1:1,000 dilution), pmTOR (1:1,000 dilution), pAKT (1:1,000 dilution), pMAPK (1:1,000 dilution), pS6 (1:1,000 dilution), p4E-BP (1:1,000 dilution), β -actin (1:1,000 dilution), Beclin1 (1:1,000 dilution), ATG5 (1:1,000 dilution), ATG7 (1:1,000 dilution), LC3B (1:1,000 dilution), PARP (1:1,000 dilution), and cleaved PARP (1:1,000 dilution). The secondary antibodies used were anti-rabbit and anti-mouse IgG (horseradish peroxidase-linked, species-specific whole antibodies; GE Healthcare), both of which were used at a 1:5,000 dilution.

2.4. Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks of samples were cut to a thickness of 5 μ m, placed on glass slides, and deparaffinized in xylene and a graded series of alcohol baths for 10 minutes. The sections were incubated in 10 mmol/L sodium citrate buffer, pH 6.0, for 10 minutes in a 95°C water bath. Endogenous peroxidase was then blocked with 0.3% (v/v) hydrogen peroxide in methanol. The slides were rinsed with TBS containing 0.1% (v/v) Tween 20 and blocked with normal goat serum for 60 minutes. The sections were incubated with a 1:200 dilution of anti-CD31 antibody (DIA310; Dianova) overnight at 4°C. The sections were next incubated with biotinylated

anti-rabbit antibodies and an avidin-biotinylated horseradish peroxidase conjugate for 10 minutes (LSAB 2 Kit, Dako Cytomation) and then reacted with 3,3'-diaminobenzidine. The three areas containing the highest numbers of stained cells within a section were selected for histological quantitation by microscopy at 400-fold magnification.

2.5. Statistical analysis

Statistical analysis was performed using the SPSS Base SystemTM and Advanced StatisticsTM programs (SPSS, Chicago, IL). Group differences were compared using Student's t-test. Survival time was defined as the period from initiation of treatment to death. Overall survival curves were calculated using the Kaplan–Meier method. P-values ≤ 0.05 were considered to be statistically significant.

3. Results

3.1. Everolimus was effective regardless EGFR mutation status.

Sensitivity to everolimus of lung cancer cell lines was examined at first. Everolimus exhibited similar efficacy on A549 cells (EGFR wild-type), H3255 cells (with the L858R mutation), PC-9 cells (with an exon 19 deletion mutation) and RPC-9 cells (with both the exon 19 deletion mutation and the T790M mutation). The curves of

concentration versus surviving fraction were similar and all IC₅₀s were around 0.01 μM (Figure 1A, 1B).

3.2. Everolimus suppressed pmTOR and pS6.

Next, effect of everolimus on EGFR-related signals was examined using immunoblotting. pmTOR and pS6 in H3255 cells were suppressed by treatment with everolimus, whereas pAKT increased in level, presumably due to negative feedback from mTOR inhibition (Figure 1C). Similar results were obtained in PC-9 and RPC-9 cells (Supplemental figure 1). However, in Figure 1C, both pS6 and mTOR levels were increased with increasing concentrations of everolimus. Thus, high dose of everolimus might increase pS6 and pmTOR as a result of negative feedback from AKT or mTORC2 [23, 24]. Furthermore, everolimus had no effect on p4E-BP (Figure 1C, Supplemental figure 1). Although rapamycin decreased p4E-BP levels in NSCLC lines [25], everolimus had a variety of effects (increase, decrease or no change) on p4E-BP, dependent on treatment dose and duration [18]. A matter of degree of AKT activation by inhibiting mTOR might cause such results [24, 25].

3.3. The lung tumors in EGFR transgenic mice responded to everolimus.

Firstly, proteins downstream of EGFR were evaluated in transgenic mice. Figure 2A showed that pmTOR, pS6 and p4E-BP were overexpressed. Thus, the mTOR

might be a therapeutic target in EGFR transgenic mouse models although mTOR is not the only protein downstream EGFR to be activated (Figure 1C, 2A).

Secondary, antitumor effect of everolimus on EGFR transgenic mice which received either everolimus (10 mg/kg/day) or vehicle from 5 to 20 weeks of age (15 weeks-treatment) was evaluated. The mean numbers (\pm SD) of superficial lung tumors (of long axis exceeding 1 mm) in the everolimus-treated and control group were 1.9 ± 0.9 (n = 14) and 9.4 ± 3.2 (n = 13) (Student's t-test, $p < 0.001$), respectively (Figure 2B). Hematoxylin-eosin (H.E.) staining revealed that everolimus prevented progression of lung tumors (Figure 3A). pS6 was suppressed during everolimus treatment, although the pmTOR and p4E-BP levels were similar (Figure 3B). When the mice having more advanced lung tumors at age of 15 weeks were treated with everolimus (10 mg/kg/day) or vehicle for one week, pS6 and pmTOR expressions were decreased and p4E-BP expression was similar in the everolimus-treated mice compared with the vehicle-treated mice (Supplemental figure 2). In this situation, the tumors did not respond to one week-treatment with everolimus (data not shown). These results suggest that treatment duration and timing of administration of everolimus may affect the proteins downstream of EGFR.

Thirdly, we treated the transgenic mice with everolimus (10 mg/kg/day) or

vehicle for 5 weeks. They had early (at age of 5 weeks) or advanced (at age of 12 weeks) lung tumors and received either drug from 5 to 10 weeks of age (n =4 for vehicle and n = 7 for everolimus) or from 12 to 17 weeks (n = 3 for vehicle and n = 4 for everolimus), respectively. The lung tumors in the sacrificed mice at 10 weeks of age and at 17 weeks of age responded to everolimus (Supplemental figures 3 and 4, respectively).

3.4. Everolimus prolonged survival in EGFR transgenic mice.

Subsequently, efficacy of everolimus on survival of EGFR transgenic mice was evaluated. The median survival time of the everolimus-treated group (58.0 weeks) was significantly longer than that of the vehicle group (31.2 weeks) (logrank test, $p < 0.001$) (Figure 4A). The body weights of the everolimus-treated and vehicle groups did not significantly differ (Figure 4B). No apparent adverse event was noted in everolimus-treated mice.

3.5. Everolimus inhibited angiogenesis.

Finally, mechanisms of the anti-tumor effect of everolimus on EGFR-driven tumors were examined. Treatment with mTOR inhibitors was reported to induce autophagy and apoptosis [26-30]. The expression levels of Beclin 1, ATG5, ATG7, LC3B, and PARP in everolimus-treated H3255 cells are shown in Figure 5A. Cleaved

PARP was noted, and a cell transition from LC3B-I to LC3B-II observed. Thus, apoptosis and autophagy seemed to occur *in vitro*. Next, proteins expressed in the lungs of transgenic mice are shown in Figure 5B. Unexpectedly, neither LC3B-II status nor the level of cleaved PARP increased in everolimus-treated tumors compared with vehicle-treated tumors, although the experiment was performed several times. As the effects of everolimus in transgenic mice did not seem to be mainly attributable to apoptosis or autophagy, we next evaluated angiogenesis in these mice.

Immunohistochemical staining of CD31 revealed that 15 weeks-treatment of everolimus inhibited angiogenesis in lung tumors of transgenic mice (Figure 6A). The mean numbers (\pm SE) of CD31- positive cells in the everolimus-treated and control groups (3 fields/lung sections of 4 mice, each) were 23.1 ± 6.9 and 72.2 ± 4.2 , respectively (Student's t-test, $p < 0.05$) (Figure 6B). We performed another experiment, in which the mice were treated with vehicle alone ($n = 3$) or everolimus (10 mg/kg/day, $n = 3$) from 5 to 10 weeks of age and were sacrificed at 10 weeks of age. The mean numbers (\pm SE) of CD31- positive cells in the everolimus-treated and control groups were 34.8 ± 2.1 and 70.3 ± 2.0 , respectively (Student's t-test, $p < 0.05$) (supplemental figure 5). Two different treatment durations (15 and 5 weeks) suppressed the angiogenesis.

4. Discussion

We found that everolimus was similarly efficacious, *in vitro*, on cells harboring various EGFR mutations. The EGFR-tyrosine kinase inhibitor resistance-related T790M secondary mutation seemed to be neutralized by everolimus (Figure 1B). In addition, everolimus suppressed lung tumors in transgenic mice expressing the L858R mutation of EGFR and prolonged the overall survival of these mice.

Signaling pathways of AKT/mTOR that include S6, 4E-BP, or the eukaryotic translation initiation factor complex 4E (eIF4E), have been reported to be associated with prognosis of NSCLC. Anagnostou et al. found that a high mTOR protein level were an independent favorable prognostic factor for surgically resected adenocarcinoma of the lung [31]. Yoshizawa et al. found that expression of pEIF4E, in addition to pAKT, predicted poor prognosis of surgically resected NSCLC but pmTOR expression was not a prognostic factor [32]. Dhillon et al. reported that high-level mTOR expression significantly predicted poor survival in resected stage IA-IIIB NSCLC patients [33]. Liu et al. found that the levels of mTOR, pmTOR, pAKT, and pS6 in surgically resected primary NSCLC were higher than in the wild-type lung [34]. In this study, overall survival was significantly shorter in cases positive for pmTOR, pPDK1, and pS6. Thus,

although AKT/mTOR-related proteins are often overexpressed in NSCLC, the prognostic implications remain unclear. In our EGFR transgenic mice, mTOR and 4E-BP were activated (Figure 2A). Everolimus suppressed pmTOR and pS6 but not p4E-BP, and increased pAKT (via negative feedback) *in vitro* (Figure 1C). Also, everolimus suppressed pS6 but not pmTOR and p4E-BP *in vivo* (Figure 3B). Conceivably, everolimus may act on different signaling pathways *in vitro* and *in vivo*. Our results using transgenic mice suggest that marked suppression of pS6 by everolimus might be important in this respect.

Apoptosis and autophagy were enhanced in response to exposure to mTOR inhibitors [26-30]. Tumor inhibition mediated by mTOR inhibitors seemed to involve antiangiogenic activity, correlating with impaired vascular endothelial growth factor (VEGF) production and blockage of VEGF-induced vascular endothelial cell stimulation by enhancement of hypoxia inducible factor-1 (HIF-1) degradation [17, 35]. Although apoptosis and autophagy were not induced in everolimus-treated EGFR transgenic mice (Figure 5B), angiogenesis was suppressed (Figure 6). Indeed, even EGFR-TKIs induced both apoptosis and autophagy in EGFR-mutated NSCLC cells *in vitro* [36, 37]. However, whether autophagy causes cancer cell death or confers a survival advantage on tumor cells under stressful conditions remains unclear [37, 38].

Everolimus may control autophagy in cancer cells although we do not know why the *in vitro* and *in vivo* data differ. Recently, two mTOR inhibitors (temsirolimus and everolimus) were shown to suppress the growth of EGFR mutant lung cancer cells *in vitro* and *in vivo* [39]. Also, temsirolimus markedly inhibited cell angiogenesis in both PC-9- and EGFR-TKI-resistant (PC-9/HGF) xenograft tumors. We found that everolimus exhibited similar efficacy when added to cultures of four EGFR-TKI-sensitive and -insensitive cells *in vitro*, and everolimus suppressed tumor growth and prolonged survival in EGFR-mutated transgenic mice. Thus, our present data support their findings. Although some reports have shown that combination of an mTOR inhibitor with EGFR-TKI was an effective therapy [40-42], the possible value of everolimus monotherapy of EGFR-mutated lung cancer cells has been shown only by Ishikawa et al. [39] and us. A strength of our study is that we first showed that lung tumors in EGFR-transgenic mice were sensitive to everolimus, and the drug prolonged survival. However, it is very unlikely that EGFR mutant lung cancer will ever be treated without the presence of an EGFR-TKI [14]. Naturally, gefitinib was effective for the EGFR transgenic mice [20]. The median survival in the mice (n = 13) treated with gefitinib was more than 100 weeks (Supplemental figure 6). EGFR mutant lung cancer patients who were refractory to EGFR-TKIs might have benefits from the treatment

with everolimus. Thus, a weakness of our study is that we could not discover whether EGFR-TKI-resistant transgenic mice with a T790M secondary mutation were also sensitive to everolimus alone because we did not have such mice.

In a phase II study [43], advanced NSCLC patients, who had undergone two or fewer prior chemotherapy regimens, one platinum-based (stratum 1); or both chemotherapy and EGFR-TKI treatment (stratum 2); received everolimus (10 mg/day). Eighty-five patients were enrolled, 42 in stratum 1 and 43 in stratum 2. The overall response rate was 4.7% (7.1% in stratum 1; 2.3% in stratum 2) and the overall disease control rate 47.1%. Only 11 cases were evaluated for EGFR mutations and none was detected. Although everolimus showed modest clinical activity when given to heavily pretreated patients with advanced-stage NSCLC, the clinical activity of everolimus in NSCLC patients harboring EGFR mutations should be further evaluated. In conclusion, our present study suggested that everolimus might be effective to treat EGFR-mutated lung cancer and had an indirect effect on tumor formation by inhibiting angiogenesis.

Figure legends

Figure 1

- A) B) Sensitivity of lung cancer cell lines to everolimus. Everolimus was similarly efficacious on H3255 cells (with the L858R mutation), PC-9 cells (with an exon 19 deletion mutation), RPC-9 cells (with the exon 19 deletion mutation and the T790M mutation), and A549 cells (EGFR wild-type). Bars indicate SD.
- C) Western blotting for EGFR, AKT, MAPK, PI3K, mTOR, S6, 4E-BP, and β -actin in H3255 cells. The cells were treated with various concentrations of everolimus for 6 h. p mTOR and pS6 were suppressed by treatment with everolimus, whereas the pAKT level was increased, presumably due to negative feedback from mTOR inhibition.

Figure 2

- A) Western blotting for EGFR, mTOR, S6, 4E-BP, and β -actin in the lungs of 20 week-old EGFR transgenic and wild-type mice. Proteins downstream of EGFR, including mTOR, were activated.
- B) The numbers of superficial lung tumors (long axis exceeding 1 mm) in the everolimus-treated and control groups were 1.9 ± 0.9 and 9.4 ± 3.2 (Student's t-test,

p<0.001), respectively. Bars indicate SD.

Figure 3

A) Hematoxylin-eosin (H.E.) staining revealed that everolimus suppressed lung tumors in EGFR transgenic mice.

B) Western blotting for EGFR, AKT, MAPK, PI3K, mTOR, S6, 4E-BP, and β -actin in the lungs of transgenic mice. pS6 was suppressed during everolimus treatment.

Figure 4

A) The median survival time in the everolimus-treated group was significantly longer than that in the vehicle group (log-rank test, p<0.001).

B) There was no significant difference in the body weights of the everolimus-treated and vehicle-treated group. Bars indicate SE.

Figure 5

Expression of proteins associated with apoptosis and autophagy.

A) Western blotting for Beclin 1, ATG5, ATG7, LC3B, and PARP in everolimus-treated H3255 cells. Cleaved PARP, and the transition from LC3B-I to LC3B-II, were observed.

B) Western blotting for Beclin 1, ATG5, ATG7, LC3B, and PARP in transgenic mice.

Neither the LC3B-II state, nor cleaved PARP, increased in everolimus-treated compared with vehicle-treated tumors.

Figure 6

Evaluation of angiogenesis in transgenic mice

A) Immunohistochemical staining of CD31 revealed that everolimus inhibited angiogenesis in lung tumors of EGFR transgenic mice.

B) The numbers of CD31-positive cells in the everolimus-treated and vehicle-treated group were 23.1 ± 6.9 and 72.2 ± 4.2 , respectively (Student's t-test, $p < 0.05$). Bars indicate SE.

Supplemental Figure legends

1. Western blotting for EGFR, AKT, MAPK, PI3K, mTOR, S6, 4E-BP, and β -actin in PC-9 cells (with an exon 19 deletion mutation) and RPC-9 cells (with the exon 19 deletion mutation and the T790M mutation). The cells were treated with 2 μ M of everolimus for 6 h. pmTOR and pS6 were suppressed by treatment with everolimus.

2. The transgenic mice having lung tumors at age of 15 weeks were treated with everolimus (10 mg/kg/day) or vehicle for one week. pS6 and pmTOR expressions were decreased and p4E-BP expression was similar in the everolimus-treated mice compared with the vehicle-treated mice.

3. The transgenic mice were treated with vehicle alone (n = 4) or everolimus (10 mg/kg/day, n = 7) from 5 to 10 weeks of age. All the mice were sacrificed at 10 weeks of age. The tumors in mice treated with everolimus had anti-tumor effect.

4. The transgenic mice were treated with vehicle alone (n = 3) or everolimus (10 mg/kg/day, n = 4) from 12 to 17 weeks of age. All the mice were sacrificed at 17 weeks of age. The tumors in mice treated with everolimus had anti-tumor effect.

5. Evaluation of angiogenesis in transgenic mice with vehicle alone (n =3) or everolimus (10 mg/kg/day, n = 3) from 5 to 10 weeks of age.

A) Immunohistochemical staining of CD31 revealed that everolimus inhibited angiogenesis in lung tumors of EGFR transgenic mice (X 400).

B) The numbers of CD31-positive cells in the everolimus-treated and vehicle-treated group (3 fields/lung sections of 3 mice, each) were 34.8 ± 2.1 and 70.3 ± 2.0 , respectively (Student's t-test, $p < 0.05$). Bars indicate SE.

6. The survival curve of the EGFR transgenic mice treated with gefitinib.

The median survival in the mice (n = 13) treated with gefitinib was more than 100 weeks.

Conflict of interest

N. Takigawa and K. Kiura have honoraria from speakers' bureau from AstraZeneca.

Other authors have no conflict of interest.

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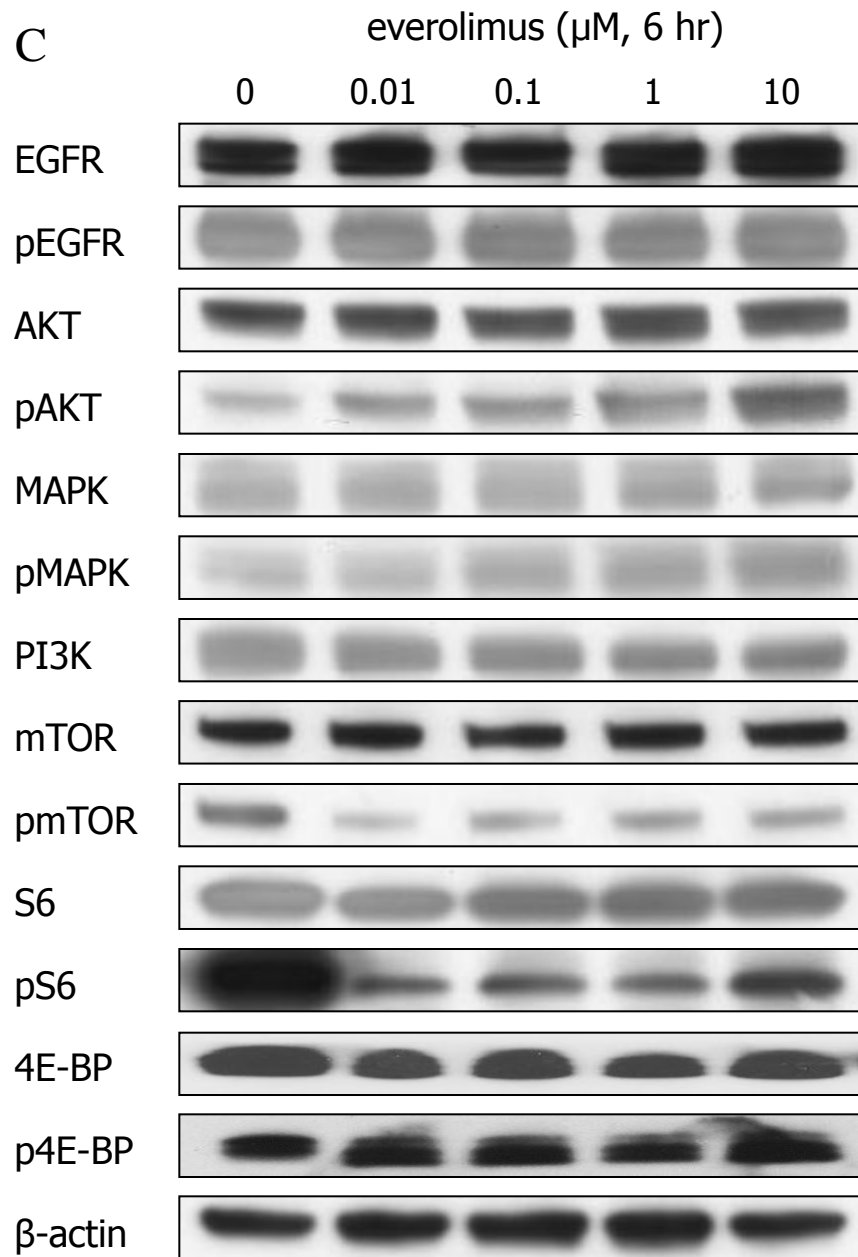
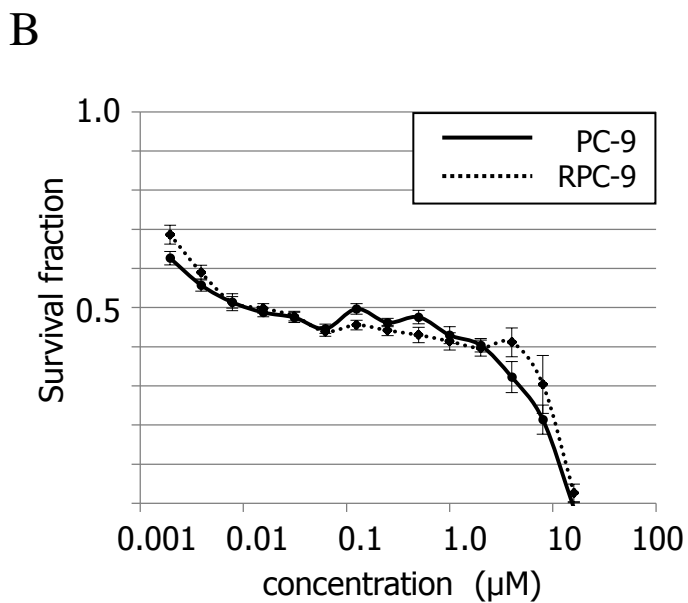
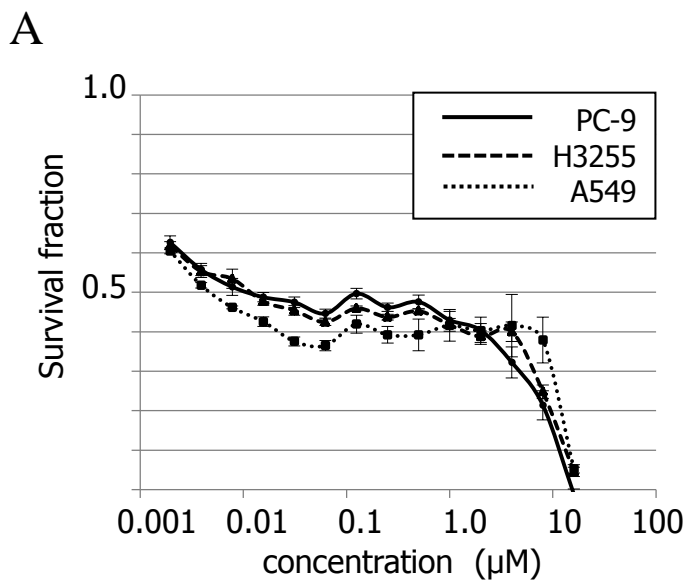


Figure 1

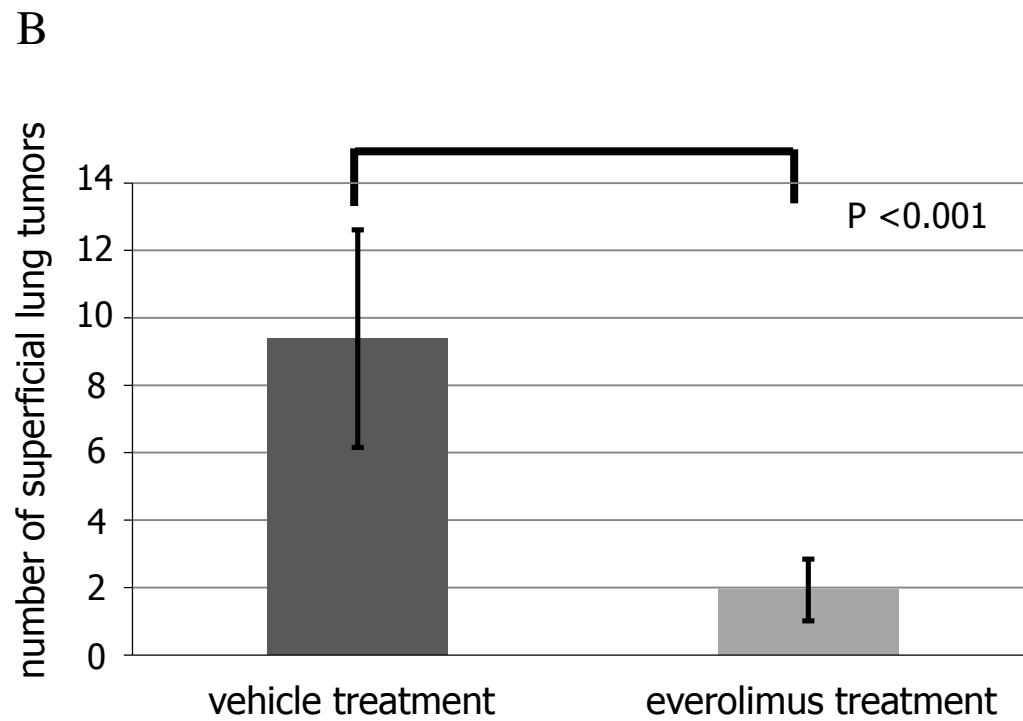
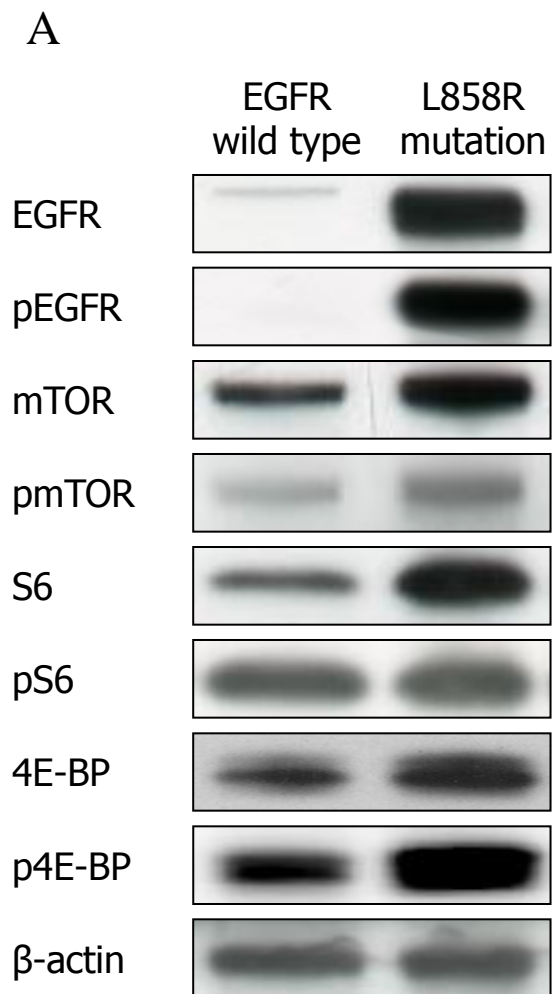


Figure 2

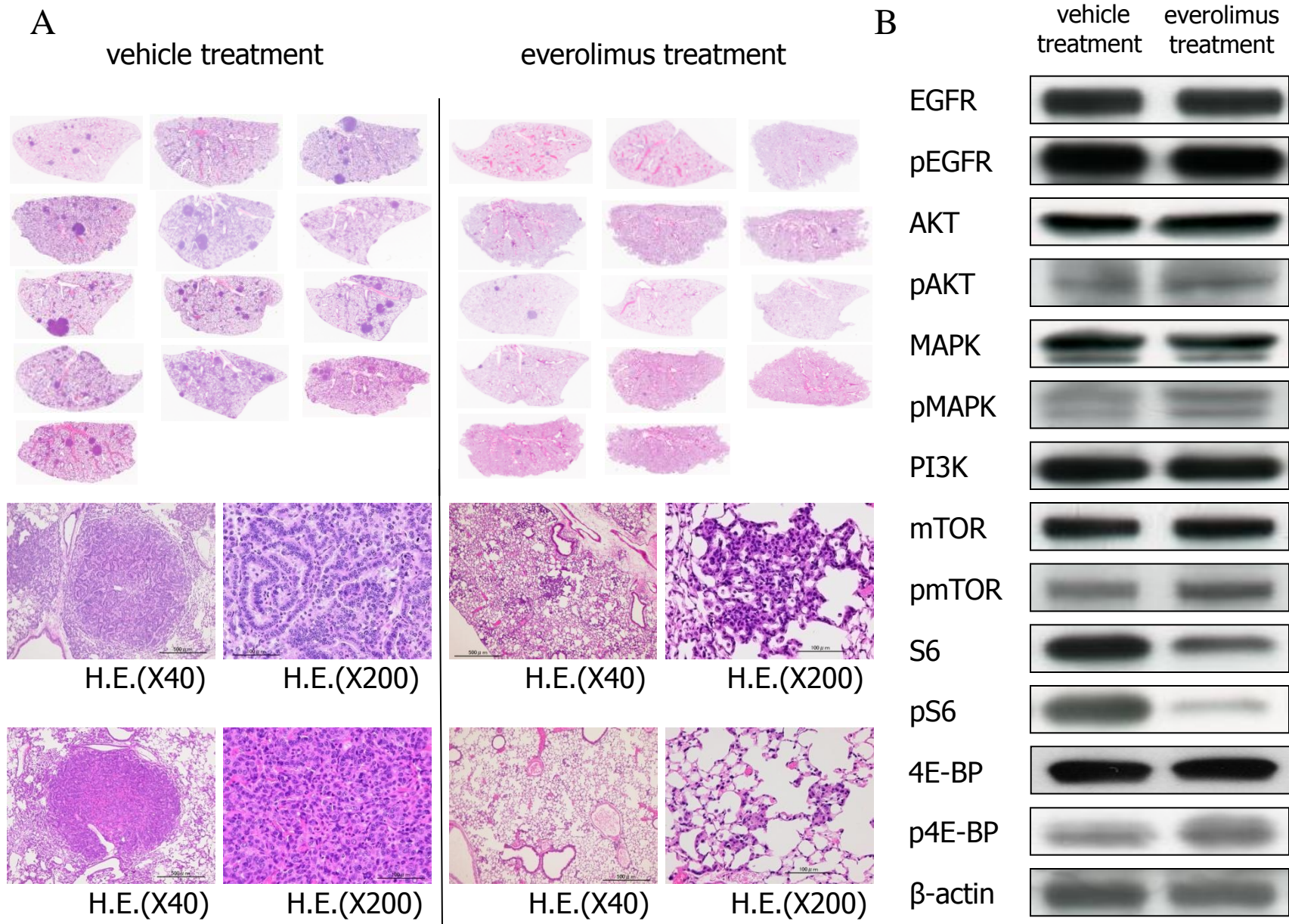


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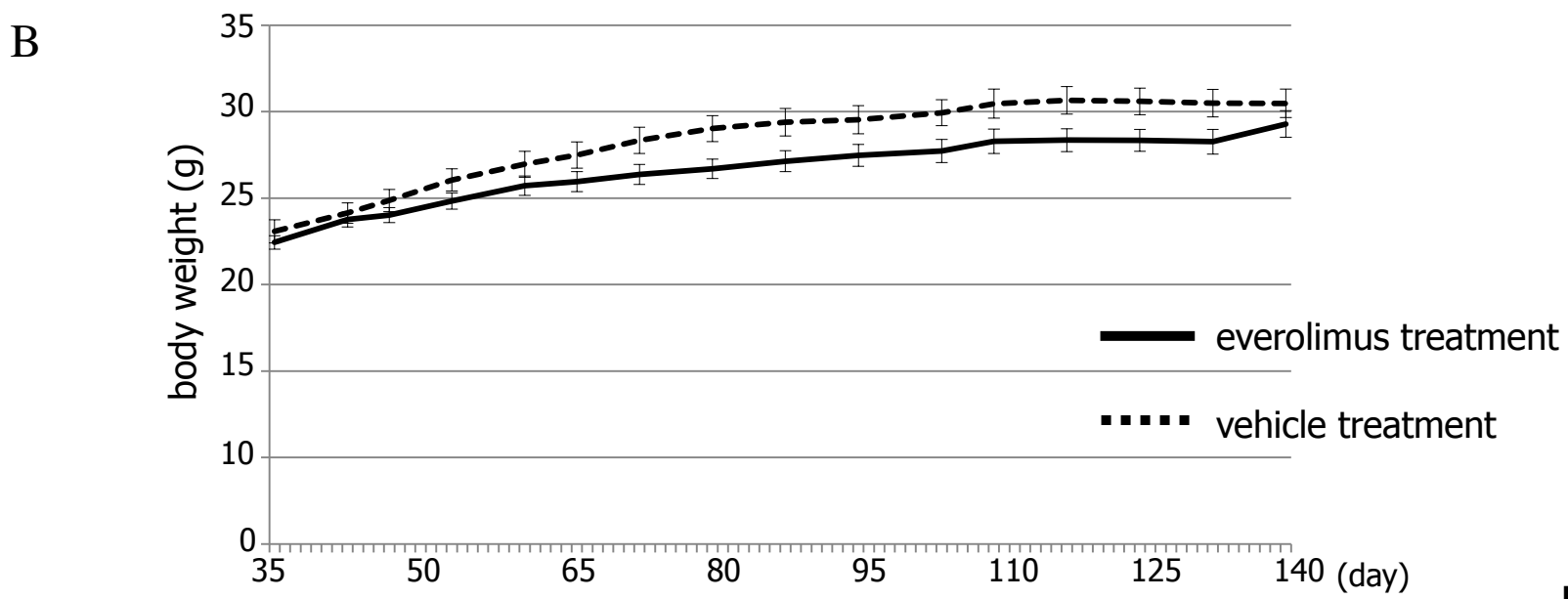
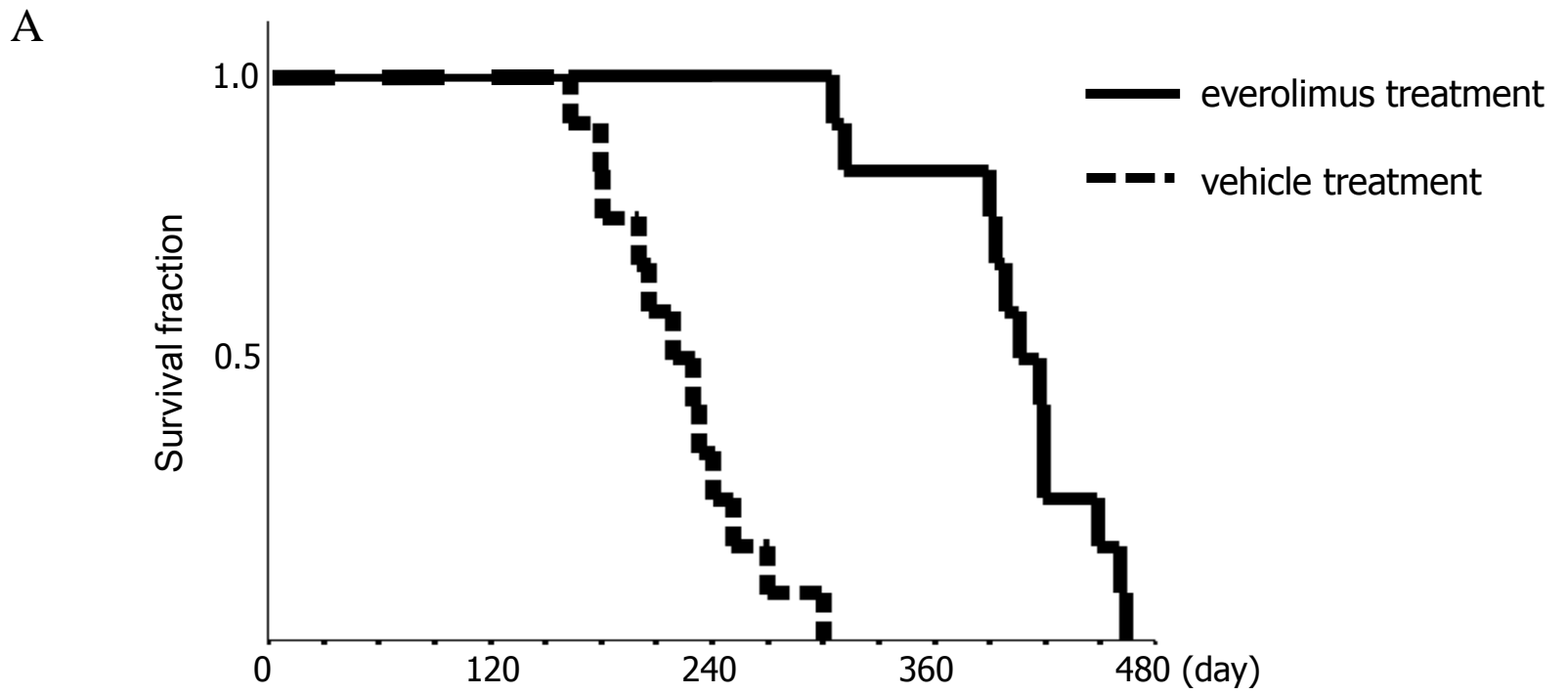


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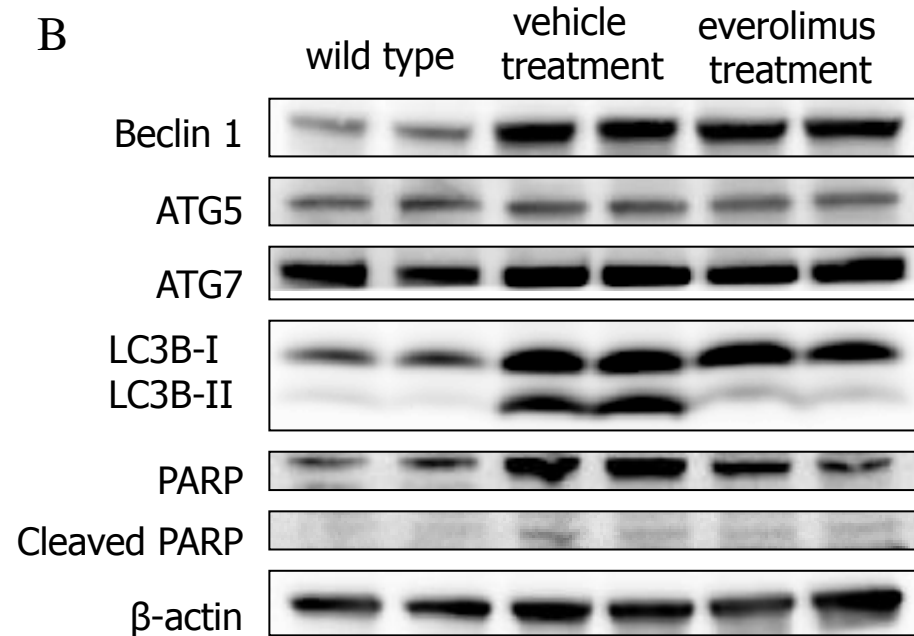
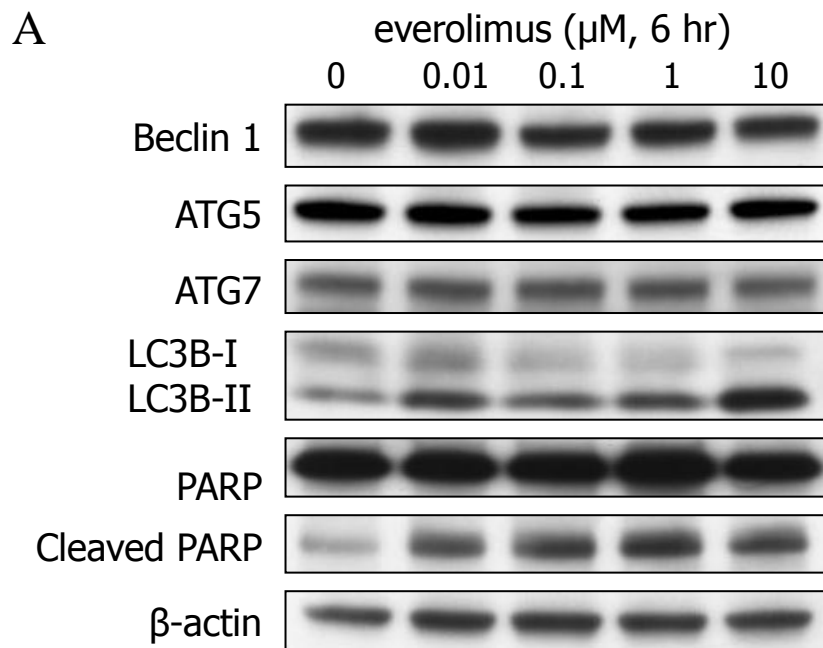
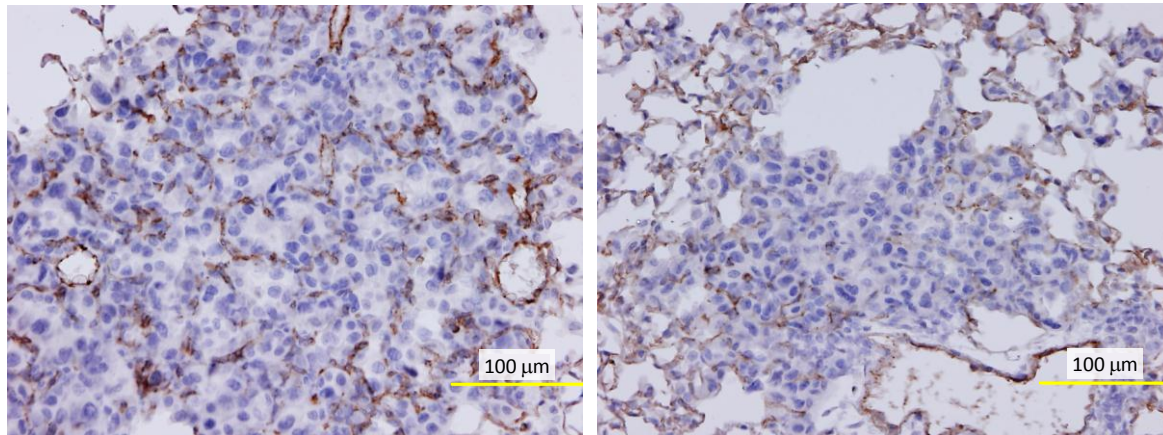


Figure 5

A

vehicle treatment

everolimus treatment



B

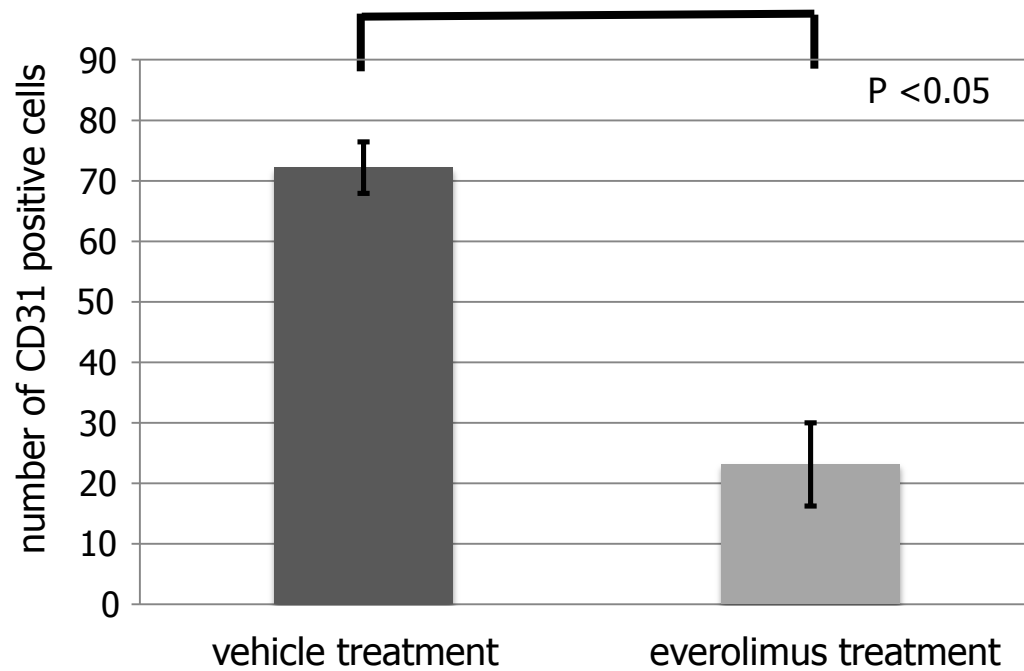
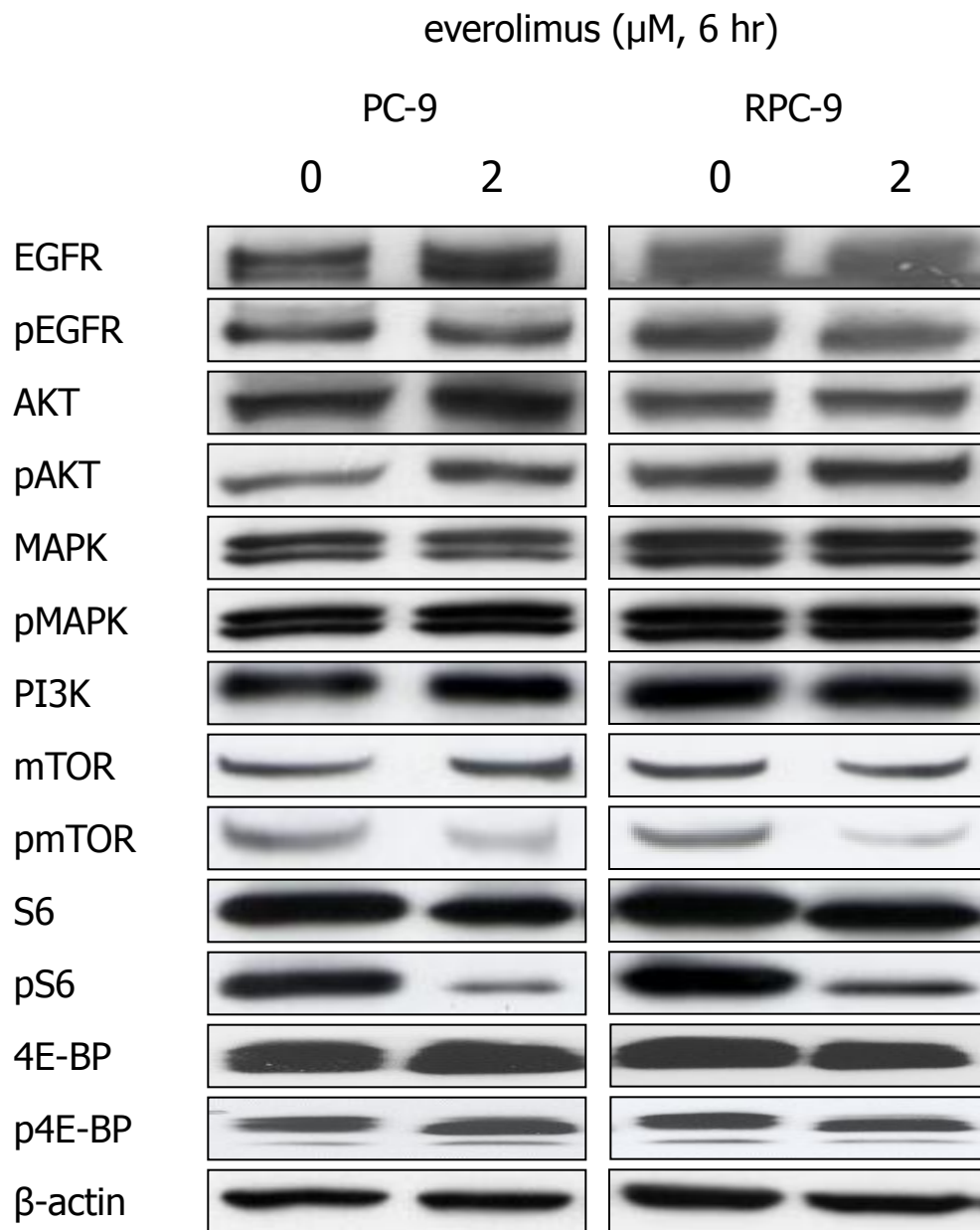
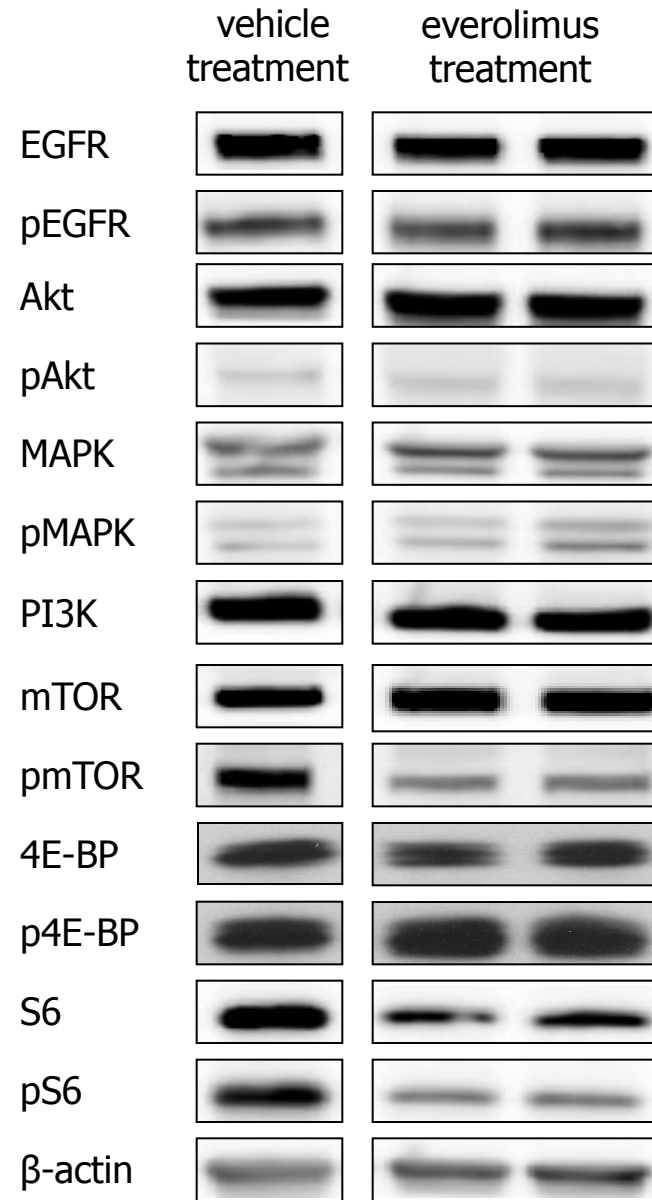


Figure 6

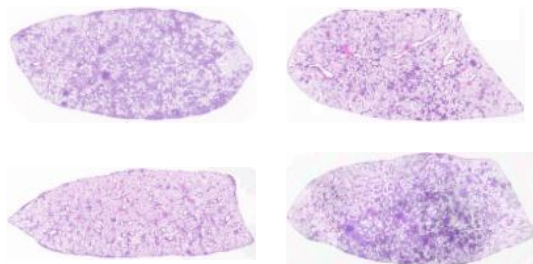


Supplemental Figure 1

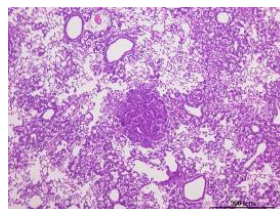
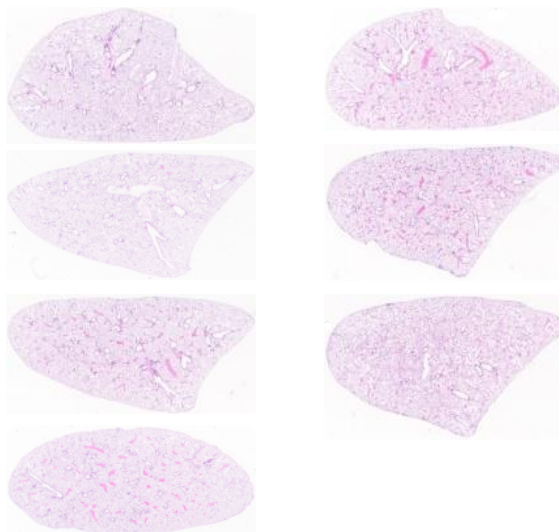


Supplemental Figure 2

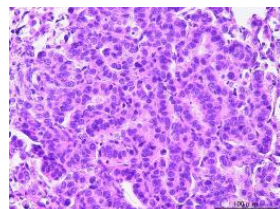
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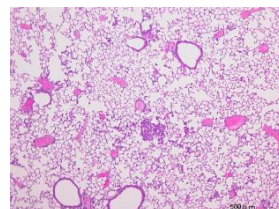
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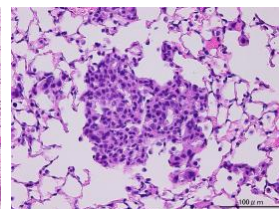
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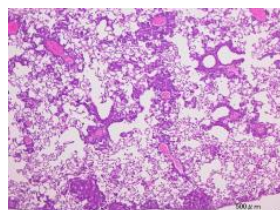
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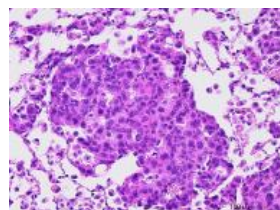
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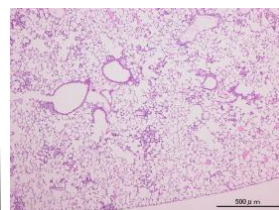
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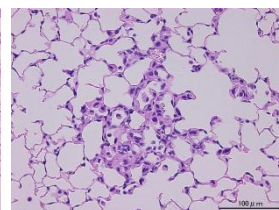
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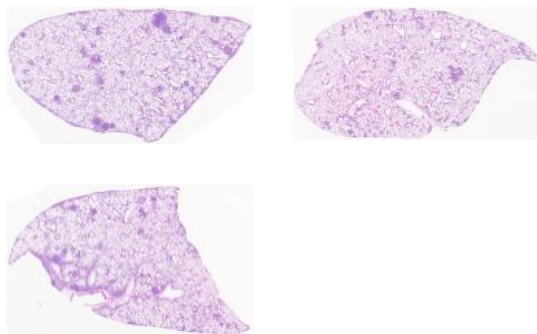


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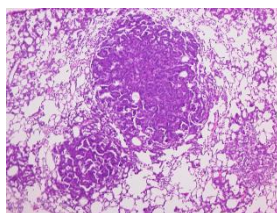
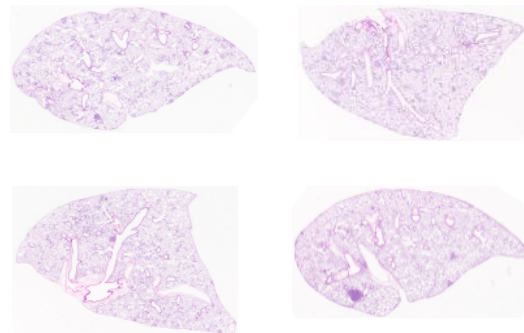


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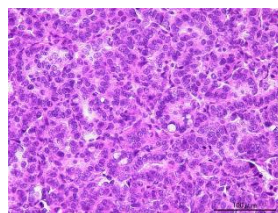
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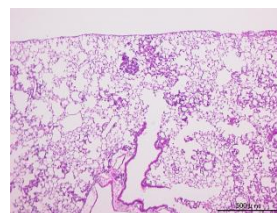
everolimus treatment



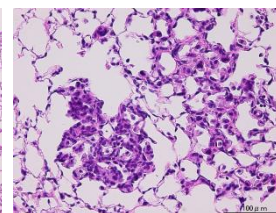
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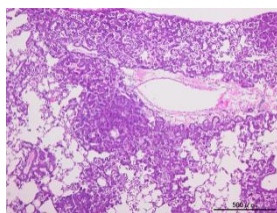
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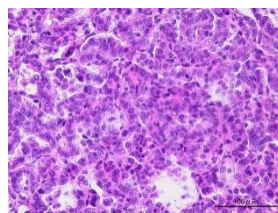
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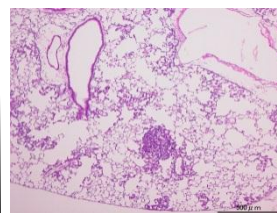
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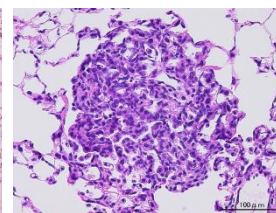
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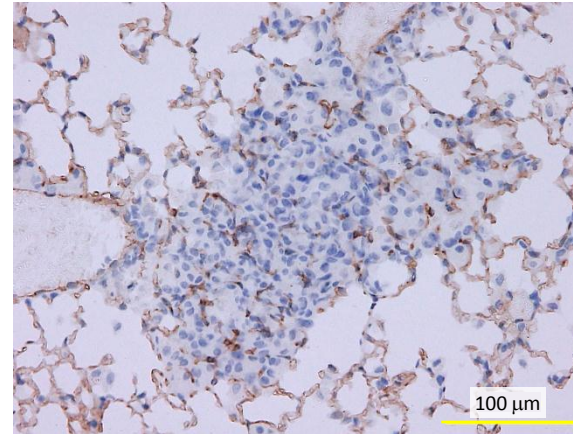
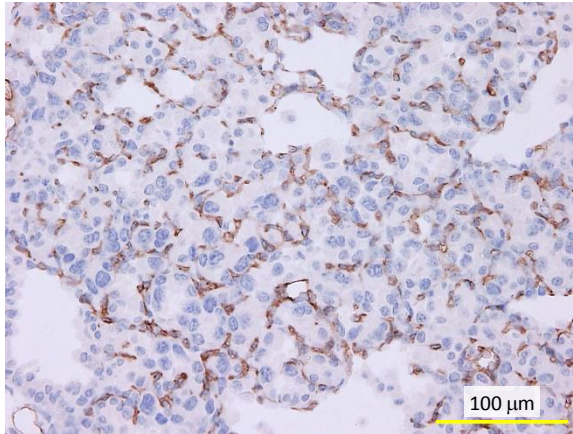


H.E.(X200)

A

vehicle treatment

everolimus treatment



B

