Inhibition of VEGF receptors induces pituitary apoplexy: an experimental study in mice

Yoshito Sugita[1, 2¶], Shigeki Takada[1, 2¶], Kenji Tanigaki[2*], Kazue Muraki[2], Munehiro Uemura[2], Masato Hojo[1, 3*], Susumu Miyamoto[1]

[1]Department of Neurosurgery, Kyoto University Graduate School of Medicine, Kyoto, Japan

[2]Research Institute, Shiga Medical Center, Shiga, Japan

[3]Department of Neurosurgery, Shiga General Hospital, Shiga, Japan

*Corresponding authors

E-mail: mhojo7@gmail.com (MH), tanigaki@res.med.shiga-pref.jp (KT)

[¶]These authors contributed equally to this work.

Abstract

Anti-vascular endothelial growth factor (VEGF) therapy has been developed for the treatment of a variety of cancers. Although this therapy may be a promising alternative treatment for refractory pituitary adenomas and pituitary carcinomas, the effects of anti-VEGF agents on the pituitary gland are not yet well understood. Here, we found that mice administered with OSI-930, an inhibitor of receptor tyrosine kinases including VEGF receptor 1 and 2, frequently exhibited hemorrhage in the pituitary gland. This is the first report that anti-VEGF therapy can cause pituitary apoplexy. C57BL/6 mice were daily injected intraperitoneally with 100 mg/kg body weight of OSI-930 for one to six days. Pituitary glands were immunohistochemically examined. Four of six mice treated for three days and all of five mice treated for six days exhibited hemorrhage in the pituitary gland. In all cases, the hemorrhage occurred just around Rathke's cleft. In OSI-930-administered mice, the vascular coverage and branching were reduced in the anterior lobe, and capillary networks were also decreased in the intermediate lobe in a treatment-day dependent manner. Few blood vessels around Rathke’s cleft of the intermediate lobe express VE-cadherin and are covered with platelet-derived growth factor receptor-β

(PDGFR-β)-positive cells, which suggests that capillaries around Rathke's cleft of the intermediate lobe were VE-cadherin-negative and not covered with pericytes. The reduction of capillary plexus around Rathke's cleft was observed at the site where hemorrhage occurred, suggesting a causal relationship with the pathogenesis of pituitary hemorrhage. Our study demonstrates that anti-VEGF agents have a risk of pituitary apoplexy. Pituitary apoplexy should be kept in mind as an adverse effect of anti-VEGF therapy.

Introduction

Anti-angiogenic therapy has been developed for the treatment of a variety of cancers. Vascular endothelial growth factor A (VEGF-A) is well known to be the main molecular driver of tumor angiogenesis [1]. Currently, bevacizumab, a recombinant monoclonal antibody targeting VEGF-A, is widely used for the treatment of high-grade gliomas [2, 3]. Hemorrhage is one of the most common adverse events of anti-VEGF therapy for cancer [4, 5]. The mechanisms of hemorrhagic complications induced by anti-VEGF agents are complex and remain to be clarified. Inhibition of VEGF decreases the renewal

capacity of endothelial cells when vessels are damaged, which might increase the risk of hemorrhage. However, life-threatening hemorrhage cannot be fully explained by endothelial cell defects alone [4, 6].

Interestingly, it has been reported that capillaries in the pituitary gland are more sensitive to VEGF inhibition than those in the brain [7]. Anti-VEGF therapy also may be a promising alternative therapy for refractory pituitary adenomas and pituitary carcinomas that are resistant to conventional treatments [8]. However, the effects of anti-VEGF therapy on the pituitary gland are not yet well understood. Pituitary apoplexy is an acute condition that causes hemorrhage and ischemic changes in the pituitary gland, and early diagnosis and treatment are important for good prognosis [9, 10]. Kasl et al. have reported a case of pituitary adenoma resulting in apoplexy after intravitreal injection of ranibizumab, a VEGF inhibitor [11]. In this case, the VEGF inhibitor was not considered to be the cause of bleeding. Pituitary apoplexy often causes hormonal dysfunction, but symptoms such as headache and fatigue are difficult to distinguish from common side effects in cancer therapy. In addition, steroids are often used in combination with cancer therapy, which may mask adrenal insufficiency.

OSI-930 is an inhibitor of receptor tyrosine kinases, including VEGF receptor 1 (VEGFR1) and 2 [12]. Clinical studies have demonstrated that OSI-930 is a well-tolerated agent which have a clinically relevant antitumor activity [13, 14]. Here, we show that OSI-930 administration induced bleeding in the pituitary gland, but not in the brain. This is the first report that anti-VEGF therapy can cause pituitary apoplexy, although it is a basic study.

Materials and Methods

Animals

Experimental animals C57BL/6N male mice (8-12-week-old, 22-28g) were purchased from Japan SLC (Shizuoka, Japan) and housed under a 12-hour light/dark cycle (8:00–20:00 on, 20:00–8:00 off) with free access to normal autoclaved diet and water. Mouse room temperature was maintained at 23 ºC. All animals used in this study were maintained and handled in accordance with the protocols approved by the Committee on Animal Research at Research Institute, Shiga Medical Center (Protocol No. R4-03). We followed NIH guidelines to treat mice and made every effort to minimize the suffering of

the mice and the number of mice used for the experiments. The study was designed and carried out in compliance with the ARRIVE guidelines [15].

Male C57BL/6 mice were randomly divided into six groups with three mice, and injected intraperitoneally with 100 mg/kg body weight of OSI-930 daily for 1, 3, or 6 days. Because of additional experiments, the total number of mice in three-day and six-day OSI-930 treated groups was six and five, respectively. The dosage of OSI-930 was determined based on the study in xenograft models [12]. As control, vehicle was injected. Mice were sacrificed 24 hours after the last injection. To harvest tissues, mice were placed under 5% isoflurane until all reflexes were absent and euthanized via cardiac puncture with care to minimize pain. Subsequently, pituitary glands were subjected to H&E and immunohistochemical staining.

Immunohistochemical analysis

Mice were perfused transcardially with 4% paraformaldehyde in PBS. Pituitary glands were isolated and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek) and cut at 30 μm thickness for immunohistochemistry. For H&E staining, the pituitary glands

were fixed in formalin for 2 days and sections were made at 10 μm thickness. Immunostaining was performed with the following antibodies as previously described: mouse anti-platelet endothelial cell adhesion molecule 1 (PECAM1) / CD31 (1:10; clone 390, 102402, BioLegend, SanDiego, USA), mouse anti-plasmalemma vesicle associated protein (PLVAP; 1:100; ab27853, Abcam, Cambridge, UK), mouse anti-platelet-derived growth factor receptor-β (PDGFR-β)/CD140b (1:100; 136002, BioLegend, SanDiego, USA), and rat anti-VE-cadherin/CD144 (1:100; clone 11D4.1, 555289, BD, NJ, USA) [16]. Alexa Fluor 568-conjugated isolectin GS-IB4 from *Griffonia simplicifolia* (1:100; I21412, Thermo Fisher Scientific, MA, USA) was used to label endothelial cells. Briefly, cryosections were incubated with primary antibodies for 24 h at 4 ℃, and then with secondary antibodies for 1 h at room temperature. Donkey anti-species IgG conjugated with Alexa 488 (A21206, A21208, Thermo Fisher Scientific, MA, USA) was used for a secondary antibody. Samples were then treated with DAPI. Sections were analyzed with Leica SP8 confocal laser scanning microscopy (Leica, Wetzlar, Germany).

Vascular analysis

Sagittal sections of pituitary glands were stained using isolectin B4 conjugated with Alexa 568. To analyze vasculature, ImageJ software was used. For vessel branching analysis, capillaries around Rathke's cleft were excluded and only the anterior lobe was included. The images were processed with "tubeness" filter (sigma: 3), and then binarized images were created with near peak values of thresholds to match blood vessels. After measuring the vascular area (%), binarized images were skeletonized and the number of vascular branches and branching points per 10000 μm^2 in the anterior lobe was counted by "analyze skeleton" [17]. In the intermediate lobe, the number of vascular networks was counted. All statistical analyses were performed using EZR software [18]. The data were analyzed by Student's t-test and considered to be significant when $P < 0.05$. Results are given as means ± S.E.M.

Quantitative real time RT-PCR analysis

Total RNA was extracted from the pituitary gland using RNA easy Mini kit (Qiagen, Hilden, Germany). Complementary DNA was obtained using a PrimeScript 1st strand cDNA synthesis kit (Takara, Shiga, Japan). Gene expressions were quantified using TB

Green Premix Ex Taq II (Tli RNaseH Plus) (Takara, Shiga, Japan). Quantitative real-time PCR was performed on a LightCycler 480 system (Roche, Basel, Switzerland). *Actb* was used as a control (Mouse Housekeeping Gene Primer Set (Takara, Shiga, Japan)). The following primers were used for qPCR: *Pecam1* forward: tcggcagacaagatgctcctggctc, *Pecam1* reverse: cagggtcagttgctgcccattcatcacc, *Plvap* forward: gctatcatcctgagcgagaagcagtgcc, *Plvap* reverse: tgccttctccttggccacctccatc, *Flt1* forward: ctgcgaagccaccgtcaacggg, *Flt1* reverse: gtggcggtgcagttgaggacaagag, *Flk1* forward: cgtaccgggacgtcgacatagcctc, *Flk1* reverse: ggtgatgtacacgatgccatgctggtc, *VE-cadherin (Cdh5)* forward: ctgccctcattgtggacaagaacaccaac, *VE-cadherin (Cdh5)* reverse: gacatctctggcacagatgcgttgaatacctg.

Results

OSI-930 administration causes pituitary apoplexy

To evaluate adverse effects of OSI-930, we first examined the brain and pituitary gland of OSI-930-administered mice. As for the brain, no hemorrhagic change was observed in control or OSI-930 groups. In contrast, hemorrhage was frequently detected in the

145 pituitary gland of OSI-930 groups (Fig 1). In control and groups treated with OSI-930 for
146 one day, no bleeding occurred in the pituitary gland (Fig 1A). Strikingly, two of three
147 three-day OSI-930 treated mice and all of six-day OSI-930 treated mice exhibited
148 hemorrhage in the pituitary gland (Table 1, Figs 1B and C). In all cases of pituitary
149 apoplexy, the hemorrhage occurred just around Rathke's cleft (Figs 1D-F). In three-day
150 OSI-930-treated group, bleeding occurred around Rathke's cleft, and accumulated within
151 the cleft (Fig 1E). In six-day OSI-930-treated group, bleeding was more massive and also
152 observed in wider areas of anterior and intermediate lobes (Fig 1F). These results suggest
153 that pituitary apoplexy was observed in a treatment-day dependent manner.
154
155 **Fig 1. OSI-930 causes pituitary apoplexy around Rathke's cleft.** H&E stained sagittal
156 images of pituitary glands in control (A and D), three-day OSI-930-treated (B and E), and
157 six-day OSI-930-treated (C and F) groups. Boxed regions in A-C are enlarged in D-F,
158 respectively. In three-day OSI-930-treated group, hemorrhage occurred around Rathke's
159 cleft (arrows; E). In six-day OSI-930-treated group, hemorrhage was exacerbated around
160 Rathke's cleft (asterisk; F), and many small bleeds were observed throughout the anterior

161 lobe (C and F). Bars, 200 μm. AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe.

162

163 **Table 1. Rates of hemorrhage in pituitary grands after OSI-930 administration**

	Duration of OSI-930 administration (days)		
	1	3	6
OSI-930	0% (0/3)	66.7% (4/6)	100% (5/5)
Control	0% (0/3)	0% (0/3)	0% (0/3)

164

165 VEGFR inhibition disrupts pituitary vasculature

166 To investigate how the vasculature of the pituitary gland is affected by OSI-930, we
167 stained endothelial cells with isolectin B4 (Fig 2). The capillary plexus was well
168 developed around Rathke's cleft, a remnant space between the anterior and intermediate
169 lobes, in control mice (Figs 2G and J), where pituitary stem cells has been reported to be
170 enriched. To examine whether this isolectin B4-positive structures are blood vessels or
171 not, we performed immunohistochemical analysis of PECAM1 (CD31, Figs 3A-H) and

PLVAP (Figs 3I-P). Isolectin B4 staining were observed around PECAM1-positive or PLVAP-positive blood vessels in the MCL (Figs 3A-P), suggesting isolectin B4-positive structures are blood vessels.

Fig 2. VEGFR inhibition disrupts pituitary vasculature. (A-F) Representative frontal sectional images of pituitaries labelled by isolectin B4 staining in control (A and D), three-day OSI-930-treated (B and E), and six-day OSI-930-treated (C and F) groups. Boxed regions in A-C are enlarged in D-F, respectively. **(G–L)** Representative sagittal images of pituitaries labelled by isolectin B4 staining in control (G and J), three-day OSI-930-treated (H and K), and six-day OSI-930-treated (I and L) groups. Boxed regions in G-I are enlarged in J-L, respectively. White dots indicate capillary plexus with a palisade-fashion arrangement in the intermediate lobe. In OSI-930-treated groups, hematoma cavities were observed around Rathke's cleft (arrows; B**,** C, K, and L), and capillary networks in the intermediate lobe were decreased compared to control (dots: J-L). In addition, capillaries around Rathke's cleft were diminished compared to control. **(M-P)** Quantitation of vascular area, branches and branching points in the anterior lobe, and

188 vascular networks in the intermediate lobe. The vascular are (M), branches (N) and
189 branching points (O) were decreased in the anterior lobe, and the number of capillary
190 plexus with a palisade-fashion arrangement was decreased in the intermediate lobe (P) in
191 a treatment-day dependent manner (* $P < 0.05$, t-test). Bars, 200 μm. AL, anterior lobe;
192 IL, intermediate lobe; PL, posterior lobe; C, control.
193
194 **Fig 3. Comparison of isolectin B4 staining with PECAM1 and PLVAP**
195 **immunostaining.** Representative confocal immunofluorescence sagittal images with
196 orthogonal views. Boxed regions in A-D and I-L are enlarged in E-H and M-P,
197 respectively. Arrows and arrow heads indicate the intersection points of the XZ and YZ
198 planes. **(A-H)** The pattern of isolectin B4 staining (red) was extremely similar to that of
199 PECAM1 immunostaining (green), including the surrounding area of Rathke's cleft. **(I-**
200 **P)** A significant number of cells positive for isolectin B4 staining were positive for
201 PLVAP. Bars, 200 μm. AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe.
202
203 After OSI-930 administration, the capillary plexus around Rathke's cleft was disrupted

(Figs 2J-L). In the anterior lobe, the vascular coverage, vascular branches and branching points were decreased in a treatment-day dependent manner (Figs 2M-O). These OSI effects on blood vessels were also detected by the change in the expression levels of VEGFR1 and VEGFR2 but not of PECAM1, PLVAP, and VE-cadherin (Fig 4). In the intermediate lobe, the number of capillary networks with a palisade-fashion arrangement was also decreased in a treatment-day dependent manner (Fig 2P). The reduction of capillary plexus around Rathke's cleft was mainly observed at the site where hemorrhage occurred (Figs 2J-L), suggesting a causal relationship with the pathogenesis of hemorrhage.

Fig 4. Quantitative real-time RT-PCR analysis of vascular markers. Expression levels of PECAM1 (A), PLVAP (B), VEGFR1 (C), VEGFR2 (D), and VE-cadherin (E) were analyzed. *Actb* was used as a control (* $P<0.05$, t-test).

Contribution of pericytes and VE-cadherin to pituitary apoplexy

220 To analyze how the vascular structure is affected by OSI-930 in pituitary apoplexy, we
221 examined the expression of PDGFR-β, a marker for pericytes, and VE-cadherin in
222 pituitaries. PDGFR-β immunoreactivity were present along vessels in anterior,
223 intermediate and posterior lobes of pituitaries of mice (Figs 5A-H). Interestingly,
224 PDGFR-β immunoreactivity was not present along capillaries around Rathke's cleft,
225 where hemorrhagic changes were frequently observed after OSI-930 administration (Figs
226 5C and G). In addition, PDGFR-β expression was decreased after OSI-930 administration
227 (Figs 5I-P). This finding was more prominent after six-day OSI-930 treatment than after
228 three-day treatment.
229
230 **Fig 5. Pericytes are not detected around Rathke's cleft. (A-P)** Representative sagittal
231 images of pituitaries stained with isolectin B4 (red) and anti-PDGFR-β antibody (green).
232 Boxed regions in A-D and I-L are enlarged in E-H and M-P, respectively. Although
233 PDGFR-β-positive pericytes were identified along vessels throughout pituitaries, they
234 were hardly detected around Rathke's cleft in control (arrowheads; G). In OSI-930-
235 treated group, hematoma cavities were observed around Rathke's cleft (arrows; I-L), and

capillary networks in the intermediate lobe were decreased compared to control (dots: F and N). In addition, capillaries around Rathke's cleft were diminished compared to control (arrowheads; F and N). In OSI-930-teated group, pericytes were decreased compared to control (C, G, K and O). Bars, 200 μm. AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe.

VE-cadherin was expressed in vessels of anterior and posterior lobes (Figs 6A-F). However, Few blood vessels of the intermediate lobes express VE-cadherin. As with pericytes, distribution of VE-cadherin was sparse in capillaries around Rathle's cleft, where hemorrhagic change was frequently observed after OSI-930 administration (Figs 6G-M). Taken together, undeveloped pericytes and VE-cadherin may have influenced the susceptibility to bleeding in capillaries around Rathke's cleft.

Fig 6. Few blood vessels of the intermediate lobes express VE-cadherin. (A-M) Representative frontal sectional images of pituitaries stained with isolectin B4 (red) and

252 anti-VE-cadherin antibody (green). Boxed regions in A-C and G-I are enlarged in D-F
253 and K-M, respectively. Although VE-cadherin was identified in both anterior and
254 posterior pituitaries, it was hardly detected in the intermediate lobe and around Rathke's
255 cleft in control (arrowheads; D-F). In OSI-930-treated group, hematoma cavities were
256 observed around Rathke's cleft (arrows; G and K). In addition, capillaries around
257 Rathke's cleft were diminished compared to control (open arrowhead; K). Bars, 200 μm.
258 AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe.
259

260 # Discussion

261 In our study, OSI-930 administration induced bleeding in the pituitary gland, but not
262 in the brain. Kamba et al. have reported that capillary regression was observed in multiple
263 organs of mice treated with AG-013736, a small-molecule VEGFR tyrosine kinase
264 inhibitor [7]. Capillary regression was found more frequently in anterior (24%) and
265 posterior (41%) pituitaries than in brains (5%). However, details of vascular changes were
266 not examined in pituitaries. These data may explain the reason why OSI-930 induced
267 bleeding in the pituitary, but not in the brain.

VEGFR-2 (Flk-1) was expressed in endothelial cells in rat and human pituitary glands [19, 20]. Furube E et al. have reported that administration of AZD2171, a receptor tyrosine kinases inhibitor for VEGFRs, decreased vascular density and the proliferation of endothelial cells in posterior pituitaries of mice [21]. However, pituitary apoplexy was not observed in their study. Only posterior lobes, but not anterior or intermediate lobes were examined. In contrast, we examined vascular changes in both anterior and intermediate lobes after administration of OSI-930. Capillary regression was more prominent in the vascular plexus around Rathke's cleft, which might be the reason why pituitary apoplexy often occurs around Rathke's cleft after OSI-930 treatment. Since MCLs and folliculo-stellate cells are also positive for isolectin B4 in the pituitary gland[22], we compared the pattern of isolectin B4 staining with those of CD31 and PLVAP immunostaining. The pattern of CD31 immunostaining was extremely similar to that of isolectin B4 staining, including the MCL portion. Isolectin B4 staining has the limitation that non-endothelial cells may also be positive. However, in the present study, this staining method was considered feasible for the purpose of analyzing pituitary vessels.

In the human pituitary gland, VEGFR1 and VEGFR2 were expressed in endocrine cells

284 and vascular structures, respectively[20]. In the rat pituitary gland, VEGFR1 and
285 VEGFR2 were also expressed in endocrine and endothelial cells [19, 20]. In our study,
286 the expression of VEGFR1 was decreased after OSI-930 administration. This may reflect
287 hormonal cell destruction by pituitary apoplexy. The expression of VEGFR2 was also
288 decreased. This may reflect the vascular regression in pituitaries.

289 Pericytes cover the microvascular wall, and contribute to the mechanical stability of
290 the capillary wall [23]. PDGF-B or PDGFR-β deficient mouse embryos lack
291 microvascular pericytes and develop numerous microvascular microaneurysms resulting
292 in hemorrhage[23, 24]. VE-cadherin has a role in the maintenance of cell-cell junction
293 stabilization and regulation of vascular barrier integrity [25, 26]. In our study, hemorrhage
294 was frequently observed in the vascular plexus of intermediate lobes around Rathke's
295 cleft, where pericytes did not exist and few vessels express VE-cadherin. The VE-
296 cadherin-negative capillaries without pericytes around Rathke's cleft might be
297 susceptible for bleeding. The capillaries distributed in the pituitary gland are
298 characteristically rich in fenestrations [27, 28]. It has been reported that VEGFR
299 inhibitor administration reduced endothelial fenestrations in thyroid perifollicular

capillaries, renal glomerular capillaries, and capillaries in islet-cell tumors of RIP-Tag2 transgenic mice [7, 29]. To date, no studies have been reported on fenestration changes after VEGFR inhibitor administration in pituitary capillaries. Due to lack of access to an electron microscopy, we could not examine ultrastructural changes in pituitary capillary endothelial fenestrations. Pituitary apoplexy may be caused due to changes of endothelial fenestration with OSI-930 treatment.

Our study has a limitation that should be noted. We performed experiments with higher doses than in clinical trials in order to more clearly evaluate the adverse effects of OSI-930. For this reason, it is likely that the incidence of pituitary apoplexy induced by anti-VEGF therapy will be lower in actual clinical practice. However, we consider that our study is meaningful, because it raises awareness of adverse effects on the pituitary gland during clinical application of anti-VEGF agents.

Conclusion

Our study demonstrates the possibility that anti-VEGF agents have a risk of pituitary apoplexy. Regarding adverse effects of anti-VEGF therapy for cancer, no case of pituitary

316 apoplexy has been previously reported in either basic or clinical studies. This is the first
317 report that anti-VEGF therapy can cause pituitary apoplexy, although it is a basic study.
318 Pituitary apoplexy should be kept in mind as an adverse effect of anti-VEGF therapy. If
319 patients treated with anti-VEGF agents complain of symptoms such as headache and
320 fatigue, brain imaging and hormonal examinations should be considered.
321

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Figure1

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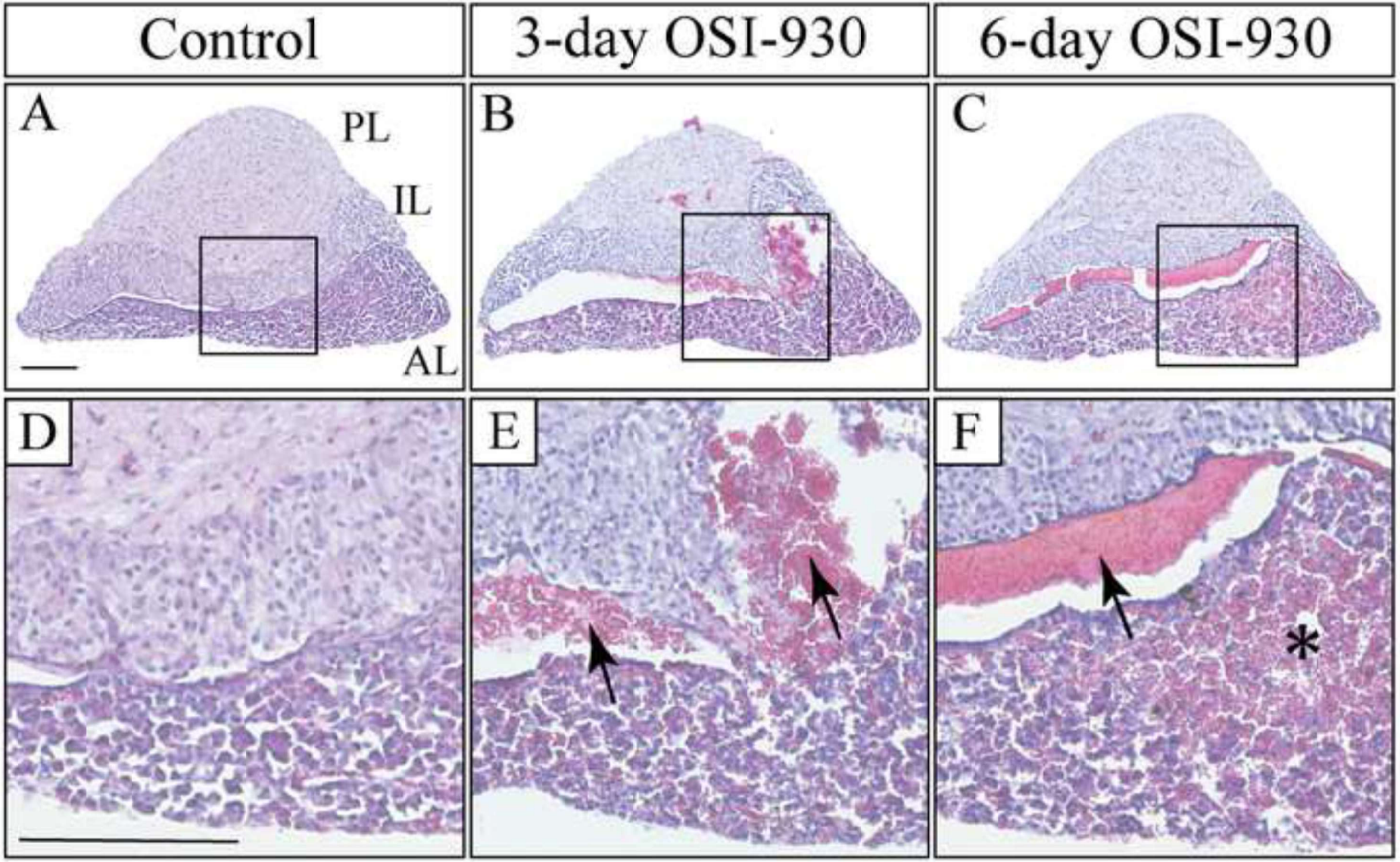

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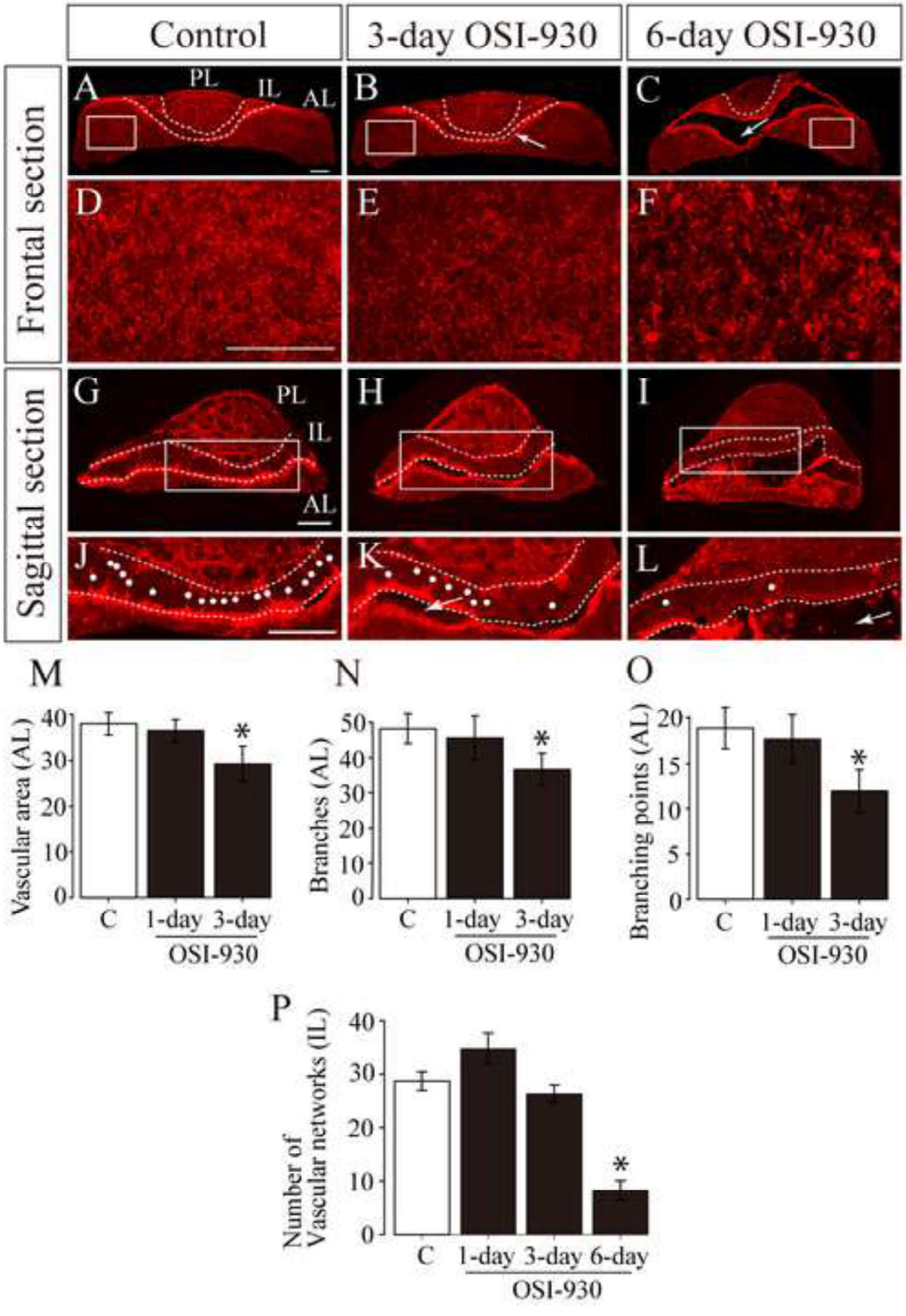

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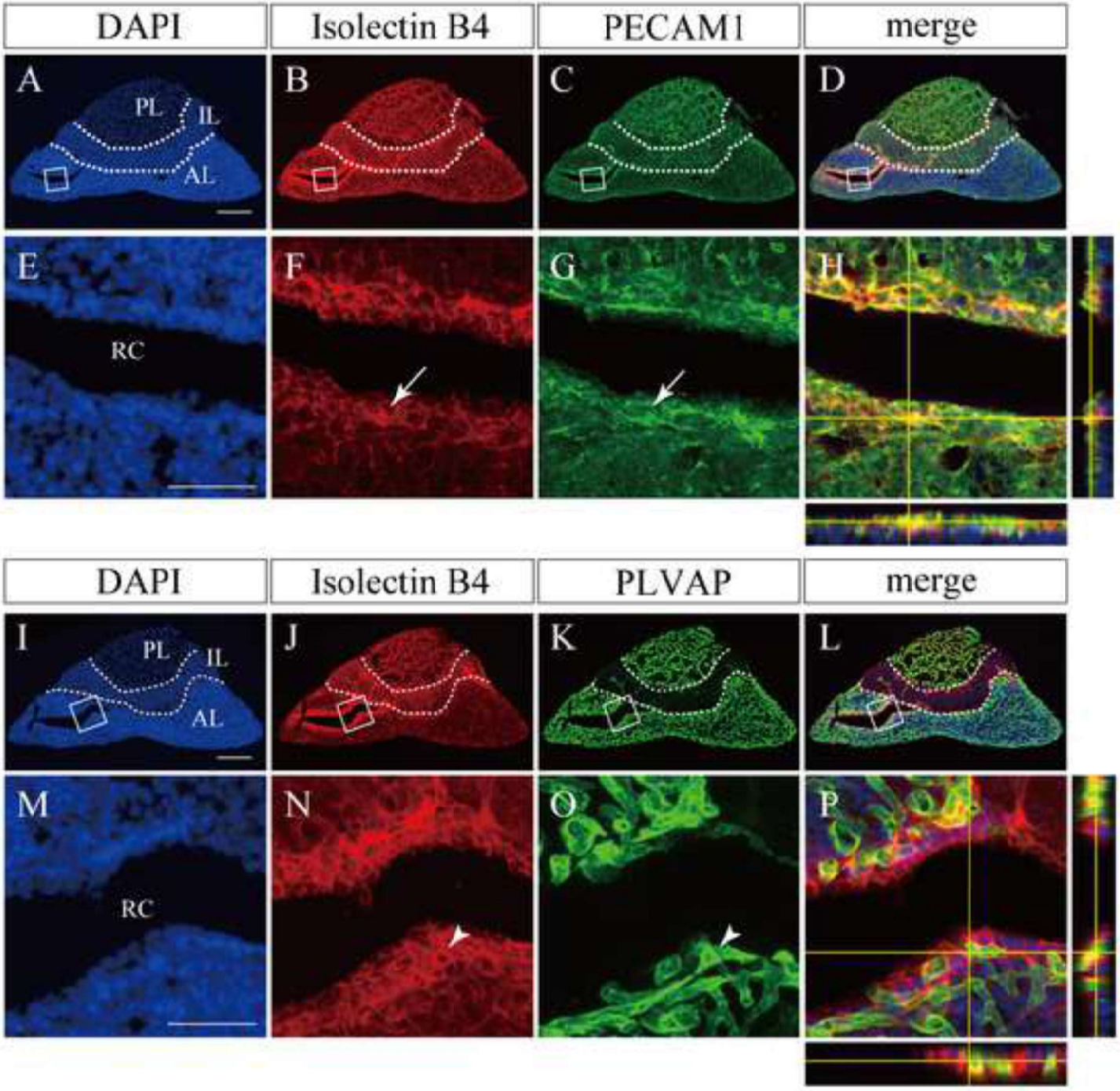

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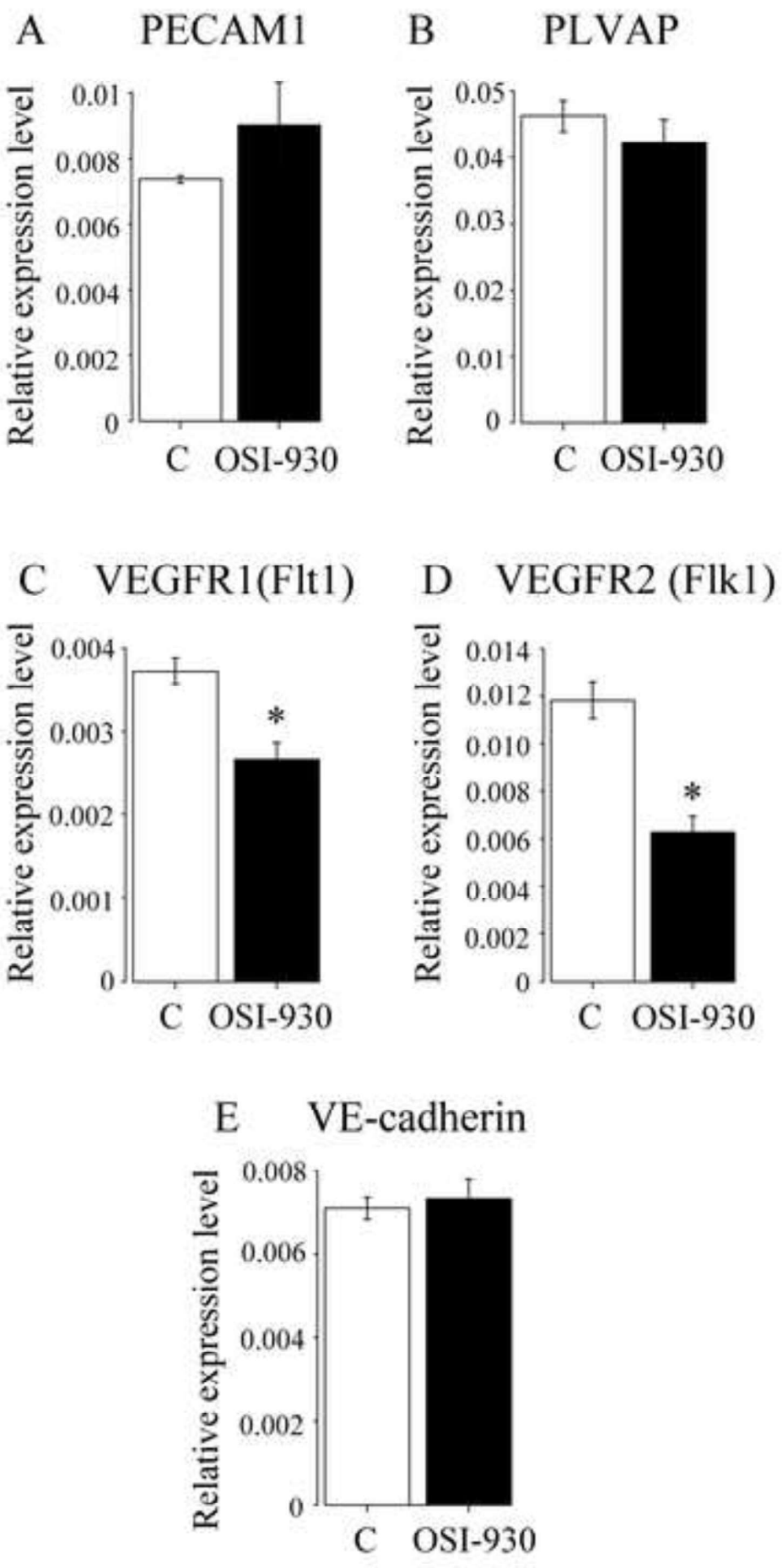

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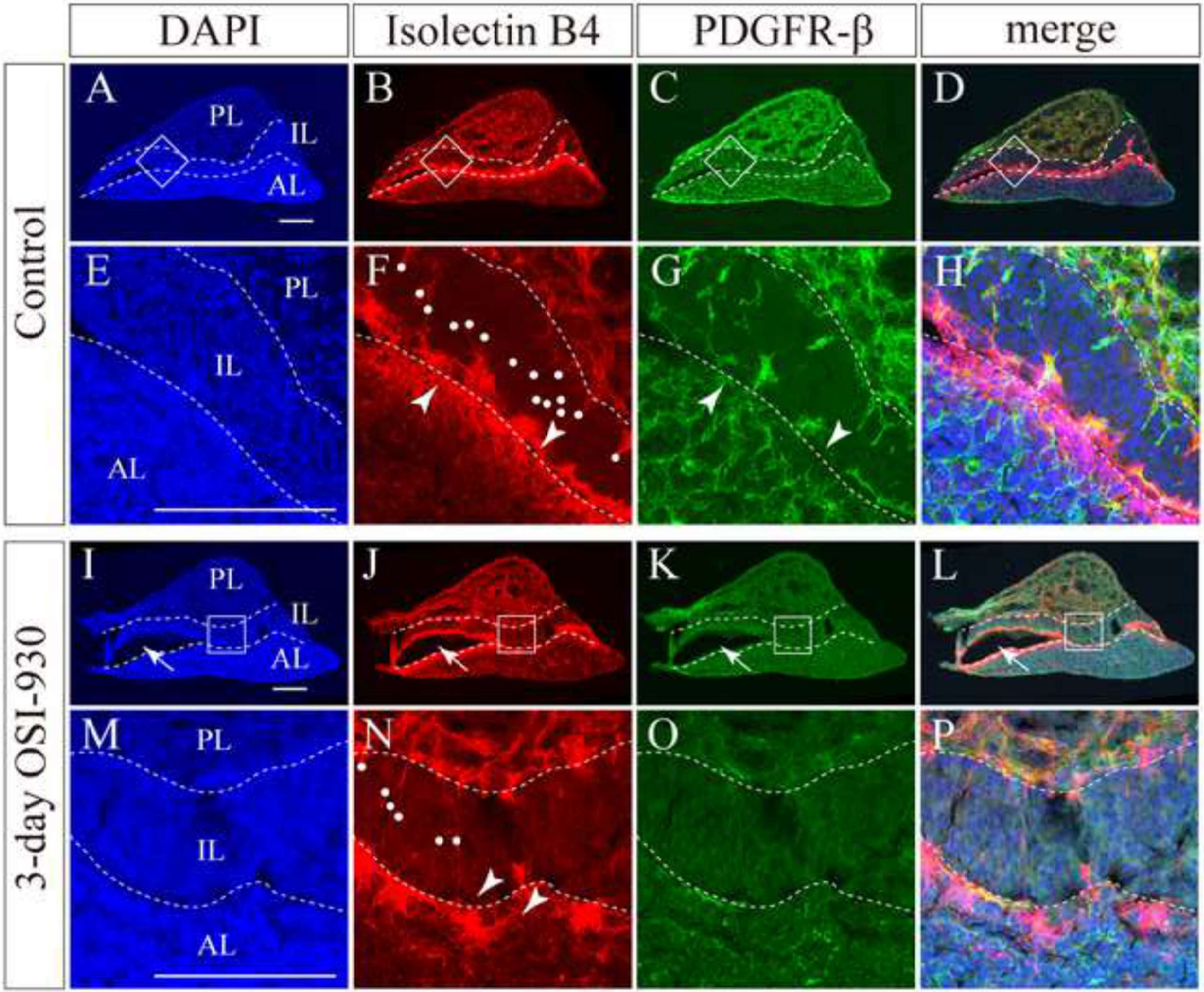

Figure6

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