이학석사 학위논문

KAI1/CD82 on mural cell suppresses angiogenesis by keeping vessel quiescent

혈관주위세포에서 발현하는
KAI1/CD82의 혈관신생 억제자로서의
기능에 관한 연구

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Abstract

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Blood vessel is a critical organ, spread all over the body for survival of every other organ. The key process forming network of those vessels is called angiogenesis. In healthy adult, angiogenesis is physiologically well-controlled by interacting between two components of vascular cell (endothelial cell and mural cell). However, in disease such as cancers or retinopathies, aberrant angiogenesis occurs out of control and becomes one of the critical factors worsening disease prognosis. As a strategy to block those pathological angiogenesis, endothelial cell has been generally targeted as an

anti-angiogenic drug. However, several studies reported the clinical cases of cancer patients resistant to them. Recently, because of those obstacles, mural cell has been emerged as a new target of anti-angiogenic drug. In addition to that, it is still elusive that how interaction between endothelial cell and mural cell regulates angiogenesis to keep quiescent state at the molecular level.

Here, we discovered that one of the tetraspanins, KAI1, on mural cell is a new distinct suppressor of angiogenesis in quiescent niche. From the developmental stage to the adult, *Kai1*$^{-/-}$ mice showed better angiogenic ability than wild type (WT) mice *in vivo* and *in vitro*. Furthermore, even though KAI1 molecules expressed both on endothelial cells and mural cells, majority of them were on the mural cell and only mural KAI1 played a role as a functional angiogenic suppressor. We also figured out that KAI1 on mural cell is involved in signaling transduction for anti-angiogenic effect in quiescent niche, by up-regulating and secreting LIF, which is an anti-angiogenic soluble factor, via SRC-p38/Akt pathway. We expect that this finding could contribute to establish a new axis of anti-angiogenesis in academic and clinical field.

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keywords : KAI1/CD82, Mural cell, Angiogenesis, Tetraspanin, Quiescent vessel, Leukemia Inhibitory Factor (LIF)

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Ⅰ. Introduction

Blood vessel is a critical organ, spread all over the body for survival of every other organ. It supplies various nutrients and metabolites and collects waste products through blood in a systemic way. From the embryonic stage, developing complex of blood vessel follows two main processes of vessel production. At first, *de novo* formation of vascular plexus is initially derived from vascular progenitor cells and forms new blood vessels, which is called vasculogenesis. Followed by this process, new vessel network branches and expands from pre-existing vessel by angiogenesis (Patan, 2004). Most of both processes occur in the developmental and postnatal stage, and pause as quiescent state in adult. When the vessel is in quiescent niche, it lays in a physiological environment with none or negligible amount of angiogenic stimulants.

Even in the adults, angiogenesis can be activated physiologically and pathologically at angiogenic niche, in response to various angiogenic stimuli. The physiological angiogenesis plays an essential role with controlled regulation in wound healing or menstrual cycle (Goth et al., 2003). However, uncontrolled angiogenesis may trigger several diseases due to vascular overgrowth by pathological angiogenesis as in cancer and retinopathies (Yoo and Kwon, 2013). In case of cancer, overgrown blood vessels appeared around tumorigenic niche to form a tumor vasculature, fostering the abnormal growth of cancer cells.

To regulate this abnormal angiogenesis, previous studies evidenced that both mechanical and chemical stimulations

contribute to control angiogenesis, but most well-known pathway is by growth factors, such as vascular endothelial growth factor (VEGF), angiopoietin-2, fibroblast growth factor (FGF) (Gualandris et al., 1996; Gupta and Qin, 2003; Li and Harris, 2005). VEGF is one of the most effective angiogenic cytokines, transducing signals related to proliferation and migration of vascular endothelial cell (EC) via VEGF receptor (VEGFR) and promoting angiogenesis. At the same time, mural cell (MC), another main vessel component covering EC, is also responsive to VEGF. In response to VEGF, MC is detached from vessels and let EC migrate and proliferate to branch new vessels (Fujimoto et al., 2004).

As briefly mentioned above, a blood vessel consists of two main type of cells; vascular EC and mural or perivascular cell (collectively, MC). Each of them takes individual roles in function of blood vessel. EC is primarily responsible for creating barriers of vessels. It also involved in controlling inflammation and blood pressure. On the other hand, MC has contractile features and regulates the healthy vasculature by stabilizing vessels (Michiels, 2003; Bergers and Song, 2005; van Dijk et al., 2015). Angiogensis is a joint work of them on top of their individual roles. The interaction between EC and MC plays an important role in vascular formation, stabilization, remodeling, and function (Armulik et al., 2005). When exposed to angiogenic condition, such as hypoxia, nitric oxide (NO), or VEGF, vessel is destabilized and permeablized by detaching MC from vessel walls. Then, EC could sprout toward stimulants and make new vessels. After branching, vessel became re-stabilized by recruitng MC around EC. In terms of angiogenesis, both

mechanical and paracrine signaling exchange between EC and MC have been studied for over decades, but the key regulator of angiogenesis between their interactions still remains elusive (Gaengel et al., 2009).

Meanwhile, tetraspanins are family of membrane proteins, having four transmembrane domains (also known as transmembrane 4, TM4). It contains two loops at extracellular domains, one of which is small (small extracellular loop, SEL) and another is long (long extracellular loop, LEL). The tetraspanin family is highly conserved during evolution, so there is no significant difference between species (Hemler, 2005). In early days after its discovery, they were just regarded as molecular facilitators by scaffolding proteins to stabilize them or guiding multiple proteins to its cluster, called tetraspanin-enriched microdomain (TEM) on the cell membrane (Maecker et al., 1997; Hemler, 2005). However, it became revealed that tetraspanins have its own roles regulating cell motility, invasion, fusion and signaling (Maecker et al., 1997; Levy et al., 1998; Boucheix et al., 2001; Hemler, 2003; Tarrant et al., 2003). Also, it affects to cell proliferation, apoptosis and tumor metastasis (Hemler, 2005).

KAI1/CD82 is an intensively studied tetraspanin, since Dong and his colleagues discovered that KAI1 on cancer cells as a tumor metastasis suppressor (Dong et al., 1995). Few years later, its mechanism of action to suppress tumor metastasis was discovered as activation of the FAK-SRC-p130Cas-CRKII pathway (Klemke et al., 1998; Gu et al., 1999). In addition to the well-established function of KAI1 in tumor cells, there have been many findings related to other roles in various cells. For

instance, recently, we reported that the novel role of KAI1 on long-term hematopoietic stem cell maintains quiescence by interacting with DARC on macrophage in bone marrow (Hur et al., 2016).

Here, we discovered another distinctive role of KAI1 on mural cell in angiogenesis. By performing various experiments *in vivo* and *in vitro*, including gain-of-function and loss-of-function assays, we revealed that KAI1/CD82 on mural cell is a new distinct suppressor of angiogenesis in quiescent niche.

Ⅱ. Materials and Methods

Mice

Kai1 knock-out (*Kai1*$^{-/-}$) mice (C57BL/6 background) and C57/BL6 mice were used in this study. *Kai1*$^{-/-}$ mice is generated as separately described below and adult C57/BL6 mice were purchased from Jackson Laboratories served as control. 6-to-12-week-old mice were used for every adult animal study. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) in Seoul National University Hospital and were performed in compliance with the "Guidelines for the Care and Use of Laboratory Animals" from the National Research Council.

Generation of *Kai1*$^{-/-}$ Mice

To create a conditional targeting vector in which exon 5 and exon 6 of the *Kai* gene were flanked by loxP sites, the genomic region from exon 4 to exon 7 used to construct the targeting vector was first subcloned from a BAC clone (Source BioScience) into a pBluescript phagemid system. The FRT-flanked neomycin cassette containing a loxP sequence was inserted at the 3' end of exon 6, and a single loxP site was inserted at the 5' of exon 5. 20mg targeting vector was linearized using NotI restriction enzyme and transfected into E14Tg2A ES cells (BayGenomics) by electroporation. After neomycin selection, surviving clones were expanded to identify recombinant embryonic stem cell (ESC) clones by Southern blot analysis. Following EcoRI digestion, the bands representing WT and targeted alleles are 11.5 kb and 7.7 kb, respectively. The

DNA probe used in Southern blot analysis was a short fragment contained in exon 4. Targeted ESCs were microinjected into C57BL/6 blastocysts, which were used to generate chimeras. Male chimeras were mated to C57BL/6 female mice to obtain F1 heterozygous offspring. The neomycin selection cassette was deleted by crossing targeted heterozygous F1 with FLP deleter mice (The Jackson Laboratory, strain 003946). Genotypes were verified by PCR and Southern blot. The PCR primers used in genotyping were as follows:

primer A : 5' -GGGTCCCCTAGGAAATTCAA-3'

primer B : 5' -ATGATGCAGATGTTCTCTCAGGGTG-3'

primer C : 5' -ACAGGGGACTCACCC TACAAGG-3'

All mice were backcrossed to C57BL/6 for at least ten generations. Prm-cre transgenic mice were purchased from Taconic. All procedure is identical to our previous published paper, which proceeded with this experiment together to share this knock-out mice (Hur et al., 2016). This study was reviewed and approved by the institutional animal care and use committee of the National Cancer Center Research Institute.

Mouse embryo harvest

Mice were mated at night time and the morning when a vaginal plug was detected in female mice was regarded as embryonic day 0.5 (E0.5). At E11.5, embryos were harvested and histologically observed as previously described (Zeeb et al., 2012).

Mouse retina

Mice were put under anesthesia and their eyeballs including the optic nerve were taken from mice at the postnatal day 4 and 5

and week 15. Adult mice are perfused with heparin/PBS before taking the eyeballs. The retina were harvested, processed as previously described (Pitulescu et al., 2010). The retinas were stained with Lectin from Bandeiraea simplicifolia (Griffonia simplicifolia) FITC conjugate (Sigma), α-SMA TRITC conjugate (Sigma), KAI1 (Santacruz), where necessary and then examined under confocal microscopy (Zeiss).

Aortic Ring assay

Aorta was harvested from WT and *Kai1*$^{-/-}$ mice. The aorta explanted on GFR-Matrigel (BD Biosciences) coated confocal dish (Ibidi) with DMEM (Gibco) supplemented with 5% (v/v) FBS (Gibco) media. At day 5 to 7, the number of sprouts and branching points were measured by ImageJ (National Institutes of Health).

Tail wound healing assay

Full-thickness wounds were made as previously described (Cho et al., 2006). Briefly, identical excisions were made on the dorsal surface of the tail of 12-week-old male WT and *Kai1*$^{-/-}$ mice using a surgical blade. At day 0, 3, 5, 7, 11, 14, the tails were photographed and wound area was analyzed with ImageJ (National Institutes of Health).

Cells

MS1 (mouse endothelial cell line) was cultured in DMEM high glucose (Gibco) supplemented with 5% FBS (Gibco) and 1X Antibiotics-antimycotic (Gibco). C3H/10T1/2 (mouse mural cell line) was maintained in RPMI 1640 HEPES (Gibco) supplemented with 10% FBS (Gibco) and 1X Antibiotics-antimycotic (Gibco).

Mouse primary aortic mural cells (MAMCs) and mouse primary aortic endothelial cells (MAECs) were harvested as previously described (Kobayashi et al., 2005) and expanded in DMEM low glucose (Gibco) supplemented with 10% FBS (Gibco) and EGM-2MV (Lonza), respectively. MS1, MAEC were cultured on 1.5% gelatin-coated dishes. MAMC was cultured on 0.1% gelatin-coated dishes.

Matrigel tube formation assay

Confocal dishes (Ibidi) were coated with 80㎕ of growth factor-reduced Matrigel (GFR-Matrigel, BD biosciences) and incubated for 30 minutes in 37℃ incubator for polymerization. As for the EC-MC co-culture experiment, $3x10^4$ MS1 and $1x10^4$ WT/*Kai1*$^{-/-}$ MAMCs were then seeded on polymerized GFR Matrigel with 5% FBS supplemented EBM media. As for EC-conditioned medium set, $3x10^4$ MS1 was seeded on GFR-Matrigel (BD biosciences) coated confocal dishes (Ibidi) with conditioned media of MAMCs from WT or *Kai1*$^{-/-}$ mouse. Random fields were measured in terms of tube length and number of branching point by ImageJ (National Institutes of Health).

Virus transduction

Adenoviral plasmid carrying mouse *Kai1* was created with the pAdEasy vector system. The mouse *Kai1* plasmid was purchased from R&D systems. m*Kai1* cloned adenoviral or mock plasmid were transfected into HEK293A cell and harvested as reference (Lee et al., 2014). Purified adenovirus was titrated using the PFU (Plaque Forming Unit) assay and transduced into the target

cell (MS1 or 10T1/2 cell).

Immunofluorescence imaging

Cells were fixed in 2% cold paraformaldehyde (Wako) for 10 minute on ice. Mouse eyes (retina), upon removal, were immediately fixed in 2% paraformaldehyde for overnight at 4℃. After washed with PBS for overnight at 4℃, samples were blocked with 1% BSA and 0.1% Triton X-100 for overnight at 4℃ with rotation, followed by staining with primary antibodies against molecules of interest. When necessary, samples were incubated with secondary antibodies. The nucleus was stained with DAPI. For immunohistochemistry of frozen sections, samples were frozen in OCT embedding medium (Sakura) or embedded in paraffin, and then prepared as microscope slides, which were stained with appropriate primary and secondary antibodies. Confocal images were acquired using Zeiss LSM-710 META confocal microscope and ZEN 2008 analysis software.

RNA sequencing analysis

RNA sequencing reads were aligned to the mouse genome build mm9 (NCBI37)(Pruitt et al., 2007) using TopHat 2.0.9 (Trapnell et al., 2009) and Bowtie 0.12.9 (Langmead et al., 2009) with a segment-length of 21, which allowed 2 mismatches per read. Expression levels for 23,170 RefSeq genes were measured by reads per kilobase per million mapped reads (RPKM)(Mortazavi et al., 2008). Differentially expressed genes between conditions were tested by Cuffdiff. Differentially expressed genes were defined as having more than a 2-fold change and an adjusted p-value less than 0.05. Hierarchical clustering of samples was

performed by R (www.R-project.org). Gene lists related with a specific function annotation of GO (Gene Ontology) were obtained from the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org). Most of RNA sequnecing protocol is same with our previous published paper (Hur et al., 2016).

Protein and RNA analysis

Cells were lysed with lysis buffer (Thermo Fisher) containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Thermo Fisher). Total protein was immunoblotted with primary antibodies against KAI1, LIF (Santa Cruz Biotechnology), tSRC, pSRC (Tyr416), pSRC (Tyr527) (Cell Signaling), followed by incubation in proper HRP-conjugated secondary antibodies (Jackson Laboratories). ACTIN (Santa Cruz Biotechnology) was used as an internal control.

RNA was extracted using either TRIzol (Invitrogen) or an RNeasy RNA isolation kit (Qiagen). Total RNA was reverse transcribed into cDNA with amfiRivert cDNA synthesis premix (Gendepot). Semi-quantitative PCR was performed with Maxime PCR Pre-Mix (Intron) according to manufacturer' s instructions and real-time PCR was performed with POWER SYBR® Green PCR Master Mix (Applied Biosystems) using an ABI PRISM TM 7500 Sequence Detection System (Applied Biosystems). Information on primers for RT-PCR and RT-qPCR is provided in supplementary tables.

Statistical analysis

Data are expressed as means ± S.D. The statistical significance

of the difference between two groups was evaluated with an unpaired t-test and the significance among three groups was analyzed with one-way analysis of variance (ANOVA) followed by Bonferroni's method. In all cases, multiple experiments were performed at least 3 times independently to verify the reproducibility. Any randomization or blinding was not used in animal models and mice, except sick animal irrelevant to experiment, were not excluded. We used 3 to 6 mice for each experiment. A two-sided probability value $p<0.05$ was considered statistically significant. The number of asterisks on top of the each graphs indicate the significance of statistics as follows; "*", "**", "***", "****" means that p-vaule ranges is 0.01 to 0.05, 0.001 to 0.01, 0.0001 to 0.001 or less than that, respectively. Statistical package SPSS version 18 (SPSS Inc) were used for performing statistical tests.

Ⅲ. Result

1. *Kai1/Cd82* knock-out mice are more angiogenic than wild type mice from the embryo to the adult stage.

As we reported in our recent paper (Hur et al., 2016), we designed the strategy generating *Kai1/Cd82* knock-out ($Kai1^{-/-}$) mice to discover distinctive roles of KAI1 *in vivo* (**Figure 1A**). Homozygous wholebody knock-out was not embryonic lethal. However, compared to wild type (WT) mice, $Kai1^{-/-}$ mice showed different phenotypes from the developmental stage. In terms of morphological appearance, $Kai1^{-/-}$ mouse embryos showed faster body growth, particularly in the head. In addition, growth of vasculature and hemorrhage, especially in the heart, enhanced at embryonic day 11.5. When analyzed by immunofluorescence (IF) imaging, $Kai1^{-/-}$ mice showed a broader and higher expression of CD31 than WT counterpart (**Figure 1B**).

At postnatal days 4 and 5, different aspect of vascular growth was exhibited between retinal vasculature of WT and $Kai1^{-/-}$ mice similar to the difference at embryonic stage (**Figure 1C**, data for postnatal day 4 is not shown). In terms of morphology, the difference of the vasculature between adult WT and $Kai1^{-/-}$mice was not significant (data not shown). However, according to *in vivo* and *ex vivo* angiogenic functional assays including aortic ring assay and tail wound healing model, angiogenesis of $Kai1^{-/-}$ group was more prominent than that of

WT (**Figure 1D and 1E**). In conclusion, even though WT mice finally caught up the vascular morphology of *Kai1*$^{-/-}$ mice at the adult stage, they still showed a different angiogenic capacity. Hence, we concluded that *Kai1*$^{-/-}$ mice have better angiogenic ability than WT mice.

2. Expression of KAI1 is higher in mural cells than in endothelial cells.

Based on our result, we speculated that this observation would be caused by retarded function of vascular component cells (EC or mural cell, MC). First of all, to check the expression pattern of KAI1 in vasculature, we stained adult retina of WT mice for IF analysis. As expected, KAI1 was expressed stronger on MCs than on ECs (**Figure 2A**).

To dissect the basal KAI1 expression in each vascular cell type, we isolated primary mouse aortic endothelial cell(MAEC) and aortic mural cell(MAMC) from WT mice. For cell lines, we used MS1 as an endothelial cell line and 10T1/2, mesenchymal precursor cell line, as mural cell line, as previously reported (Hirschi et al. 1999). All ECs and MCs were verified by proper marker expression anlaysis, respectively (**Figure 2B**).

Based on those marker expressions, mural cells expressed higher KAI1 level than endothelial cell, both in mRNA and protein level (**Figure 2B, 2C** and **2D**). These data show that KAI1 expression is much higher in MCs than in ECs.

3. KAI1 on mural cell plays role as a functional angiogenic suppressor via secretory soluble factor.

Higher expression of KAI1 on mural cell, which is involved in vessel stabilization and maturation, let us regard mural cell as a potential cause of *Kai1*$^{-/-}$ mice' s phenotype. To confirm whether KAI1 on ECs or MCs is important for angiogenesis, we performed co-culture tube formation assay using mock or *Kai1*-overexpressed (*Kai1* O/E) MS1 and 10T1/2 cells. Surprisingly, *Kai1* O/E in MCs (10T1/2) strongly suppressed tube formation, whereas that in ECs (MS1) had a relatively minor effect (**Figure 3A**).

This result led us to compare the sprouting capacity of ECs cultured with primary WT or *Kai1*$^{-/-}$ MC as a loss-of-function experiment. Consistent with the result above, *Kai1*$^{-/-}$ MCs group showed longer tube length and more branching points than WT MCs (**Figure 3B**). From those results, we concluded that KAI1 on mural cell is a major player to exhibit anti-angiogenic effect.

Next, we were curious about its mechanism to suppress vessel sprouting by mural KAI1. Assuming that it would be mediated by soluble factor secreted from MC, EC (MS1) was cultured with WT or *Kai1*$^{-/-}$ MC conditioned media. Notably, even without co-culture, WT mural cell conditioned media could suppress tube formation of EC, while *Kai1*$^{-/-}$ MC conditioned media could not (**Figure 3C**). The results indicate that there is certain angiogenic soluble factor, secreted from MC and enhanced by mural KAI1.

4. KAI1 induces expression and secretion of LIF, an anti-angiogenic secretory soluble factor.

Using RNA sequencing of WT and *Kai1*$^{-/-}$ MCs, we decided to find this soluble factor, which is under control of mural KAI1. 4 genes are selected, which is significantly down-regulated in *Kai1*$^{-/-}$ MCs. To narrow down the target molecule, we utilized angiogenesis related gene onotolgy. While gene expression level of positive angiogenic regulators (Gene Ontology (GO) : 0045766) was similar in the two samples, the expression of negative angiogenic regulators (GO : 0016525) was significantly decreased in *Kai1*$^{-/-}$ MCs. And in combination with negative regulation of cell cycle to find the factor that surppress proliferation of EC (GO : 0045786) and the genes related to blood vessel remodeling (GO : 0001974), we selected LIF as a target of anti-angiogenic soluble molecule in the downstream of KAI1 (**Figure 4A**). In *Kai1*$^{-/-}$ and over-expressed (O/E) MCs, expression pattern of LIF protein was manually confirmed by western blotting and ELISA assay, which was well-matched with RNA sequencing result. As expected, LIF expression was positively correlated with KAI1 expression on MCs (**Figure 4B and 4C**).

To be more precise demonstration, we performed further experiment neutralizing LIF in tube formation assay. As a result, paracrine effect of anti-angiogenic LIF was effectively reversed by neutralizing LIF in supernatant of *Kai1* O/E 10T1/2 (**Figure 4D**). In conclusion, KAI1 on MCs shows anti-angiogenic effects toward ECs via secretion of LIF.

5. KAI1 up-regulates LIF via SRC-p38/AKT pathway.

To dissect which signaling pathway is involved in transducing signal from KAI1 molecule to induce LIF secretion, we performed inhibitor assay by RT-PCR. Several chemical inhibitors related to angiogenic signaling molecules were selected based on prevalent VEGFR signaling pathways (Ivy et al., 2009). When we treated these inhibitors to *Kai1* O/E MC (10T1/2), LIF expression was dramatically suppressed only by SRC, p38 and Akt inhibitors (**Figure 5A**).

Among those effective signaling molecules, we focused on SRC protein, known as working at upstream of p38 and Akt signaling (Yen et al., 2005). Since SRC has two different forms, active and inactive, we checked expression of each form in *Kai1*$^{-/-}$ and O/E MCs. Interestingly, expression of active SRC (Tyr416-phosphorylated; pY416) was higher in *Kai1* O/E MCs and lower in *Kai1*$^{-/-}$ MCs. Expression of inactive SRC (Tyr527-phosphorylated; pY527) showed the opposite pattern, while total SRC remained unchanged (**Figure 5B**). Quantification of those observations for mock and *Kai1* O/E 10T1/2 showed what we explained above more clearly (**Figure 5C**). From those experiments, we discovered that mural KAI1 regulates LIF expression and secretion via SRC-p38/Akt pathway.

Ⅳ. Discussion

In this study, we discovered functional role of KAI1 molecule on mural cell (MC) as a braker of angiogenesis in quiescent niche. From the developmental stage to the adult, *Kai1* knock-out (*Kai1*$^{-/-}$) mice showed better angiogenic ability than wild type (WT) mice *in vivo* and *in vitro*. Even though our knock-out mouse model has limitation in terms of its cell type specificity, we overcame it by isolating primary MCs from WT and *Kai1*$^{-/-}$ mice and comparing them. As a result, even though KAI1 molecules expressed both on endothelial cells (ECs) and MCs, majority of them were on the mural cell and mural KAI1 only played a role as a functional angiogenic suppressor. To elaborate the function of KAI1 *in vivo*, we should do make mural cell specific knock-out mice for further studies.

And for the first time, we also figured out that KAI1 on mural cell is involved in signaling transduction, finally showing anti-angiogenic effect. As mentioned in introduction, KAI1 has been actively studied as a suppressor of tumor metastasis. Mashimo and his colleague reported that KAI1 on tumor cells is directly activated by p53 (Mashimo et al., 1998). Inspired from that, we can speculated that mural KAI1 may also directly activate its downstream signaling, which is discovered as SRC-p38/Akt pathway at quiescent niche in our result. It implies important message that KAI1 on mural cell becomes functional molecule, not just a facilitator of other proteins. However, how KAI1 itself is activated and how its expression level would be regulated is still elusive. As previous studies speculated, we also

think that the over-expression of KAI1 may induce its activation (Wu et al., 2011; Odintsova et al., 2013).

From our result, KAI1 could transduce the signaling via SRC-p38/Akt pathway to secret leukemia inhibitory factor (LIF) as an anti-angiogenic soluble factor. In previous reports, LIF was already confirmed as anti-angiogenic molecules (Pepper et al., 1995; Kubota et al., 2008). However, it is novel that LIF is secreted by mural cells and regulated by KAI1 molecule on mural cell. We are currently studying how this LIF affects EC to show anti-angiogenic phenotype in molecular level. Not only the paracrine effect, but also the possibility of direct contact between mural KAI1 and some endothelial molecule should be investigated to elaborate on the anti-angiogenic mechanism of mural KAI1.

Most of anti-cancer drugs is traditionally derived from the anti-angiogenic drug, which blocks the nutrient supply to cancer via angiogenesis. Concept blocking VEGF pathway has been widely developed and used in clinical fields as an anti-angiogenic therapeutic target. Bevacizumab (trade name Avastin®) and ranibizumab (trade name Lucentis®) are the most popular anti-angiogenic drugs inhibiting VEGF (Rosenfeld et al., 2006; Shih and Lindley, 2006). However, these systemic blocking of VEGF induced various adverse effects like hemorrhage, worsening coronary artery diseases or peripheral artery diseases etc (Semenza, 2008). Some patients even showed evasive resistance to those anti-VEGF therapies in many complexities of unknown reasons (Shen and McDonald, 2012; Tranos et al., 2013). Therefore, there is still novel

priority developing new anti-angiogenic drug with different approach.

Our result can suggest KAI1 as a new target of anti-angiogenesis and anti-cancer drug, which can inhibit tumor metastasis and angiogenesis at the same time. Additionally, unlike research trend in the past, recent studies have given weight to finding anti-angiogenic mechanism in MC as much as in EC among their interactions, because the initial step of angiogenesis starts with detaching of MC from the EC. (Clapp et al., 2009). Thus, significance of mural KAI1 and its effector molecule, LIF, should be studied further in tumorigenic niche.

In conclusion, our studies discovered that KAI1 on MC, but not on EC, acts as a efficient suppressor of angiogenesis via SRC-p38/Akt pathway. And this signaling cascade caused increasing and releasing soluble anti-angiogenic factor, LIF, from MC and make EC anti-angiogenic. We expect that this finding could be translated to human case as well and contribute to establish new axis of anti-angiogenesis in academic and clinical field.

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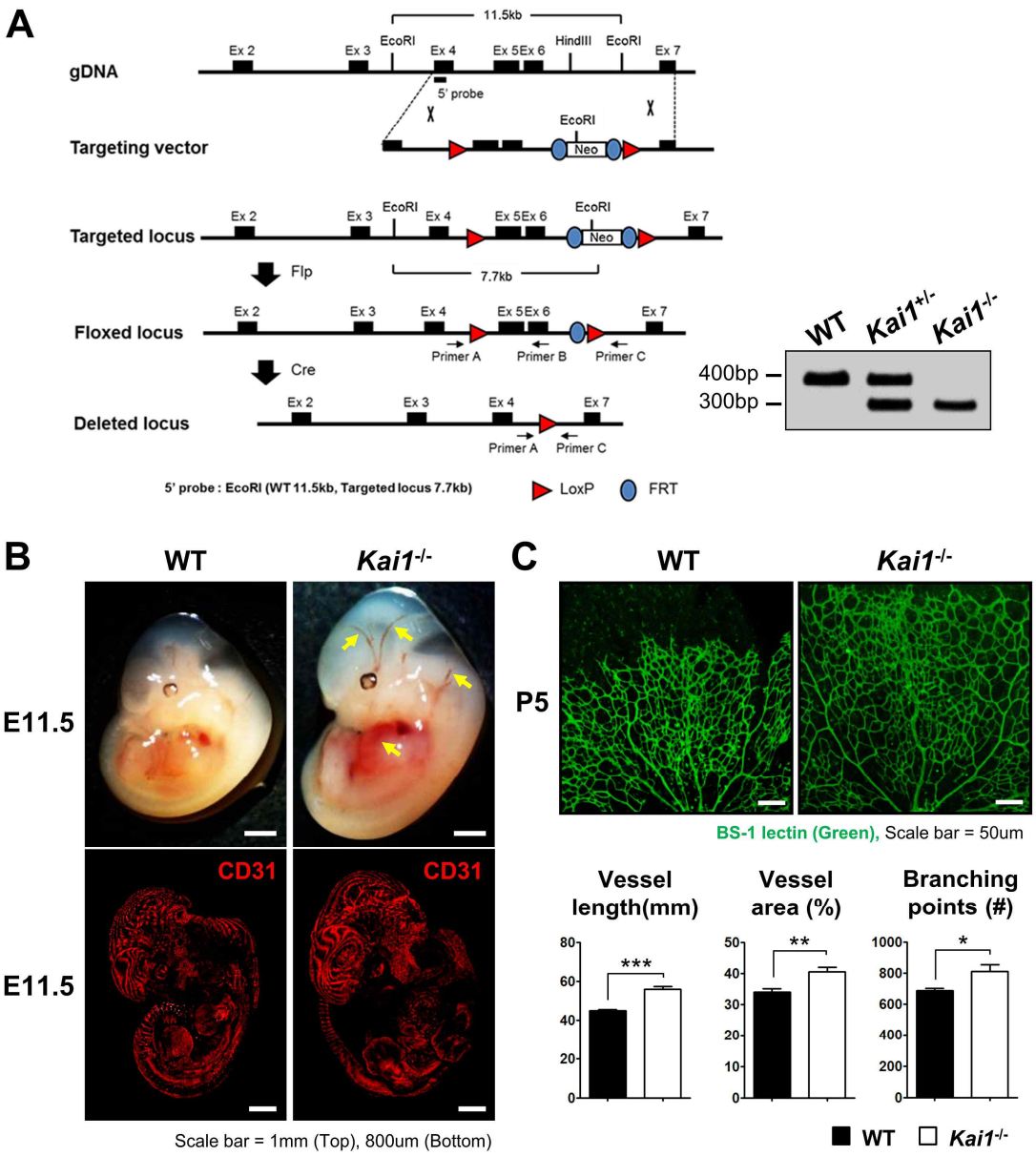
Ⅵ. Figures

[Figure 1]



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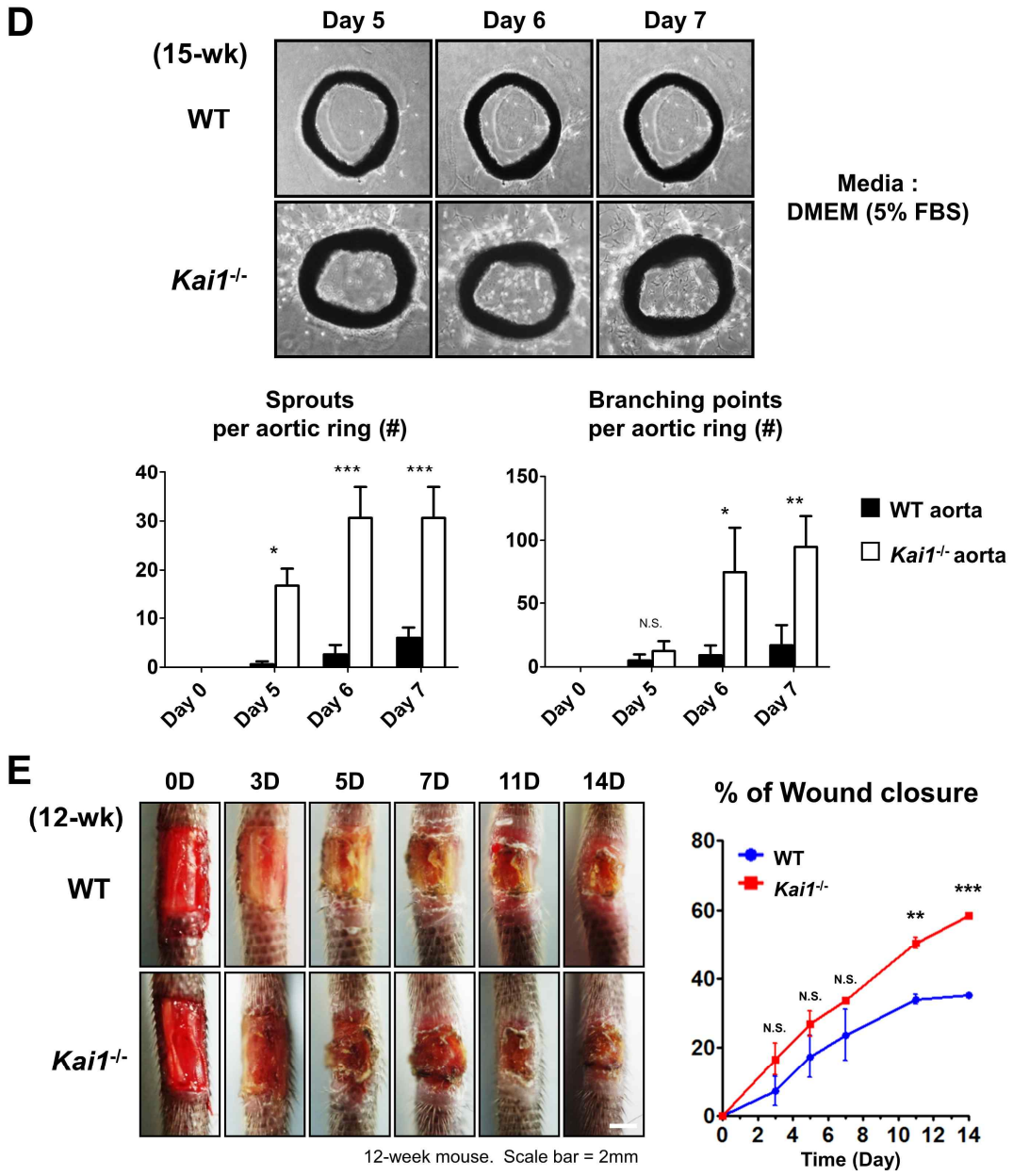


Figure 1. *Kai1/Cd82* knock-out mice are more angiogenic than wild type from embryo to the adult stage.

(A) Strategy to generate *Kai1* knock-out (*Kai1*$^{-/-}$) mice (Left) and genotyping result of possible phenotypes from their littermate (Right). From the top, each components of

schematic figure represents the *Kai1* locus, the targeting vector, and the targeted, floxed and deleted locus in order.

(B) Representative image of whole embryo at embryonic day 11.5 (E11.5) **(Top)** and its immunofluorescent image **(Bottom)** in wild type (WT) and *Kai1*$^{-/-}$ mouse. Yellow arrows indicate the excessive growth of blood vessel in *Kai1*$^{-/-}$ embryo than in WT. Embryo section is stained with CD31 (red) as an endothelial cell marker to compare the vasculature. Scale bar, 1mm(Top) and 800μm(Bottom).

(C) Retinal vasculature of WT and *Kai1*$^{-/-}$ mouse at postnatal day 5 by immunofluorescent (IF) staining **(Top).** IF image is quantified as three criteria; Vessel length(mm), Vessel area(%), Branching points(#) **(Bottom)**. BS-1 lectin (green) is stained as an endothelial cell marker. Scale bar, 50μm.

(D) Aortic ring assay of the aorta from WT and *Kai1*$^{-/-}$ mouse **(Top)**. Aorta from adult mouse (15-week) is cultured in DMEM media supplemented with 5% Fetal Bovine Serum (FBS). Quntification of this assay is performed in terms of sprouts and branching points per aortic ring **(Bottom)**.

(E) Comparison of tail wound healing ability between WT and *Kai1*$^{-/-}$ mouse **(Left)**. Percentage of wound closure is quantified by healed area **(Right)**. Wound healing assay is performed with 12-week adult mice. Scale bar, 2mm.

[Figure 2]

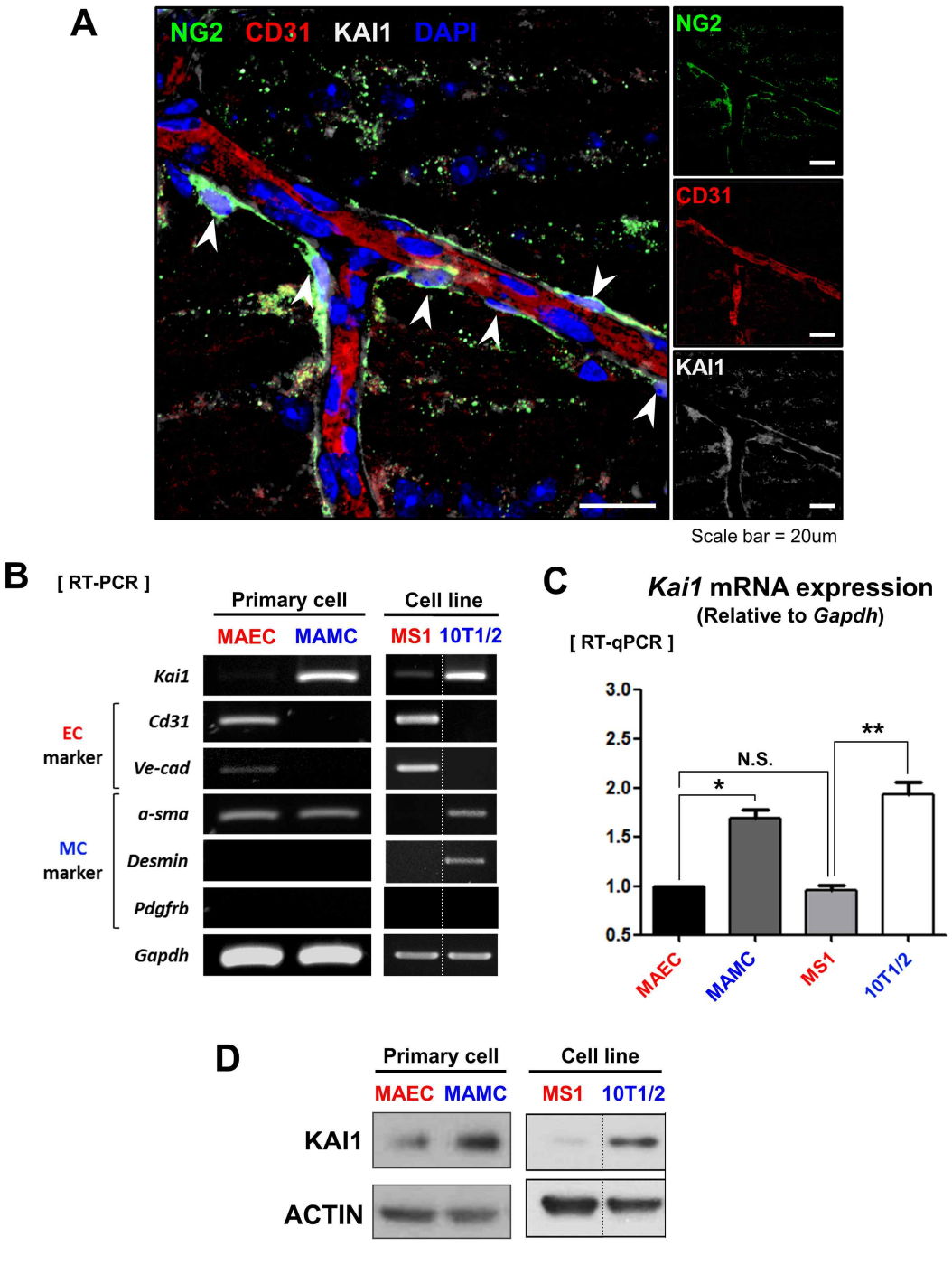


Figure 2. KAI1 is expressed higher in mural cells than in endothelial cells.

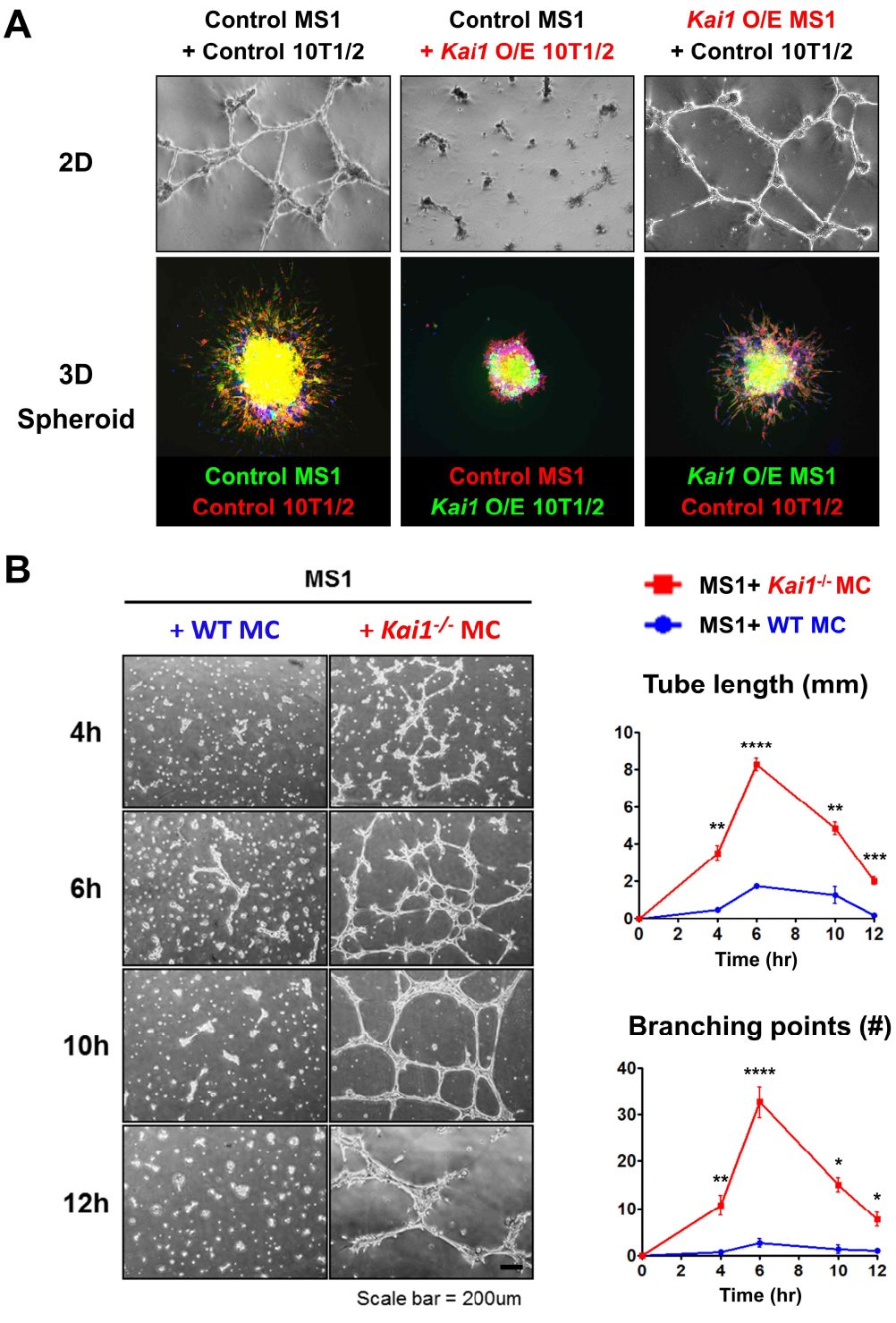
(A) Immonufluoresent image of retina from adult wild type mouse. NG2 (green, mural cell marker), CD31 (red, endothelial cell marker, KAI1 (white) and DAPI (blue, nuclear) is stained. Scale bar, 20㎛.

(B) mRNA level of KAI1 and cell type marker for endothelial cell (EC, red) and mural cell (MC, blue), using RT-PCR. Mouse aortic endothelial cell(MAEC) and mouse aortic mural cell(MAMC) is used as primary cell. For cell lines, MS1 and 10T1/2 is used. *Gapdh* is served as endogenous control.

(C) Quantitative graph of KAI1 mRNA expression in same set of primary cells and cell lines as Figure 2B. Quantification is performed by RT-qPCR and normalized to *Gapdh* expression level. *Gapdh* is served as endogenous control.

(D) KAI1 protein expression level of endothelial cell(red) and mural cell(blue) by western blotting. ACTIN is served as endogenous control.

[Figure 3]



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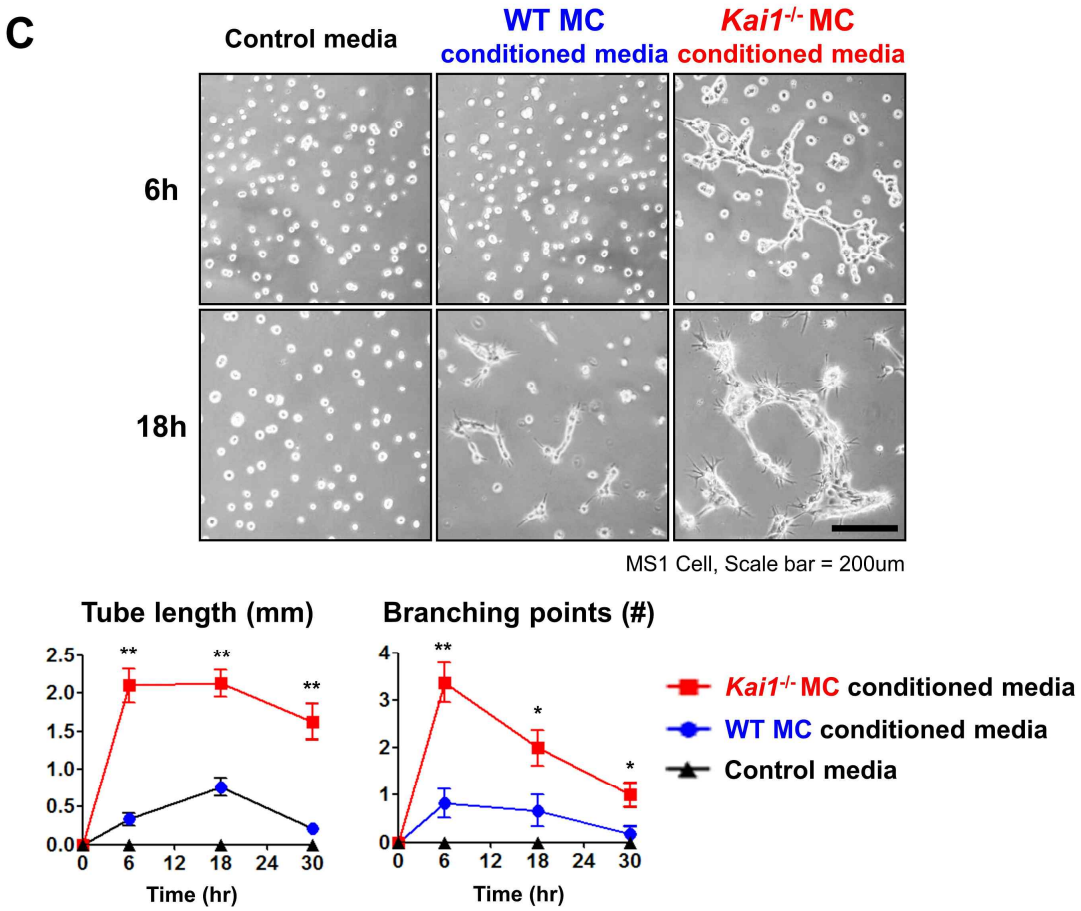


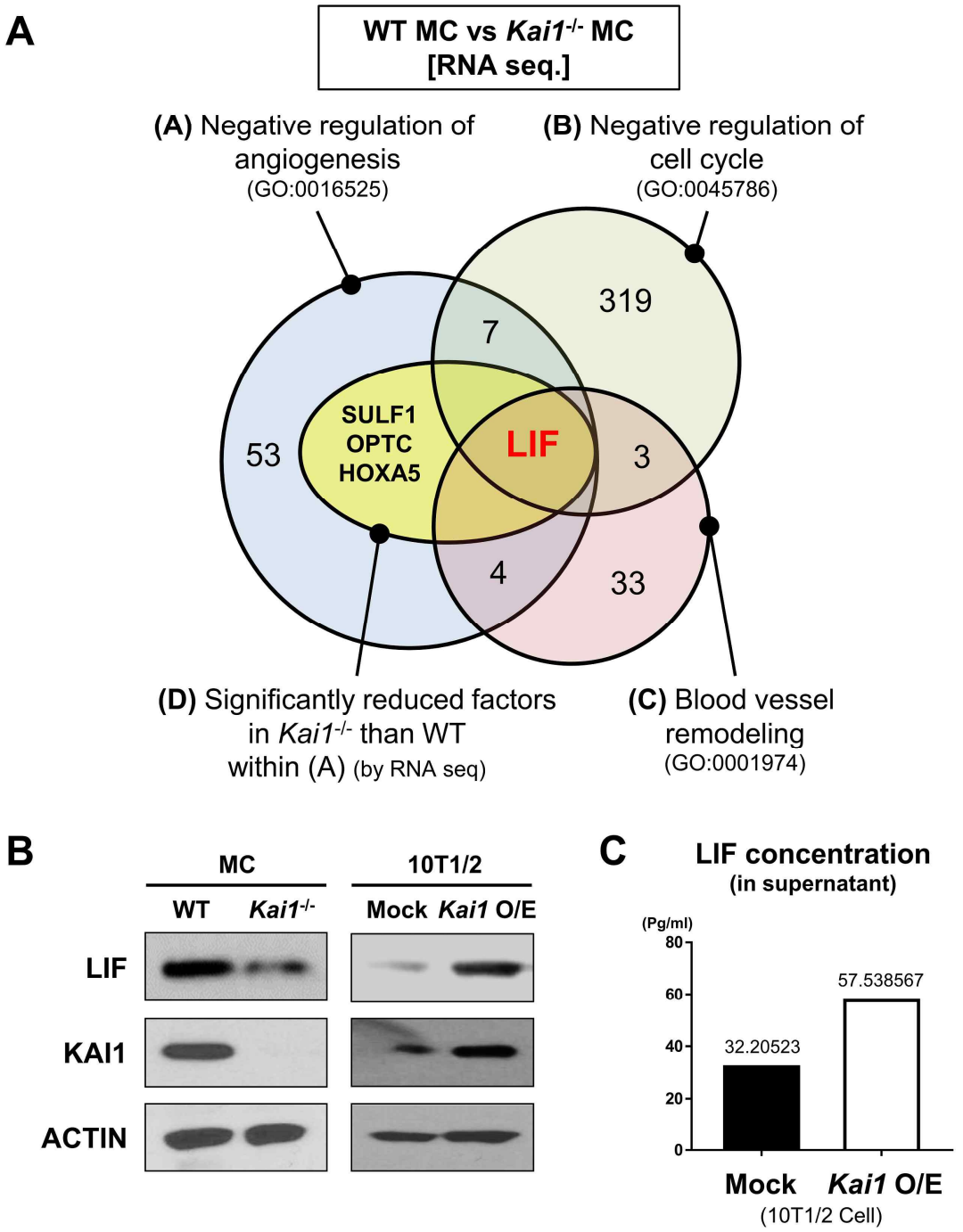
Figure 3. KAI1 on mural cell plays role as a functional angiogenic suppressor via secretory soluble factor.

(A) Tube formation assay in 2D and 3D spheroid culture. Control or *Kai1* over expressed(O/E) EC line (MS1) and mural cell (MC) line (10T1/2) is mixed in combination for each experimental set. For *Kai1* O/E cells, GFP-tagged adenovirus is transduced. Scale bar, 200 μm.

(B) Tube formation of EC line (MS1) with WT or *Kai1*$^{-/-}$ Mural cells **(Left)** and its quantification **(Right)**.

(C) Tube formation of EC line (MS1) only with conditioned media of WT or *Kai1*$^{-/-}$ Mural cells **(Top)**. Quantification is done as Figure 3B **(Bottom)**. Scale bar, 200 μm.

[Figure 4]



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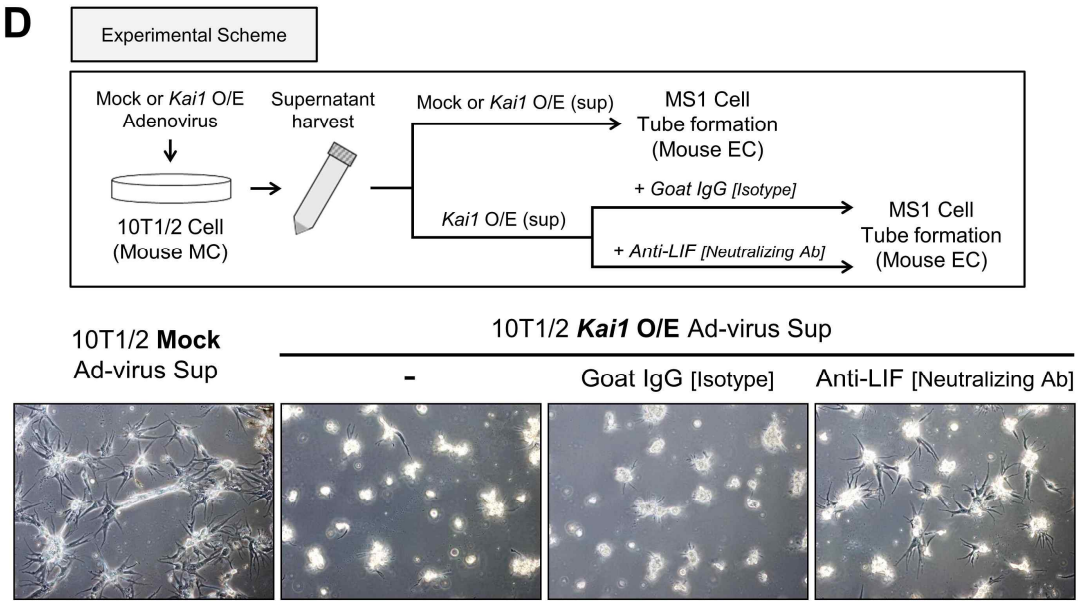

Figure 4. KAI1 induces expression and secretion of LIF, which is an anti-angiogenic secretory soluble factor.

(A) Rationale for narrowing down to LIF as an target gene of KAI1 down stream, based on RNA sequencing and gene ontology. RNA expression of WT and *Kai1*$^{-/-}$ MCs is sequenced and gene ontology categories are selected in terms of factors related to anti-angiogenesis.

(B) Protein expression level of LIF in WT and *Kai1*$^{-/-}$ Mural cells as a gain-of-function test, and in mock and *Kai1* O/E MC line (10T1/2) as a loss-of-function experiment.

(C) LIF concentration in supernatant of mock and *Kai1* O/E MC (10T1/2) line by ELISA assay.

(D) Anti-angiogenic effects of LIF by tube formation assay of EC (MS1) with conditioned media of mock or *Kai1* O/E MC (10T1/2) and its neutralizing experiment using LIF neutralizing antibody(Ab). Experimental scheme is presented on the top.

[Figure 5]

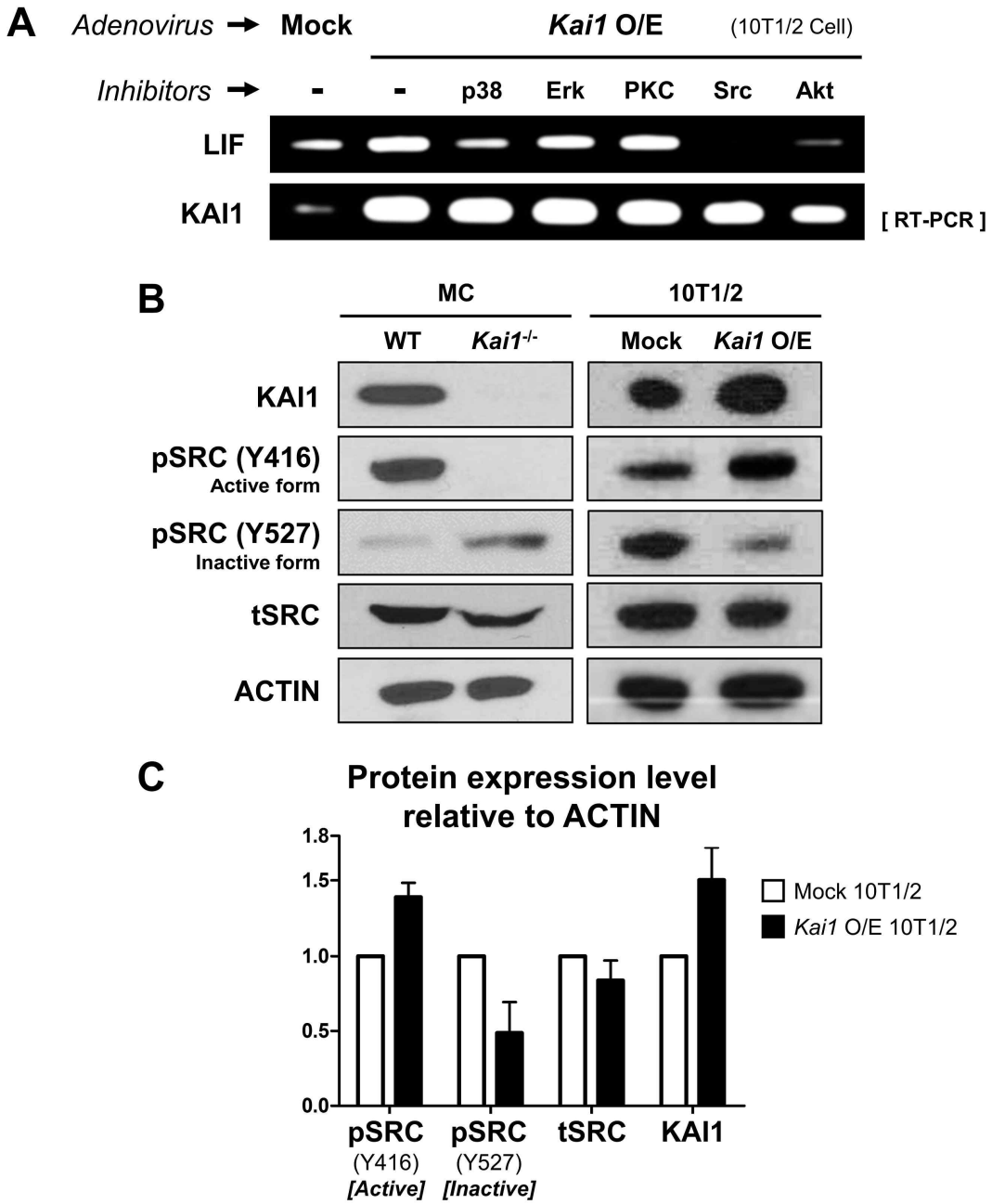

Figure 5. KAI1 upregulates LIF via SRC-p38/AKT pathway.

(A) LIF expression of Mock or *Kai1* O/E mural cell (10T1/2) treated with inhibitors of various typical signaling molecules in angiogenesis related pathways.

(B) Protein expression level of SRC family in WT and *Kai1*$^{-/-}$ Mural cells as a gain-of-function concept, and in mock and *Kai1* O/E 10T1/2 cell as a loss-of-function concept. SRC family includes Phosphorylated SRC (pSRC; Y416, active form, and Y527, inactive form) and total SRC (tSRC). ACTIN is served as endogenous control.

(C) Quantification of western blot for mock and *Kai1* O/E 10T1/2 group in Figure 5B. All expression levels are normalized to ACTIN. ACTIN is served as endogenous control.

[Figure 6]

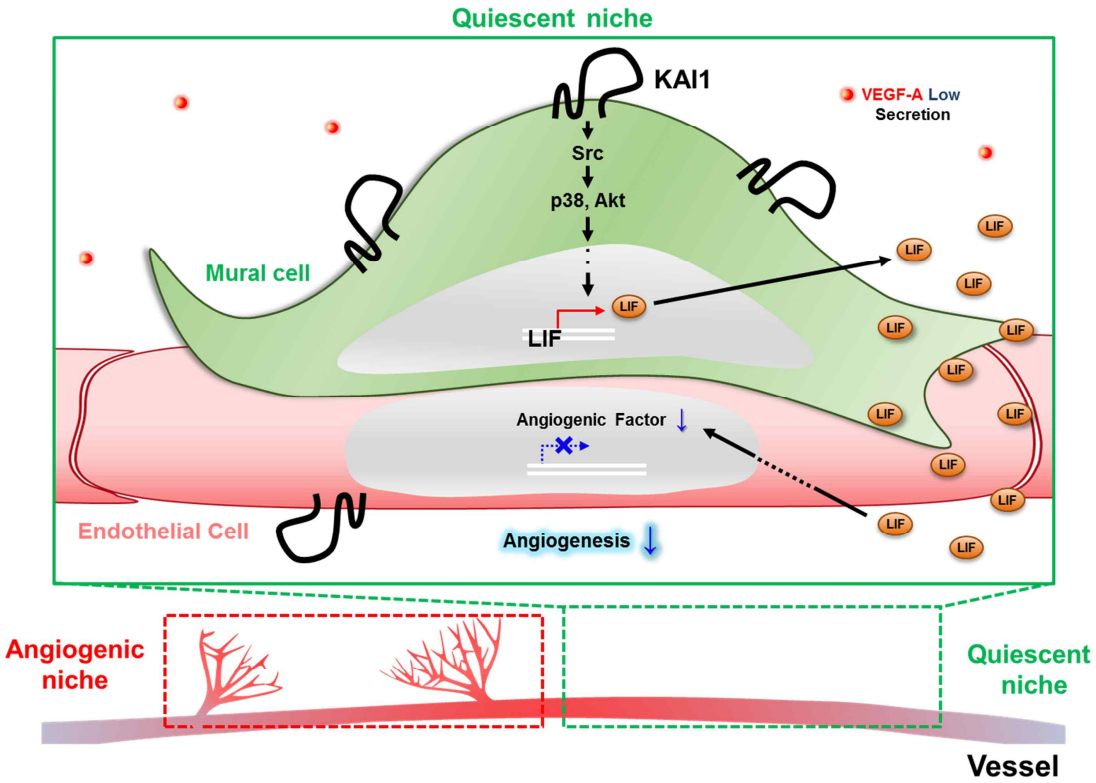

Figure 6. Schematic figure of the results

At quiescent niche, mural KAI1 up-regulates LIF via SRC-p38/Akt pathway. And by secreting LIF, EC blocked its angiogenic function and niche remain quiescent.

Ⅶ. Tables

Table 1. List of mouse primers (RT-PCR)

gene name	F,R	sequence (5' → 3')	size (b.p.)	Tm (℃)
α-sma	F	CTGACAGAGGCACCACTGAA	311	60
	R	ATCTCACGCTCGGCAGTAGTA		60
Desmin	F	TGCAGCCACTCTAGCTCGTA	393	61
	R	CTCATCAGGGAGTCGTTGGT		60
Gapdh	F	CCCTTCATTGACCTCAACTACAT	343	58
	R	CATTGCTGACAATCTTGAGTGAG		58
Kai1	F	CAGCCACTACAACTGGACAGAG	339	61
	R	TACTTGGGGACCTTGCTGTAGT		61
Pdgfrb	F	AGTCCCGGCTACCCTATCTG	426	60
	R	CAACTGGCCTCTGAGGACTAAA		60
Ve-cad	F	ATTGAGACAGACCCCAAACG	344	58
	R	ATTCGGAAGAATTGGCCTCT		57

Table 2. List of mouse primers (RT-qPCR)

gene name	F,R	sequence (5' → 3')	size (b.p.)
Kai1	F	GCCTGGGACTACGTGCAG	73
	R	CCTCGTTCTCTGTCCAGTTGT	
Gapdh	F	TGTCCGTCGTGGATCTGAC	75
	R	CCTGCTTCACCACCTTCTTG	

국문 초록

혈관주변세포에서 발현하는 KAI1/CD82의 혈관신생 억제자로서의 기능에 관한 연구

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김 태 원

모든 기관들은 혈관을 통해 영양소와 산소를 공급받고 노폐물을 배출한다. 따라서, 혈관은 모든 기관의 생존에 중요할 뿐 아니라 유일하게 전신에 퍼져 그 역할을 한다. 이러한 혈관망을 형성하는 핵심 기작을 혈관신생(angiogenesis)라고 한다. 혈관은 크게 혈관의 장벽 역할을 하는 혈관 내피세포와 혈관의 형태 유지 및 수축을 담당하는 혈관주변세포로 이루어져 있다. 건강한 신체의 경우, 이 두 가지 혈관세포가 서로 상호작용을 통해 혈관신생이 생리학적으로 잘 조절이 되어 적재적소에서만 일어난다. 하지만, 각종 암이나 망막병증 등의 질병이 생길 경우, 비정상적인 혈관신생, 즉 병리학적 혈관신생이 일어나게 되고 이는 이러한 질병들의 예후를 악화시키는 주요 원인이 된다.

지금까지의 병리학적 혈관신생을 차단하는 전략은 혈관 내피세포를 주로 항혈관신생제의 표적 세포로 여겨왔다. 하지만, 최근 여러 기초 연구 및 임상시험 결과들로부터, 기존의 내피세포를 표적으로 하는 항혈관신

생제들이 일부 암환자에게 내성이 있는 것으로 밝혀졌다. 따라서, 최근에는 이 같은 한계를 극복하고자, 혈관을 구성하는 또 다른 세포인 혈관주변세포가 새로운 항혈관신생제의 표적이 되었다. 이 뿐만 아니라, 기초 연구 분야에서도 아직 혈관 내피세포와 혈관주변세포 간의 상호작용이 어떻게 혈관이 휴면상태에 머물도록 조절하는지 분자 수준에서는 명확히 알려진 바가 없다.

이 논문에서는 테트라스파닌(Tetraspanin) 분자 중 하나인 KAI1/CD82가 휴면상태의 혈관주변세포에서 발현하면서 혈관신생을 억제하고 있는 새로운 기능을 하는 분자라는 것을 밝혀내었다. KAI1의 기능을 알아보고자 *Kai1* 유전자가 완전히 결여된 쥐를 제작하였고, 발달단계에서부터 성체까지 인비보 (*in vivo*) 실험과 인비트로 (*in vitro*) 실험에서 모두 야생형 쥐와 비교하였을 때, *Kai1*이 결여된 쥐의 혈관신생능이 월등히 높았다. 또한, 각각 혈관 내피세포와 혈관주변세포에 대한 야생형 쥐의 초대세포(primary cell) 및 세포주(cell line)에서 KAI1 분자의 발현량을 비교해 보았을 때, 두 세포 모두 KAI1을 발현하였으나, 혈관주변세포에서 그 양이 지배적으로 많았다. 양 뿐만 아니라 아데노 바이러스를 이용한 KAI1을 과발현을 이용한 혈관 신생능 비교실험에서, 혈관 내피세포가 아닌 혈관주변세포의 KAI1만이 기능적인 혈관신생 억제 분자로서의 역할을 했다. 또한, 이러한 혈관주변세포에 존재하는 KAI1의 항혈관신생 능력은 SRC-p38/Akt라는 신호 전달 체계를 거쳐, 가용성 항혈관억제 인자인 백혈병 억제 인자 (Leukemia inhibitory factor, LIF)의 발현 및 분비를 증가시켜서 혈관의 휴면 상태를 유지한다는 것을 밝혔다. 이 연구를 통해 KAI1이라는 분자가 기초 및 임상 연구에서 항혈관신생 분야의 새로운 지평을 열 것으로 기대한다.

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주요어 : KAI1/CD82, 혈관주변세포, 혈관신생, 테트라스파닌, 혈관, 항혈관신생, 백혈병 억제 인자 (LIF)

학 번 : 2014-24804