Crucial role of κ opioid receptor system in tumor angiogenesis

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Abbreviations

AM: Adrenomedullin

ANOVA: Analysis of variance

ATP: Adenosine triphosphate

cAMP: 3’5’-cyclic adenosine monophosphate

b.i.d.: bis in die

BNI: Norbinaltorphimine. 17,17'-(Dicyclopropylmethyl)-6,6',7,7'-6,6'-imino-7,7'-binorphinan-3,4',14,14'-tetrol dihydrochloride

DAMGO: [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl sulfoxide

DOR: δ-Opioid receptor

E: Embryonic day, ex) E12.5: Embryonic day-12.5

EC: Endothelial cell

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

EGM-2: endothelial cell growth medium-2

ES: Embryonic stem

FACS: fluorescence activated cell sorting

FBS: Fatal bovine serum (newborn calf serum)

FDA: Food and Drugs Administration

FGF: Fibroblast growth factor

FITC: Fluorescein isothiocyanate

Flk1: Fetal liver kinase 1

Flt1: Fms-related tyrosine kinase 1

Gapdh: Glyceraldehyde 3-phosphate dehydrogenase

GEM: Gemcitabine

Gi: Inhibitory G protein

GPCR: G protein coupled receptor

HBSS: Hank's Balanced Salt Solution

HIF: Hypoxia-inducible transcription factor

HRP: Horseradish peroxidase

HUVEC: Human umbilical vascular endothelial cell

iPS: induced pluripotent stem

KDR: Kinase insert domain receptor

KO: Knock out

KOR: κ-Opioid receptor

LLC: Lewis lung carcinoma

MoAB; Monoclonal antibody

MOR: μ-Opioid receptor

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide

Myc: Myelocytomatosis oncogene

NAc: Nucleus accumbens

NAL: Nalfurafine

NRP: Neuropilin

OCT: Optimum cutting temperature

PBS: Phosphate-buffered saline

PDAC: Pancreatic ductal adenocarcinoma

PDGF: Platelet-derived growth factor

PDYN: Prodynorphin

PE: Phycoerythrin

PGE2: Prostaglandin E2

PGF: Placenta growth factor

PKA: Protein kinase A

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

Ras: Rat sarcoma viral oncogene

RM: repeated measures

RPMI: Roswell Park Memorial Institute

SDS: Sodium dodecylsulphate

SNC80:[(+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzy]-N,N-diethylbenzamide]

TGF-β: Transforming growth factor beta

TKI: Tyrosine kinase inhibitors

TRK-820: Nalfurafine. 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[N-methyl-trans-3-(3-furyl) acrylamido] morphinan hydrochloride

TSP1: Thrombospondin-1

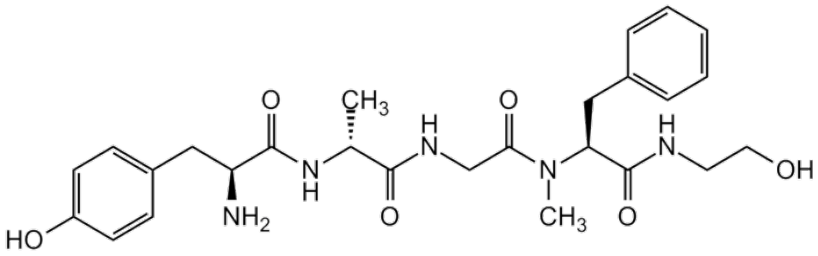
U50,488H: (-)-*trans*-(1S,2S)-U-50488 hydrochloride

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

Structures of drugs used in the present study

DAMGO: [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin



SNC80:[(+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzy

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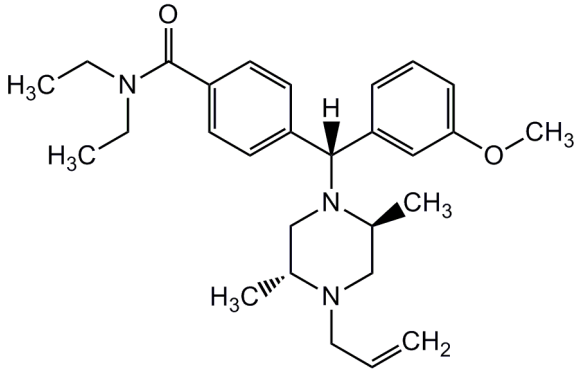
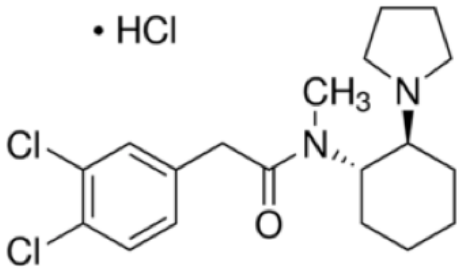
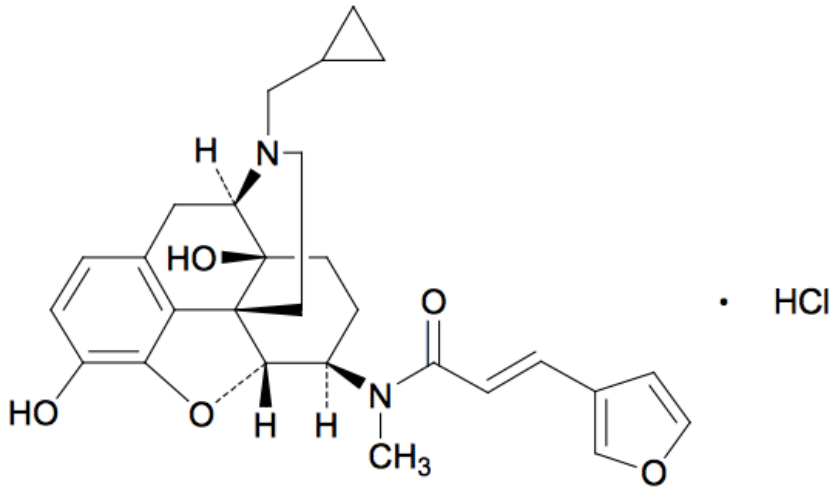


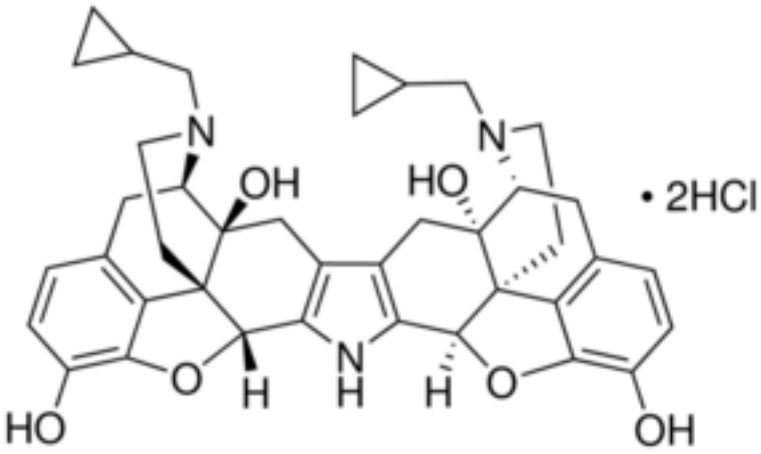
U50,488H: (-)-trans-(1S,2S)-U-50488 hydrochloride



TRK-820 (Nalfurafine): 17- cyclopropylmethyl-3,14b-dihydroxy-4,5a-epoxy-6b-[N-methyl-trans-3-(3-furyl) acrylamido] morphinan hydrochloride



BNI (Norbinaltorphimine): 17,17'-(Dicyclopropylmethyl)-6,6',7,7'-6,6'-imino-7,7'-binorphinan-3,4',14,14'-tetrol dihydrochloride



General Introduction

Angiogenesis

The development of the vascular system is one of the earliest events in organogenesis, and contributes to the formation of most organs in our bodies. The vascular system is first formed as a primitive vascular network by the differentiation and assembly of vascular progenitor cells derived from mesodermal cells. These progenitor cells undergo a complex remodeling process, in which growth, migration, sprouting and pruning lead to the development of a functional circulatory system. Earlier studies have suggested that many of the events in normal vascular formation during embryogenesis are recapitulated during *de novo* angiogenesis in adults such as tumor angiogenesis and neovascularization induced after tissue damage [1]. Furthermore, the disordered vascular function triggers the development of lifestyle-related diseases such as hypertension, diabetes and hyperlipidaemia [2-4]. Thus, a better understanding of vascular biology may lead to novel strategies for the treatment of a variety of diseases.

Role of vascular endothelial growth factor (VEGF) signaling in angiogenesis

Several factors affecting vascular formation, such as VEGF, neuropilin (NRP), angiopoetins, transforming growth factor beta (TGF-β), platelet-derived growth

factor (PDGF), fibroblast growth factor (FGF), ephrin and notch have been identified over the past few decades, mainly by the characterization of vascular mutant phenotypes in mice [5-11]. Among these factors, VEGF signaling is a key modulator of vascular development during embryogenesis and for neovascularization in the adult [12,13]. In mammals, five VEGF ligands, VEGF-A, -B, -C, -D and placenta growth factor (PGF), have been identified and have been shown to bind in an overlapping pattern to three receptor tyrosine kinases, known as VEGF receptor-1, -2, -3 (VEGFR1-3; receptor nomenclature follows Alexander *et al*.) [14], as well as to co-receptors such as heparin sulphate proteoglycans and NRPs. VEGF-A heterozygote knockout mice die early in gestation due to failure of the vascular formation [15]. On the other hand, the two- to threefold overexpression of VEGF-A from its endogenous locus results in abnormal heart formation and lethality at embryonic day (E)-12.5 to E14.0 [16], indicating that strictly balanced VEGF function is important in normal embryogenesis. Furthermore, the intensity of VEGF signaling is strictly regulated through ligand-receptor interaction. VEGFR2 (also known as fetal liver kinase 1 (Flk1) in mouse and kinase insert domain receptor (KDR) in human) is tyrosine-phosphorylated much more efficiently than VEGFR1 (also known as fms-related tyrosine kinase 1 (Flt1)) upon VEGF binding [16-18]. Although VEGFR1 tyrosine kinase-deficient homozygous mice developed normal vessels and survived [19], mice that were homozygous for point mutation at Tyr1173 of VEGFR2 (Tyr1175 in human VEGFR2)

died at E8.5 to E9.5 without any organized blood vessels or yolk sac blood islands, and hematopoietic progenitors were severely reduced, as seen with Flk-1 null mice [20]. Interestingly, VEGFR1-null mice die at midgestation with vascular overgrowth and disorganization [21]. Taken together, these findings suggest that VEGFR2 is the major receptor in endothelial cells (ECs) for VEGF-induced responses, and VEGF signal intensity on VEGFR2 is regulated by the binding of VEGF to the higher affinity receptor.

Role of 3'5'-cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling in angiogenesis

cAMP discovered in the late 1950s marked the birth of the second messenger theory and sparked signal transduction research [22]. Adenylate cyclase generates cAMP from adenosine triphosphate (ATP) in essentially all tissues in the body. This enzyme is embedded in the plasma membrane and is activated by transmembrane receptors that are coupled to trimeric G-protein [23,24]. The effects of cAMP are mediated by various downstream targets, such as PKA [25]. It has been generally accepted that cAMP plays an important role in almost every known physiological action, such as metabolism, gene expression, cell division and growth, and cell differentiation as well as secretion and neurotransmission [26,27]. In cardiovascular biology, cAMP is a critical second messenger in the modulation of vasodilatation, cardiac chronotropic and inotropic

responses, and cellular growth [28]. For example, cAMP-elevating G protein coupled receptor (GPCR) agonists, including adrenomedullin (AM), prostaglandin E2 (PGE2), and β-adrenergic agonists, reduce endothelial permeability [29,30]. In the recent study, it has been reported that cAMP signaling plays a critical role in the reconstitution of ECs through the up-regulation of VEGF receptors, VEGFR2 and NRP1, and arterial specification in vascular development [11,31-33]. Additionally, it has been considered that the activation of PKA positively modulates timing of vascular cell differentiation through epigenetic regulation [34]. These findings indicate that cAMP/PKA signaling could be a crucial mechanism in physiological and pathogenic angiogenesis *in vivo*.

Tumor angiogenesis

It has been generally accepted that tumor angiogenesis is a key event in tumor progression and metastasis, and it enables cancer cells to intake nutrients and oxygen as well as the excretion of metabolic wastes and carbon dioxide, and leads to the acquisition of mobility and invasiveness [2,35,36]. Tumor angiogenesis is caused by disruption of the balance between angiogenesis activators and inhibitors in the tumor microenvironment. There is a growing body of evidence that the several angiogenesis activators, such as VEGF, are abundantly produced in the tumor microenvironment of most cancers, indicating that these disruptions enhance tumor angiogenesis and aggravation [37,38]. Thus, these general findings suggest that anti-angiogenic therapies

could be an important approach to the cancer therapy.

Angiogenesis inhibitors in cancer therapy

The concept of using angiogenesis inhibitors as anti-cancer drugs was received with considerable scepticism when first presented by Dr. Folkman in the early 1970s [35]. Solid tumors cannot grow beyond 2 to 3 mm in diameter without being able to recruit their own blood supply. Bevacizumab, a humanized monoclonal antibody that is specific for human VEGF-A, was the first anti-angiogenic agent approved by the Food and Drugs Administration (FDA) in 2004 for the treatment of colorectal cancer, renal cell cancer, non-small cell lung cancer, and glioblastoma [39]. Furthermore, sunitinib and sorafenib were approved by the FDA in 2008 as multi-target tyrosine kinase inhibitors (TKIs) and have demonstrated efficacy against various solid tumors in clinical trials [40-42]. TKIs can interact physically with a highly conserved kinase domain shared by VEGFR1-3, as well as PDGF receptors, FGF receptors, epidermal growth factor (EGF) receptors, Raf kinase and c-Kit. Although VEGF-targeted therapy for cancer has been highly successful for the prevention of tumor angiogenesis so far, most patients eventually acquire resistance to anti-angiogenic therapy and rapid vascular regrowth in tumors occurs after the discontinuation of anti-VEGF therapy. Furthermore, treatment with VEGF-targeted drugs has side effects, such as hypertension and proteinuria-related kidney dysfunction. Thus, there

is a clear need to identify novel targets for anti-angiogenic therapeutic agents to achieve a continuous inhibition of angiogenesis for tumor therapy.

To date, approximately 30 endogenous inhibitors of angiogenesis have been identified [43]. Many endogenous inhibitors including thrombospondin1 (TSP1), which was the first protein to be recognized as an endogenous angiogenesis inhibitor, are fragments of naturally occurring extracellular matrix and basement membrane proteins [44]. The expression of TSP1 is inversely correlated with tumor progression in melanoma, lung and breast carcinoma [45]. Suppression of TSP1 augmented tumor angiogenesis through the production of matrix metalloprotease 9 and the enhancement of VEGFR2 signalling [45]. In contrast, TSP1 overexpression resulted in delayed tumor growth by the inhibition of tumor angiogenesis [46]. Although many studies on these endogenous angiogenesis inhibitors have shown that they significantly inhibit tumor angiogenesis and tumor growth, it is still difficult to accurately control their expression and to apply them in clinical practice.

Kappa opioid receptors

Opioid systems mainly consist of three different types of opioid receptors, μ, δ and κ (MOR, DOR and KOR), and the respective endogenous peptide. These opioid systems, which inhibit cAMP/PKA signaling through Gi protein activation, regulate a wide range of physiological functions such as pain, the emotional response and the

reward circuitry in neural tissues [47,48]. In particular, the KOR system has been identified as one such prominent neuromodulator system in the regulation of negative emotional behavior, pain and itch [49-55]. KORs are endogenously activated by the peptide dynorphin, which is cleaved from the precursor prodynorphin (PDYN) [56]. Dynorphin is widely known to mediate negative emotional states; for example, aversive and depression-like behavior and dysphoria in both human and animal models [57-59]. Notably, it has been reported that the direct activation of KORs and dynorphinergic neurons in the nucleus accumbens (NAc) of the basal ganglia mediates conditioned place aversion [52,60], and local antagonism of KOR in this region prevents depression-like behavioral responses [61,62]. On the other hand, numerous groups have shown that the KOR agonists induced analgetic and anti-pruritic effects [49,50,63,64], suggesting that the KOR agonist may have a therapeutic potential for the treatment of chronic pain and pruritus if it little has dysphoria-like effects. To date, a novel KOR agonist nalfurafine was recently synthesized in Japan [65] and has been clinically approved for use in hemodialysis-related uremic pruritus with less side effects.

Opioids in vascular-nerve networks

Although endogenous opioids were first characterized in the brain, these transmitters and their receptors (MOR, DOR and KOR) are found in both neural (brain and spinal cord) and extraneural tissues (ganglia, gut, spleen, stomach, lung,

pancreas, liver, heart, blood and blood vessels). Opioids and opioid receptors are present in blood vessels from the later stages of the rat embryo at E16 through to adulthood [66,67]. Treatment with opioid peptides inhibited both angiogenesis in a chick chorioallantoic membrane model [68] and DNA synthesis in rat vascular walls [66]. In adults, the endogenous opioid system has been shown to be active in hemodynamic and cardiovascular responses, such as hemorrhagic shock, sepsis and trauma [69,70]. In addition, we recently found that κ opioid peptides acted as novel anti-angiogenic modulators by suppressing the expression of VEGF receptors via inhibition of cAMP/PKA signaling during vascular differentiation in development [71,72]. These findings suggest that opioid systems play important roles in vascular functions, although their physiological roles and molecular mechanisms remain largely unknown.

Using opioids in tumor therapy

Clinically, opioid analgesics such as morphine, a MOR agonist, have been broadly applied to relieve pain associated with all types of cancer. In addition, a recent study showed that morphine suppressed tumor angiogenesis through the inhibition of hypoxia-inducible transcription factors (HIFs), which enhances the expression of VEGF-A and VEGF receptors [73]. Independent studies have shown that morphine can either decrease or increase tumor growth in mice [74,75], however, the effects of opioids

on tumor growth are still unclear. On the other hand, our recent studies indicate that KOR agonists could have a potential for tumor angiogenesis inhibitor [71,72]. These findings suggest that opioid systems play important roles in not only the cancer pain-relief but also anti-cancer therapy, therefore, it is needed to reveal the clinical utility and the scientific basis using opioids in tumor therapy.

Aim and Scope

The aim of the present study was to investigate the role of κ-opioid receptor system in tumor angiogenesis. Behavioral, biochemical and molecular biological experiments was conducted to achieve the present purpose.

The specific aims of the proposed research are as follow:

In chapter 1:

To clarify the mechanisms in suppression of *de novo* angiogenesis in the vascular endothelial cells by the activation of κ-opioid receptors, I examined the effects of κ-opioid receptor agonists on the migration and tube formation using human umbilical vascular endothelial cells.

In chapter 2:

In order to ascertain whether κ-opioid receptor system negatively regulates tumor growth and tumor angiogenesis through suppressing VEGF signaling, I investigated the effects of κ-opioid receptor ligands on tumor growth and tumor angiogenesis using tumor-bearing mice.

In chapter 3:

To ascertain the potential of κ-opioid receptor agonist nalfurafine for anti-cancer therapy, I confirmed whether nalfurafine enhances the chemotherapy-induced survival advantage in pancreatic cancer-bearing mice.

Ethics

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments. Animals were used only once in the present study.

Chapter 1

Role of κ opioid receptor systems in regulation of physiological angiogenesis

Introduction

Angiogenesis is a key event in vascular development and organogenesis in the embryo, as well as in physiological tissue remodeling and pathologic disorders, particularly tumorigenesis and lifestyle-related diseases such as hypertension, diabetes and hyperlipidaemia. The balance between endogenous angiogenesis activators and inhibitors critically maintains a normally quiescent vasculature to sustain homeostasis. It has been widely accepted that disruption of the balance between angiogenesis activators and inhibitors causes pathogenic angiogenesis. Thus, a better understanding of vascular biology may lead to novel strategies for the treatment of a variety of diseases caused by the pathogenic angiogenesis.

Opioid systems are mainly present in neural tissues and could be involved in neurogenesis during brain development [76,77]. The three opioid receptors, μ, δ and κ (MOR, DOR and KOR), mainly act as inhibitory G (Gi) protein-coupled receptors through which endogenous opioids (endorphins, enkephalins and dynorphins) regulate physiological functions [47,48]. These receptors also activate other G protein-dependent signaling such as Gβγ, and G protein-independent signaling, for instance through β-arrestin [78-80]. Previous reports by Yamashita and Yamamizu *et al.* [11,13,31-34,81,82], interestingly, showed that 3'5'-cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling contributes to the vascular development through the

up-regulation of vascular endothelial growth factor (VEGF) receptors, such as VEGF receptor 2 (VEGFR2) and neuropilin (NRP), using a novel embryonic stem (ES)/induced pluripotent stem (iPS) cell differentiation system that exhibits early vascular development using VEGFR2-positive cells as common progenitors for vascular cells. Additionally, in our recent studies, we first demonstrated that KORs, but not MOR or DOR, are highly expressed in vascular progenitors and immature vascular endothelial cells, and negatively regulate vascular development via the inhibition of cAMP/PKA signaling [71].

In this study, I investigated whether the activation of KORs could inhibit *de novo* angiogenesis in the vascular endothelial cells.

Materials and methods

Cell culture

Primary human umbilical vascular endothelial cells (HUVECs) were purchased from Lonza. HUVECs were cultured in endothelial cell (EC) growth medium (EGM-2 BulletKit, Lonza, Basel, Switzerland) supplemented with 2% fetal bovine serum (FBS). Various reagents were occasionally added to the HUVEC culture, including [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO; Sigma-Aldrich Co., St. Louis, MO, USA), [(+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide] (SNC80; Tocris Cookson Ltd, Ballwin, MO, USA), (-)-*trans*-(1S,2S)-U-50488 hydrochloride (U50,488H; Research Biochemicals International, Natick, MA), 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[N-methyl-trans-3-(3-furyl) acrylamide] morphinan hydrochloride (TRK-820 (nalfurafine); TORAY, Tokyo, Japan), and 17,17'-(dicyclopropylmethyl)-6,6',7,7'-6,6'-imino-7,7'-binorphinan-3,4',14,14'-tetrol dihydrochloride (norbinaltorphimine (BNI); Sigma-Aldrich Co.).

Boyden chamber assay

HUVECs were serum-starved with EBM2 medium (0.1% FBS, no growth factor) for 12 hr, and HUVECs (10^5 cells per well) were seeded into the upper well of a boyden chamber

system (Corning, New York, NY, USA) on polyethylene terephthalate membrane with 8-mm pores. Human recombinant $VEGF_{165}$ (R&D Systems, Minneapolis, MN, USA) was added as a chemo-attractant into the lower well at 20 ng/ml. Inhibition of VEGF-induced chemotaxis was assessed after including DAMGO, SNC80, U50,488H, nalfurafine or BNI at relevant doses. Migration through the membrane was determined after 4 hr of incubation at 37ºC by fixing, staining with hematoxylin and eosin, and counting the migrated cells in five random fields at 100 x magnification.

Wound-healing assay

HUVECs that had grown to confluence in 24-well culture plates were serum-starved with EBM2 medium (0.1% FBS, no growth factor) for 12 hr, and a portion of the cell monolayer was then scraped away with a P200 pipette tip. The remaining cells were gently washed with medium and incubated for 16 hr in EGM-2 BulletKit. EC migration from the edge of the injured monolayer was quantified by measuring the distance between the wound edges, at three random positions in one visual field, before and after incubation with the use of a computer-assisted microscope (Leica Microsystems, Heidelberg, Germany) using AxioVision (Carl Zeiss MicroImaging GmbH, Jena, Germany) and Image J (NIH, New York, NY, USA).

Tube formation assay

HUVECs (1.5 x 10^4) were cultured in a 24-well plate (Thermo Fisher Scientific, Inc., Waltham, MA, USA) coated with 150 μl Matrigel Basement Membrane Matrix GFR (BD Biosciences, Inc., NJ, USA). These experiments were performed with various reagents such as DAMGO, SNC80, U50,488H, nalfurafine or BNI. EC tube length was quantified at five random positions by Image J.

KOR knockdown using siRNA

The siRNA targeting human KOR were purchased from invitrogen (Stealth RNAi, Invitrogen Co., Carlsbad, CA, USA). Stealth RNAi for KOR (10 nM) or control (10 nM) were transfected into HUVECs using Lipofectamine RNAiMAX (Invitrogen Co.) according to the manufacture's instruction. After 2 days, HUVECs were examined by a boyden chamber assay, a wound-healing assay, and a tube formation assay.

Cell sorting for vascular endothelial cells using fluorescence activated cell sorting (FACS)

Lung tissues collected from naive mice were minced and then treated with dispase II (2.4 U/ml) and collagenase (1 mg/ml). The samples were incubated with 0.1% Trypsin/ethylenediaminetetraacetic acid (EDTA) and added DNase I. Dissociated cells were stained with combinations of phycoerythrin (PE)-conjugated CD31 antibody monoclonal antibody (MoAb, BD Biosciences, Inc.) and fluorescein isothiocyanate (FITC)-conjugated

anti-CD45 MoAb (BD Biosciences, Inc.), and then purified endothelial cells using FACS Aria and FACS Canto (BD Biosciences, Inc.).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells in HUVEC and purified EC using RNeasy kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. Reverse-transcription was performed with the SuperScript III first-strand synthesis system (Invitrogen Co.). qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems Inc., San Diego, CA, USA) and a 7300 Real time PCR system (Applied Biosystems Inc.). The amount of target RNA was determined from the appropriate standard curve and normalized relative to the amount of GAPDH mRNA. Primer sequences are shown below: MOR (forword: 5'-AGACTGCCACCAACATCTACAT-3'; reverse: 5'-TGGACCCC TGCCTGTATTTTG-3'); DOR (forword: 5'-GCTGTGCTCTCCATTGACTAC-3'; reverse: 5'- GATGTCCACCAGCGTCCAGAC -3'); KOR (forword: 5'- AGTCCCCCATTCAGATC TTCC-3'; reverse: 5'-ACAGCAATGTAGCGGTCCAC-3'); VEGFR2 (forword: 5'-GGGAT GGTCCTTGCATCAGAA-3'; reverse: 5'-ACTGGTAGCCACTGGTCTGGTTG-3'); NRP1 (forword: 5'-AGGGCCGATTCAGGACCATAC-3'; reverse: 5'-ACATGAGAGCCGGACA TGTGATAC-3'); GAPDH (forword: 5'-CCCACGGCAAGTTCAACGG-3'; reverse: 5'-CTTTCCAGAGGGGCCATCCA-3').

Western blotting

Briefly, HUVECs were lysed in lysis buffer, and the samples were run on sodium dodecylsulphate (SDS)/polyacrylamide gel electrophoresis using gradient gel (Atto Co, Tokyo, Japan) followed by electrophoretic transfer onto nitrocellulose membranes. After the blots were incubated for 1 hour in the blocking agent Blocking One (Nacalai Tesque Inc., Kyoto, Japan), they were incubated overnight with anti-VEGFR223, anti-Neuropilin1 (R&D Systems), anti-VEGFR2 phospho-Tyr951 (Cell Signaling technology Inc., Danvers, MA, USA), or anti-VEGFR2 phospho-Tyr1175 (Cell Signaling) at 4ºC. Anti-rat or goat or rabbit IgG antibodies conjugated with horseradish peroxidase (HRP) were used as secondary antibodies (1:10000). A Can Get Signal Immunoreaction Enhancer solution kit (Toyobo, Osaka, Japan) was used for signal enhancement. Immunoreactivity was detected with the Chemi-Lumi One enhanced chemiluminescence kit (Nacalai Tesque). Signal intensity was calculated with Scion Image software (Meyer Instruments Inc., Houston TX, USA).

Statistical analysis

Data are expressed as the mean with SEM. At least three independent experiments were performed. The data were subjected to a statistical analysis with unpaired *t*-test or one-way analysis of variance (ANOVA) test followed by the Bonferroni multiple comparisons test as appropriate for the experimental design. All statistical analyses were performed with Prism version 5.0 (GraphPad Software, La Jolla, CA, USA).

Results

KOR agonists inhibit the migration and capillary structure formation of endothelial cells

EC migration is a critical step to form new blood vessels during angiogenesis. To investigate the roles of opioids on EC migration, I performed a boyden chamber assay and a wound-healing assay with HUVECs. Treatment with VEGF significantly increased migrated EC cells with a boyden chamber assay. Treatment with KOR agonists, U50,488H or nalfurafine, together with VEGF decreased migrated EC cells (Fig. 1-1a). In contrast, neither the MOR agonist DAMGO nor the DOR agonist SNC80 inhibited EC migration. The consistent results was shown that treatment with U50,488H or nalfurafine significantly inhibited EC migration with a wound-healing assay (Fig. 1-1b, c). These inhibitory effects on EC migration were reversed by treatment with nor-binaltorphimine (BNI), a selective KOR antagonist, or knockdown of KOR with siRNA (Fig. 1-2a-d). Furthermore, I examined HUVEC tube formation using a 2-dimensioned matrigel assay. Both U50,488H and nalfurafine, but not DAMGO or SNC80, dramatically inhibited HUVEC tube formation (Fig. 1-3a, b). These inhibitory effects on tube formation were reversed by knockdown of KOR with siRNA (Fig. 1-3c). These findings indicate that KOR signal pathway could regulate angiogenesis *in vitro*.

KOR agonists suppress VEGFR2 expression in endothelial cells.

As shown in Fig. 1-4a and 1-4b, KOR was highly expressed in ECs purified from adult mice and in HUVECs, whereas MOR and DOR were weakly expressed. Opioid receptors transduce signals through Gi protein to inhibit adenylyl cyclase and subsequently decrease cAMP production and inactivate PKA. We previously revealed that KOR agonists inhibit EC differentiation from ES cells through suppression of VEGFR2 and Neuroplin1 expressions via inhibition of cAMP/PKA signaling [71]. Therefore, I examined VEGF receptor expression after the activation of opioid receptors. Similar, but not same to previous results [71], in HUVECs, VEGFR2 protein, but not Neuropilin1, was significantly downregulated by the addition of U50,488H or nalfurafine (Fig. 1-4c-e). Neither DAMGO nor SNC80 affected VEGFR expression in HUVECs. I further investigated the phosphorylation of VEGFR2 after the activation of opioid receptors. VEGFR2 phosphorylation at Tyrosine 951 (Tyr951) and 1175 (Tyr1175) mainly activates downstream signals and leads to EC migration and proliferation, thereby forming vasculature [83]. In this study, VEGFR2 phosphorylation at both Tyr951 and Tyr1175 was specifically suppressed by treatment with U50,488H or nalfurafine, but these suppression of VEGFR2 phosphorylation are depend on protein expression of VEGFR2 (Fig. 1-4e, f). These results indicate that KOR signaling specifically regulates VEGFR2 expression in ECs.

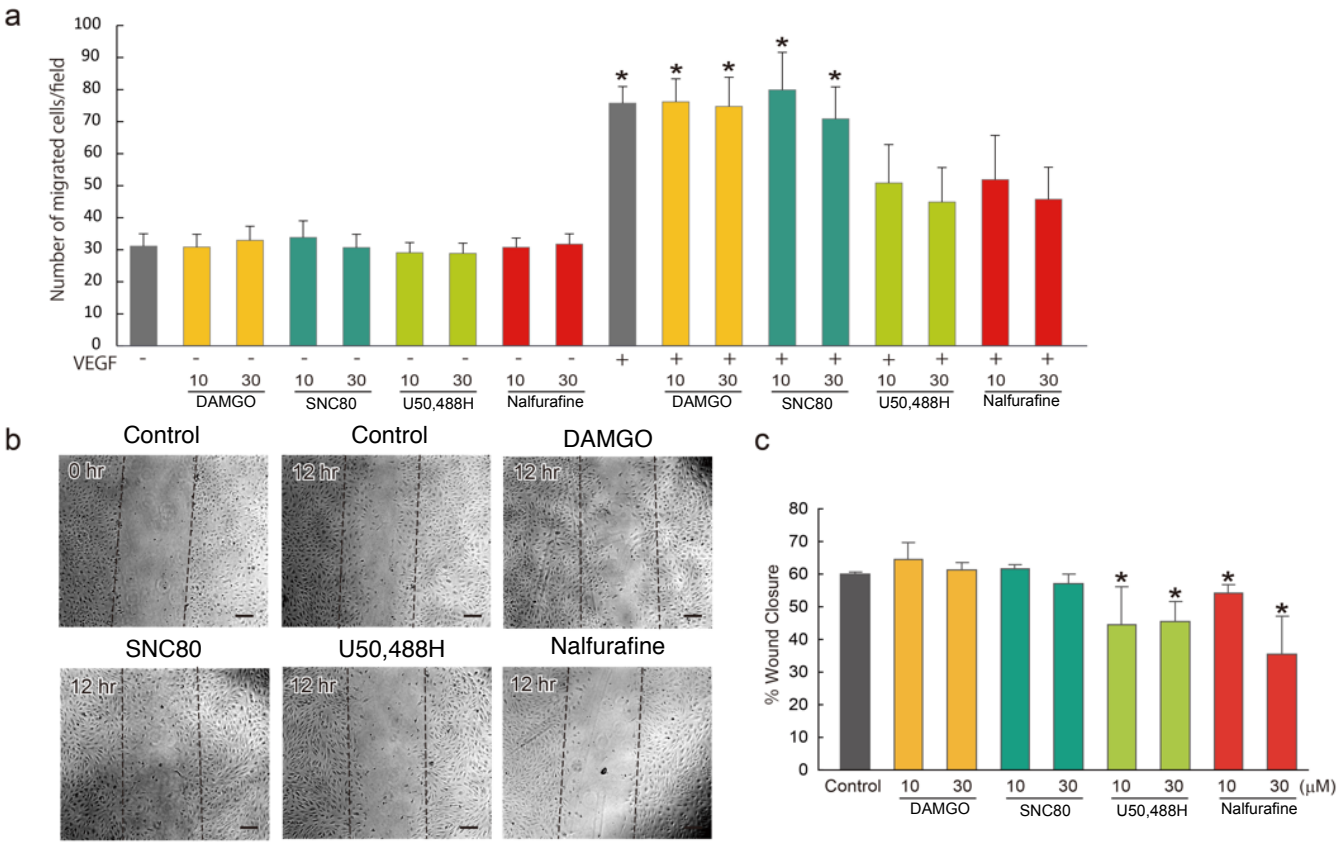


Figure 1-1. Inhibitory effects of KOR agonists, U50,488H and nalfurafine, on HUVEC migration.
(a) The boyden chamber assay. Inhibition of VEGF-induced chemotaxis was assessed after including DAMGO (10, 30 μM), SNC80 (10, 30 μM), U50,488H (10, 30 μM), or nalfurafine (10, 30 μM) (n = 3, *p<0.05 vs. Control). (b) The wound-healing assay. HUVECs were plated, scratched and then incubated with DAMGO (10, 30 μM), SNC80 (10, 30 μM), U50,488H (10, 30 μM) or nalfurafine (10, 30 μM) as indicated. Scale bars: 200 μm. (c) Quantitative evaluation of the effect of opioid receptor agonists on HUVEC wound-healing assay. Three independent experiments are shown (n = 3, *p<0.05 vs. Control).

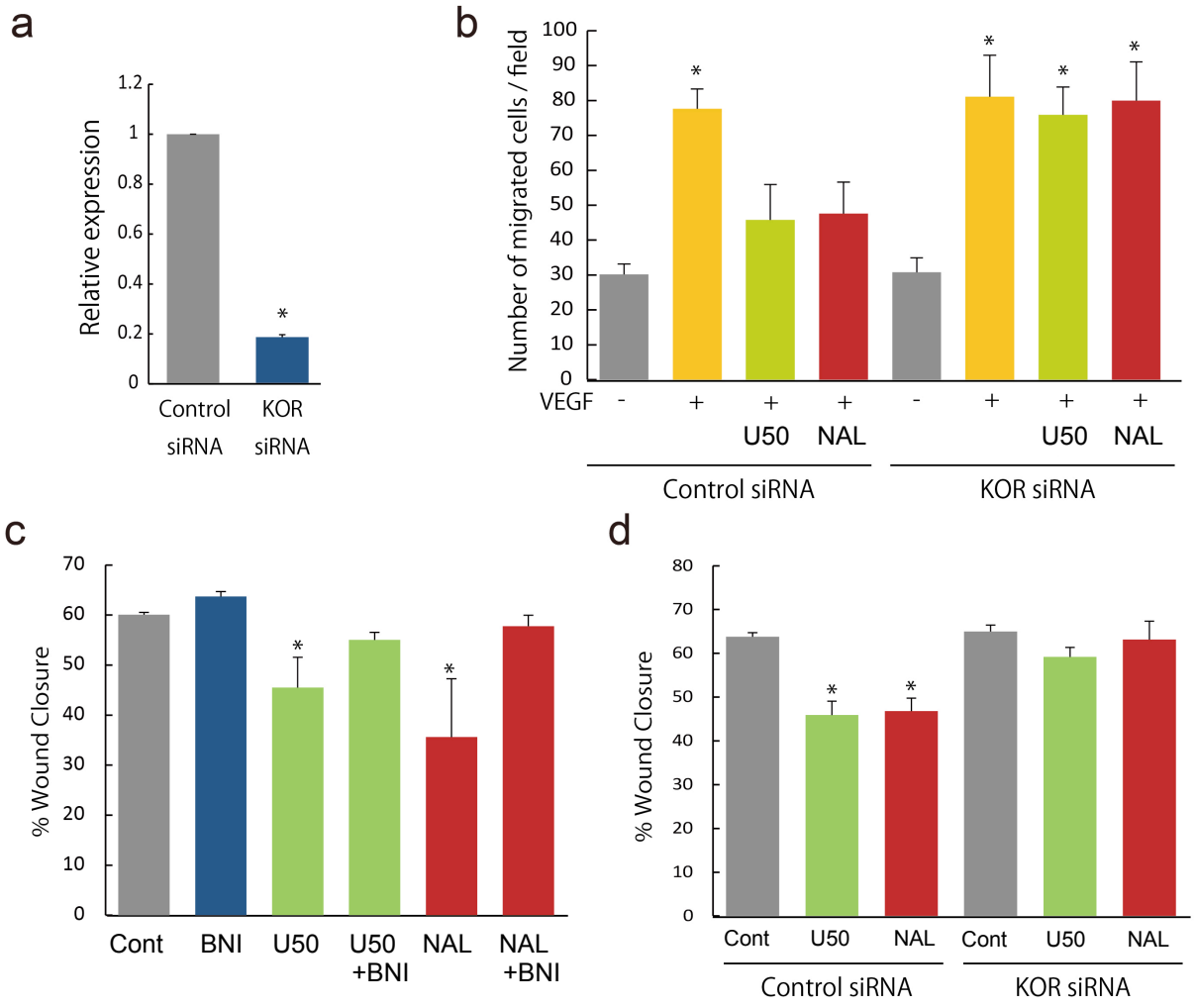


Figure 1-2. Inhibitory effects of KOR agonists, U50,488H and nalfurafine, on HUVEC migration are restored by KOR antagonists, BNI, or knockdown of KOR with siRNA.
(a) qPCR showing mRNA expression of KOR with control siRNA or KOR siRNA in HUVECs. (b) The boyden chamber assay. Inhibition of VEGF-induced chemotaxis was assessed after including U50,488H (U50, 10 μM), or nalfurafine (NAL, 10 μM) with control siRNA or KOR siRNA (n = 3, *p<0.05 vs. Control). (c) The wound-healing assay. Quantitative evaluation of the effect of KOR agonists, U50,488H (10 μM) and nalfurafine (10 μM), and KOR antagonists, BNI (10 μM) on HUVEC migration assay. Three independent experiments are shown (*p<0.05 vs. Control). (d) The wound-healing assay. Quantitative evaluation of the effect of opioid receptor agonists, U50,488H (10 μM) and nalfurafine (10 μM) with control siRNA or KOR siRNA (n = 3, *p<0.05 vs. Control).

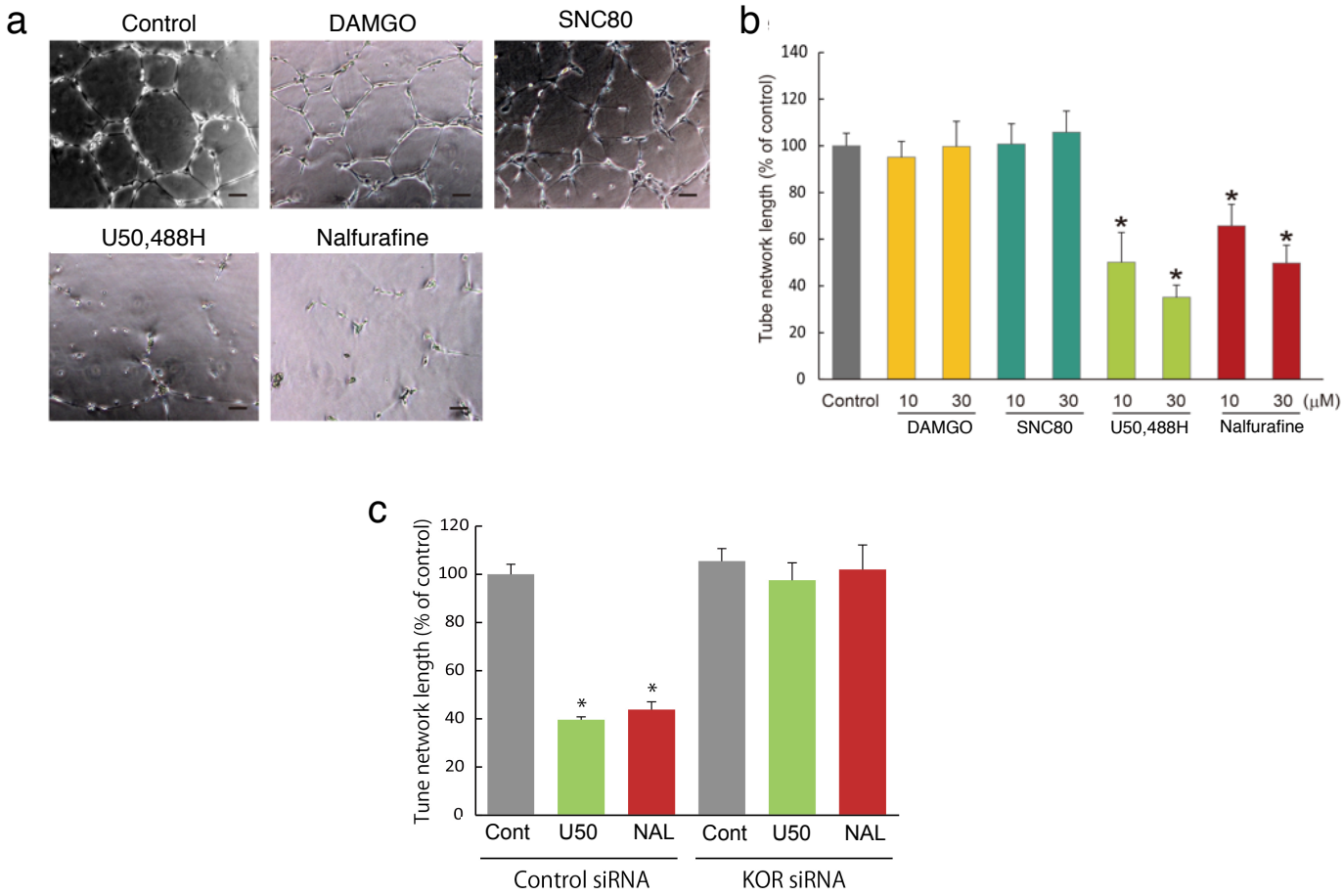


Figure 1-3. Inhibitory effects of KOR agonists, U50,488H and nalfurafine, on HUVEC tube formation via KOR activation.
(a) Representative photographs of vasculature with DAMGO (10 μM), SNC80 (10 μM), U50,488H (10 μM) or nalfurafine (10 μM) on HUVEC tube formation assay. Scale bars: 200 μm. (b) Quantitative evaluation of the effect of opioid receptor agonists on HUVEC tube formation assay. Three independent experiments are shown (n = 3, *p<0.05 vs. Control). (c) Quantitative evaluation of the effect of opioid receptor agonists, U50,488H (U50, 10 μM) and nalfurafine (NAL, 10 μM) with control siRNA or KOR siRNA on HUVEC tube formation assay. (n = 3, *p<0.05 vs. Control).

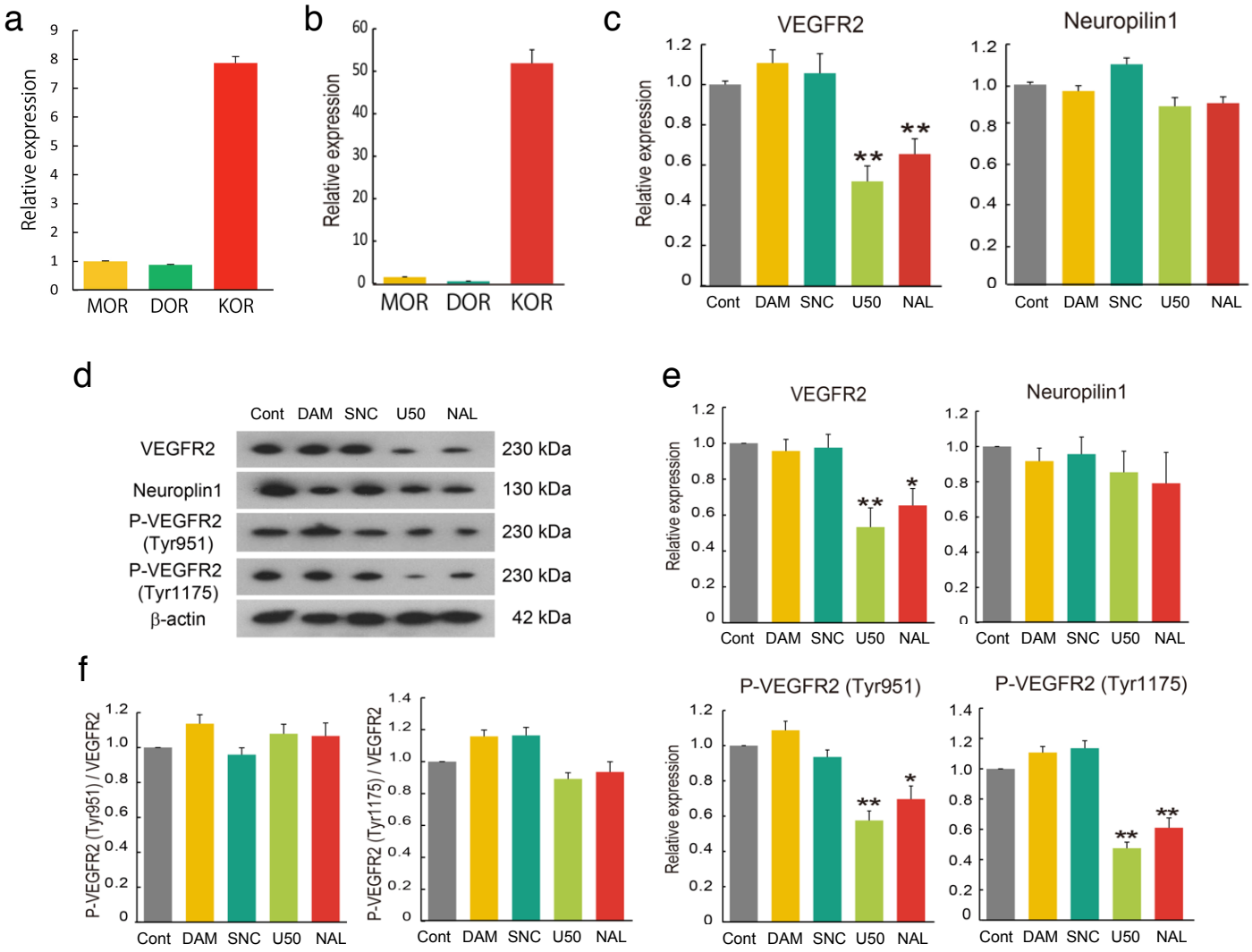


Figure 1-4. Inhibitory effects of KOR agonists, U50,488H and nalfurafine, on VEGFR2 expression in HUVECs.
(a) qPCR showing mRNA expression of MOR, DOR, and KOR in purified endothelial cells from lung in control mice (n = 2). (b) qPCR showing mRNA expression of MOR, DOR, and KOR in HUVECs. (c) qPCR showing mRNA expression of VEGFR2 and Neuropilin1 after 24 hr culture with DAMGO (DAM, 10 μM), SNC80 (SNC, 10 μM), U50,488H (U50, 10 μM) or nalfurafine (NAL, 10 μM). (d) Western blotting of VEGFR2, Neuroplin1, VEGFR2 phospho-Tyr951, VEGFR2 phospho-Tyr1175, or β-actin after 24 hr culture with DAMGO (10 μM), SNC80 (10 μM), U50,488H (10 μM) or nalfurafine (10 μM). (e) Quantitative evaluation of the effect of opioid receptor agonists by western blotting. Normalization of expression of VEGF, Neuroplin1, VEGFR2 phospho-Tyr951, or VEGFR2 phospho-Tyr1175 is addressed by expression of β-actin. Three independent experiments are shown (n = 3, **p<0.01, *p<0.05 vs. Control). (f) Quantitative evaluation of the effect of opioid receptor agonists by western blotting. Normalization of expression of VEGFR2 phospho-Tyr951 or VEGFR2 phospho-Tyr1175 is addressed by expression of VEGFR2.

Discussion

In this study, I demonstrated a novel mechanism for the regulation of EC differentiation and vascular formation through the opioid system. These results showed that KORs but not MORs or DORs, were highly expressed in HUVEC and in ECs purified from adult mice as well as vascular progenitors and immature vascular endothelial cells under the vascular development stages [71]. Additionally, treatment with the KOR agonists U50,488H and nalfurafine significantly inhibited HUVEC migration and vascular tube formation by suppressing VEGFR2 expression and phosphorylation. Furthermore, treatment with nor-BNI, a KOR antagonist, and knock down of KOR expression using siRNA blocked the inhibitory effects of KOR agonists on HUVEC migration and vascular tube formation, indicating that KOR activation suppressed *de novo* angiogenesis by suppressing VEGFR2 activation through inhibition of cAMP/PKA signaling [31,71].

Endogenous angiogenesis inhibitors, such as thrombospondin-1, endostatin, tumstatin, chondromodulin-1 and vasohibin, are naturally present in blood flow and possess antiangiogenic activity and may counterbalance angiogenesis stimulators such as VEGF, basic fibroblast growth factor among others [84,85]. Thus, a physiologic balance in angiogenesis is maintained by angiogenic and antiangiogenic factors. I demonstrated that the κ opioids acts as a novel anti-angiogenic factor by suppressing EC migration and vascular tube formation through suppressing VEGFR2 expression. We recently found that cAMP/PKA signaling in

vascular progenitors increased the VEGF-A receptors, VEGFR2 and NRP1, which enhance EC differentiation and vascular formation through increased sensitivity of vascular progenitors to VEGF [31,34]. Additionally, we found that mice lacking KORs (KOR-null) or dynorphin (an endogenous KOR ligand-null) showed a significant increase in vascular formation in early embryos. Moreover, ectopic vascular invasion into somites of E10.5 embryos accompanied by decreased plexinD1 expression in ECs was observed in both strains of null mice [71].

Taken together, these findings suggest that the KOR system may be a dual inhibitory regulator of EC differentiation and of *de novo* angiogenesis, inditcating that the κ opioid machinery is the first system identified as endogenous inhibitory machinery to cAMP/PKA function of ECs in regulation of physiological angiogenesis. Furthermore, these knowledge show that KOR agonist can be a novel anti-cancer therapy for tumor angiogenesis inhibitor.

Chapter 2

κ Opioid receptor ligands inhibit the tumor growth through suppressing tumor angiogenesis

Introduction

Tumor angiogenesis is required for tumor progression, with the intake of nutrients and oxygen as well as the excretion of metabolic wastes and carbon dioxide [2,35]. Numerous researches in the field of oncology have revealed several activators such as VEGF are highly expressed in the tumor microenvironment and strongly induce tumor angiogenesis [37,38]. Therefore, restoration of the balance between activators and inhibitors for angiogenesis is a critical treatment strategy for tumors.

VEGF plays a pivotal role in neovascularization in the embryo as well as in the adult mainly through VEGFR2 (also known as kinase insert domain receptor (KDR) in human and fetal liver kinase 1 (Flk1) in mouse). Expression of the VEGF gene has been shown to be up-regulated by hypoxia and oncogene signaling such as rat sarcoma viral oncogene (Ras) and myelocytomatosis oncogene (Myc) in cancer cells, which would lead to the formation of vasculature and the proliferation of tumors [86-88]. Consequently, numerous drugs have been developed to inhibit tumor angiogenesis by suppressing VEGF signaling. Furthermore, the blocking of antibodies against neuropilin1 (NRP1), a VEGF co-receptor, additively prevented the progression of tumors when combined with anti-VEGF drugs [89]. In clinical medicine, bevacizumab, a humanized monoclonal antibody that is specific for human VEGF, is the first anti-angiogenic agent for the treatment of colorectal cancer, renal cell cancer, non-small cell lung cancer, and glioblastoma [39]. Although therapies that inhibit tumor angiogenesis have

been highly successful for tumor therapy, most patients eventually acquire resistance to anti-angiogenic therapy. Thus, we must identify novel targets for anti-angiogenic therapeutics to achieve the continuous inhibition of angiogenesis for tumor therapy.

Opioid analgesics such as morphine, a MOR agonist, have been broadly applied to relieve pain from all types of cancer. Independent studies have shown that morphine suppresses tumor angiogenesis through the inhibition of hypoxia-inducible transcription factors, which enhances the expression of VEGF and VEGF receptors [73]. However, the effect of morphine on tumor growth is still controversial. Independent studies have shown that morphine can either decrease or increase tumor growth in mice [74] [75]. More recently, we showed that endogenous κ opioid peptides acted as novel anti-angiogenic modulators through suppressing the expression of VEGF receptors, VEGFR2 and NRP1, during vascular differentiation via inhibition of cAMP/PKA signaling [31,71]. Additionally, as shown in Chapter 1, I found that KOR agonists U50,488H and nalfurafine suppressed and *de novo* angiogenesis in ECs via suppressing the expression of VEGFR2.

In this study, I next investigated whether the endogenous KOR system could have a potential as anti-tumor angiogenic modulators, and the KOR agonist nalfurafine could be useful for anti-cancer therapy as a tumor angiogenesis inhibitor.

Materials and methods

Animals

The experiments were performed on C57BL/6J mice (Tokyo Laboratory Animals Science, Tokyo, Japan), KOR-knock out (KO) mice (The Jackson Laboratory, BarHarbor, Maine, USA). The animals were normally fed standard laboratory food and water and housed in temperature (23 ± 1ºC) -controlled rooms under a 12 h/12 h light/dark cycle.

Graft tumor growth assay

Lewis lung carcinoma (LLC) and B16 melanoma cells were kind gifts from Dr. Shibuya and Dr. Muramatsu (Tokyo Medical and Dental University, Tokyo, Japan). LLC or B16 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% antibiotics. The animals were anesthetized intraperitoneally with 70 mg/kg pentobarbital sodium (Nacalai Tesque, Kyoto, Japan). LLC or B16 cells were detached by trypsinization, collected, and counted. Cells were resuspended in a mixture of extracellular matrix (ECM) gel (Sigma-Aldrich Co.) and Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific Inc.) (ratio 3:1) at a concentration of 2 x 10^6 cells/0.5 mL, and 0.5 mL of this suspension was then inoculated subcutaneously into the right lower back of mice. Tumor size was measured using a caliper and tumor volume was calculated as (L x

W^2)/2, where L: length and W: width. The tumor weight was measured after mice were sacrificed.

Two series of experiments were conducted. First, the effect of the global deletion of KOR was determined in KOR-KO mice and wild-type mice. Second, the anti-tumor effect of the repeated administration of nalfurafine, a KOR agonist, was investigated using three groups of C57BL/6J mice and KOR-KO mice. Two days after tumor implantation, mice were injected with either 0.9% saline or nalfurafine (0.1, 1, or 10 μg/kg, *b.i.d.*) every 12 hours for 12 days. For immunohistochemical studies, tumors were placed in optimum cutting temperature (OCT) compound media (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan) and stored at -80ºC until use.

Immunohistochemistry

Six-μm sections of tumors were fixed with 4% paraformaldehyde, washed twice in phosphate-buffered saline (PBS), and blocked by 1% skim milk (BD Biosciences). Samples were incubated overnight with anti-CD31 antibody (BD Biosciences) and anti-VE-cadherin antibody (BD Biosciences) at 4ºC. For immunofluorescent staining, anti-rat IgG antibodies conjugated with Alexa488 (Invitrogen Co.) or Alexa546 (Invitrogen Co.) were used as secondary antibodies. Nuclei were visualized with DAPI (Invitrogen Co.). Stained cells were photographed with an inverted fluorescent microscope (BZ-9000, Keyence, Osaka, Japan).

FACS analysis

Cell sorting was conducted as previously described in Chapter 1. Tumors were minced and then treated with dispase II (2.4 U/ml) and collagenase (1 mg/ml), and then the samples were incubated with 0.1% Trypsin/EDTA and added DNase I. Dissociated tumor cells were stained with combinations of PE-conjugated CD31 antibody MoAb (BD Biosciences) and FITC-conjugated anti-CD45 MoAb (BD Biosciences) and then subjected to analysis or purified endothelial cells using FACS Aria and FACS Canto (Becton Dickinson).

Statistical analysis

Data are expressed as the mean with SEM. No data points were removed from the statistical analysis except as specified. The data were subjected to an unpaired *t*-test or one-way ANOVA test followed by the Bonferroni multiple comparisons test as appropriate for the experimental design. The data with time-dependent changes were analyzed using two-way repeated measures (RM) ANOVA followed by the Bonferroni post-hoc test, where appropriate. All statistical analyses were performed with Prism version 5.0 (GraphPad Software).

Results

Loss of KOR increases tumor angiogenesis

I confirmed the effects of KOR on tumor angiogenesis *in vivo* using a KOR-KO animal model. We previously demonstrated that KOR-KO mice increased vascular formation in early stages (from E8.75 to E11.5) of vascular development, but KOR-KO mice are viable by diminishing abnormal vascular formation from late stages of vascular development [71]. These results suggest that the KOR system could control neo-vascular formation *in vivo*. To investigate whether KORs are involved in tumor angiogenesis *in vivo*, I subcutaneously implanted LLC or B16 melanoma cells into KOR-KO mice or control mice. Interestingly, the tumor volumes and the tumor weights of both LLC and B16 in KOR-KO mice were significantly greater than those in control mice at 19 days after transplantation (Fig. 2-1a-d). Additionally, I found that LLC or B16 melanoma grafted in prodynorphin (PDYN)-KO mice, which are an endogenous KOR ligand-null, showed increased the tumor growth compared with those in wild-type mice (data not shown). To assess whether a loss of KOR signaling increases angiogenesis in tumors, I performed immunostaining and FACS analysis with inoculation tumors. LLC grafted in KOR-KO mice increased ECs as shown the endothelial markers, CD31 and VE-cadherin (Fig. 2-2a, b). Furthermore, FACS analysis showed that LLC graft in KOR-KO mice showed significantly enhanced CD31 positive angioendothelial cells compared to that in control mice (percentage of $CD31^{+}/CD45^{-}$ ECs; 2.59 ± 0.52 in

control mice vs. 5.89 ± 1.06 in KOR-KO mice, n = 4 (independent mice), *p<0.05, Fig. 2-2c, d). I confirmed the expression of opioid receptors and VEGFR2 in CD31-positive ECs purified from tumors. Purified ECs from LLC graft in control mice highly expressed KOR, but not MOR or DOR (Fig. 2-2e). ECs from LLC grafted in KOR-KO mice showed a significant increase in VEGFR2 compared with ECs from LLC grafted in control mice (Fig. 2-2f). These results suggest that KOR could play a critical role as anti-angiogenic mediators in tumors.

KOR agonists inhibit tumor angiogenesis and tumor growth.

Since κ opioids could act as inhibitors of tumor angiogenesis, I considered that KOR agonists would have a potential for novel tumor therapy. Using a mouse B16 graft model, we examined the inhibitory function for tumor angiogenesis by nalfurefine. The repeated intraperitoneal injection of nalfurafine (0.1, 1, or 10 mg/kg, *b.i.d.*) every 12 hr significantly decreased the tumor size from 11 days to 14 days after transplantation (Fig. 2-3a, b). The tumor volume and tumor weight in nalfurafine-treated mice were much less than those in control mice at 7 days and 14 days after transplantation (Fig. 2-3c-f). In contrast, nalfurafine had no inhibitory effects in B16 grafted in KOR-KO mice, indicating that nalfurafine specifically induced the inhibition of tumor angiogenesis and tumor growth through KOR (Fig. 2-3g). Then, I performed the immunostaining and FACS analysis to examine tumor angiogenesis. Treatment with nalfurafine remarkably suppressed the both CD31 and

VE-cadherin positive tumor vessel cells at 7 days and 14 days after transplantation (Fig. 2-4a-d). Moreover, CD31 positive ECs of B16 graft in nalfurafine-treated mice were significantly decreased compared with those of control mice at 14 days after transplantation (percentage of $CD31^{+}/CD45^{-}$ ECs; 2.78 ± 0.39 in control mice vs. 1.28 ± 0.30 in nalfurafine-treated mice, n = 4 (independent mice), *$p<0.05$, Fig. 2-4e, f). Purified ECs from B16 graft of nalfurafine-treated mice showed a significant decrease in VEGFR2 compared with ECs from B16 graft in control mice (Fig. 2-4g). Taken together, these results suggest that the KOR agonist nalfurafine could inhibit tumor angiogenesis through inhibition of VEGFR2 expression, thereby suppressing tumor growth.

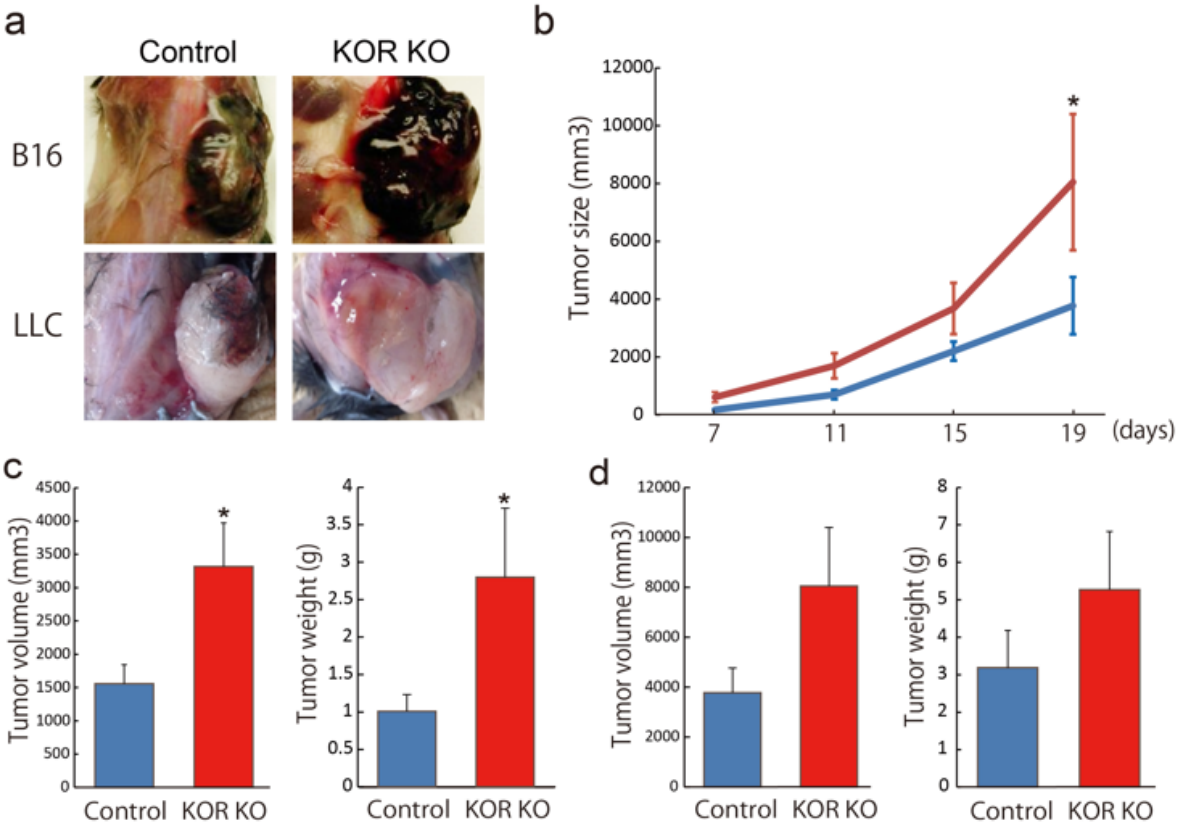


Figure 2-1. Increase of tumor growth in graft KOR KO mice.
(a) Typical example of B16 or LLC-bearing control (left) or KOR KO (right) mice. (b) Quantitative analysis of tumor size between control (n = 5) and KOR KO (n = 6) mice at 7, 11, 15, 19 days (*p<0.05 vs. Control). (c) The tumor volume and the tumor weight between B16 grafted-control (n = 10) and -KOR KO(n = 9) mice at 19 days (*p<0.05 vs. Control). (d) The tumor volume and the tumor weight between LLC grafted-control (n = 5) and -KOR KO (n = 6) mice at 19 days.

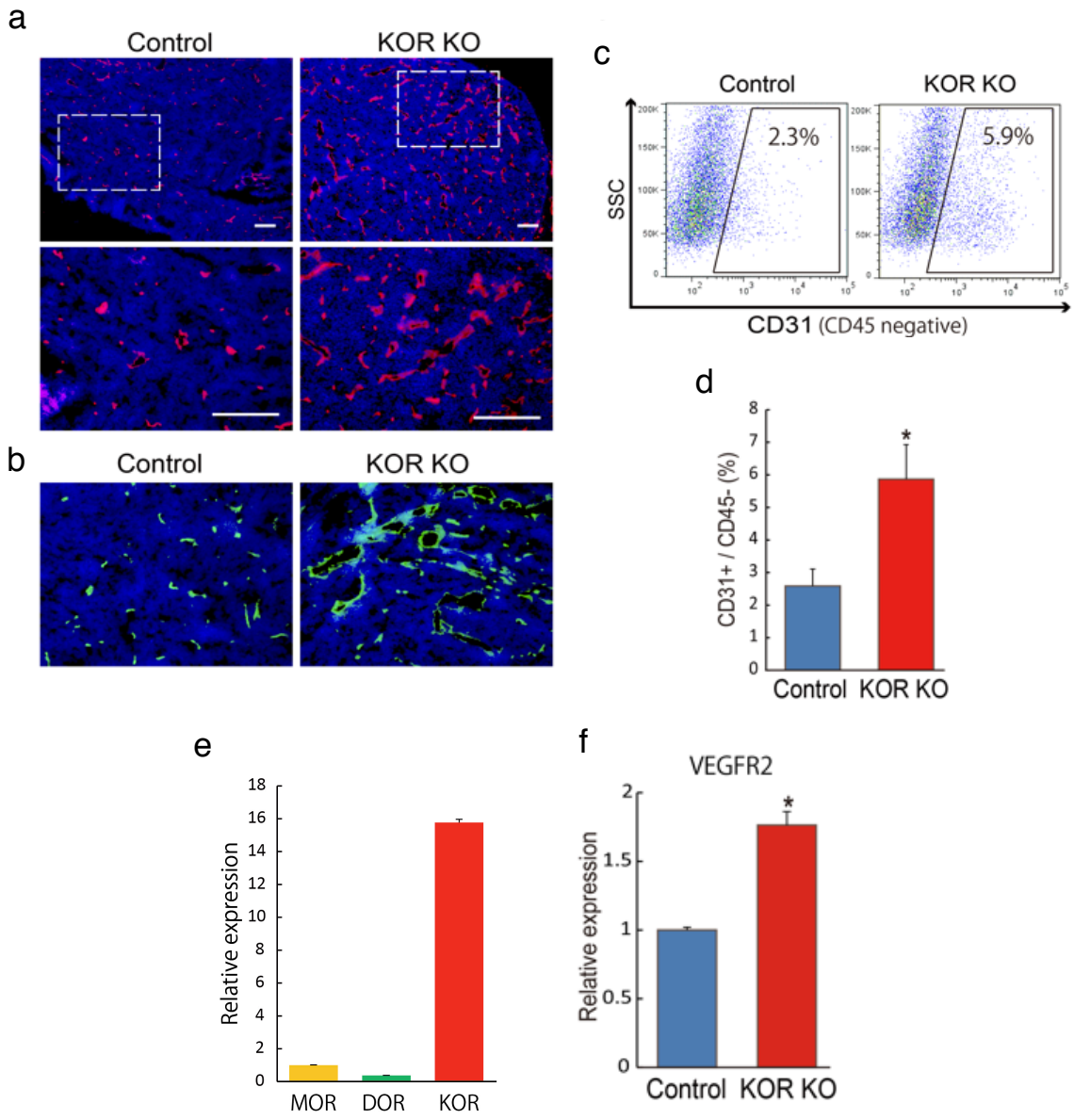


Figure 2-2. Increase of tumor angiogenesis in graft KOR KO mice.
(a) Fluorescent staining for CD31 (red) at 19 days. Nuclei are stained with DAPI (blue). Left panel, Control. Right panel, KOR KO. Scale bars: 200 µm. (b) Fluorescent staining for VE-cadherin (green) at 19 days. Nuclei are stained with DAPI (blue). Left panel, Control. Right panel, KOR KO. Scale bars: 200 µm. (c) Flow cytometry. X-axis: CD31 (CD45 negative), Y-axis: SSC. Percentages of $CD31^{+}/CD45^{-}$ ECs among tumors-dissociated cells are indicated. (d) Quantitative evaluation on $CD31^{+}/CD45^{-}$ EC population in tumors by FACS. Percentages of $CD31^{+}/CD45^{-}$ cell population among tumors-dissociated cells. Tumors transplanted Control (n = 4) or KOR KO (n = 4) mice are shown (*$p<0.05$ vs. Control). (e) qPCR showing mRNA expression of MOR, DOR, and KOR in purified tumor endothelial cells from LLC grafted in control mice. (f) qPCR showing mRNA expression of VEGFR2 in purified ECs from LLC grafted in control mice or KOR KO mice at 19 days after tumor transplantation.

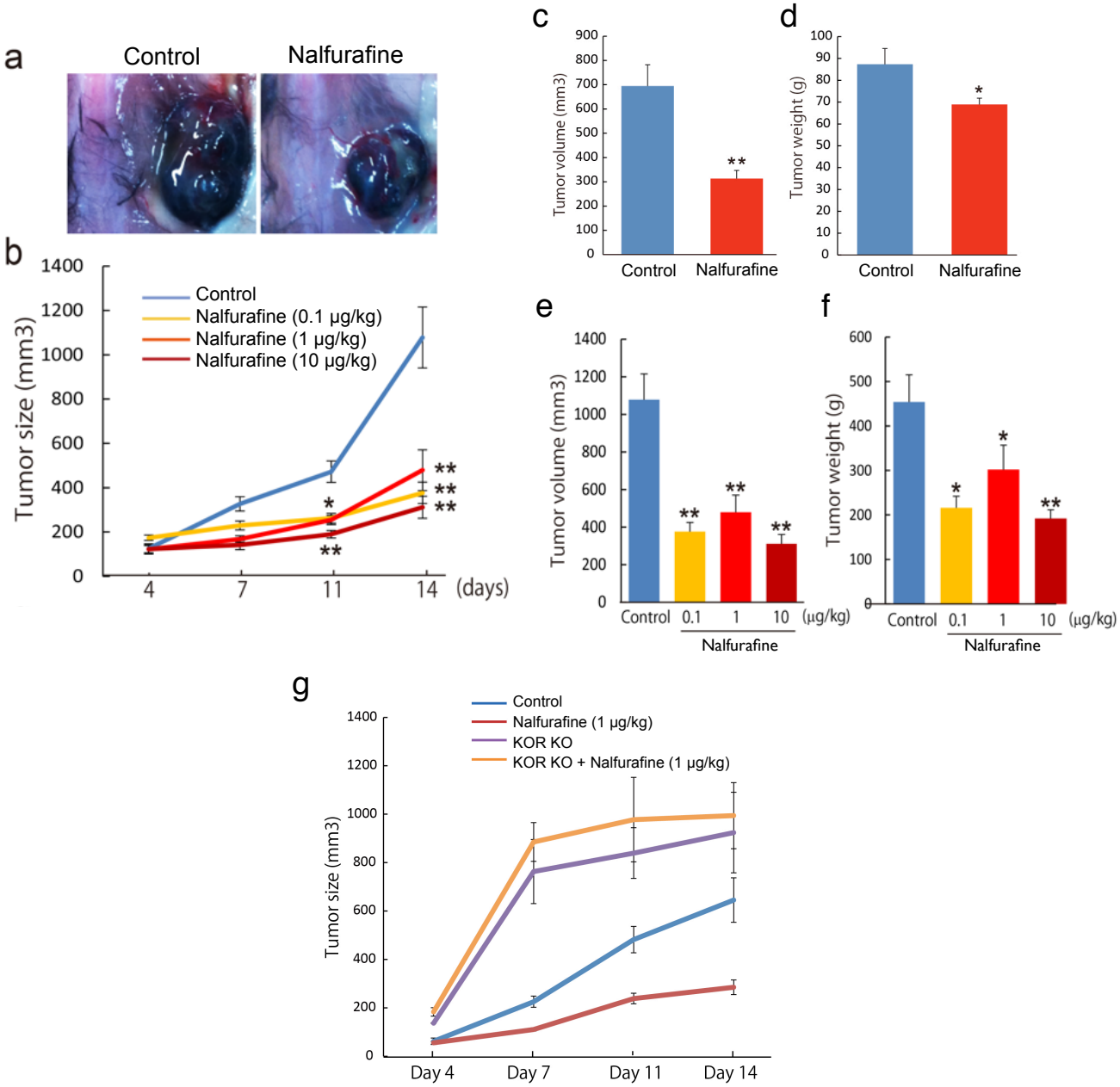


Figure 2-3. Suppression of tumor growth by a KOR agonist, nalfurafine in graft mice.
(a) Typical example of B16-bearing control (left) or nalfurafine treated (right) mice. (b) Quantitative analysis of tumor size among PBS-treated (n = 16) and nalfurafine (0.1 μg/kg (n = 9), 1 μg/kg (n = 17), 10 μg/kg (n = 9)-treated mice at 4, 7, 11, 14 days after tumor transplantation (**p<0.01, *p<0.05 vs. Control). (c, d) Quantitative analysis of tumor volume and tumor weight among PBS-treated (n = 8) and nalfurafine (1 μg/kg)-treated (n = 8) mice at 7 days after tumor transplantation (**p<0.01, *p<0.05 vs. Control).(e, f) Quantitative analysis of tumor volume and tumor weight among PBS-treated (n = 16) and nalfurafine (0.1 μg/kg (n = 9), 1 μg/kg (n = 17), 10 μg/kg (n = 9)-treated mice at 14 days after tumor transplantation (**p<0.01, *p<0.05 vs. Control). (g) Quantitative analysis of tumor size among PBS-treated (n = 4) and nalfurafine 1 μg/kg (n = 4)-treated control mice or KOR KO mice at 4, 7, 11, 14 days after tumor transplantation.

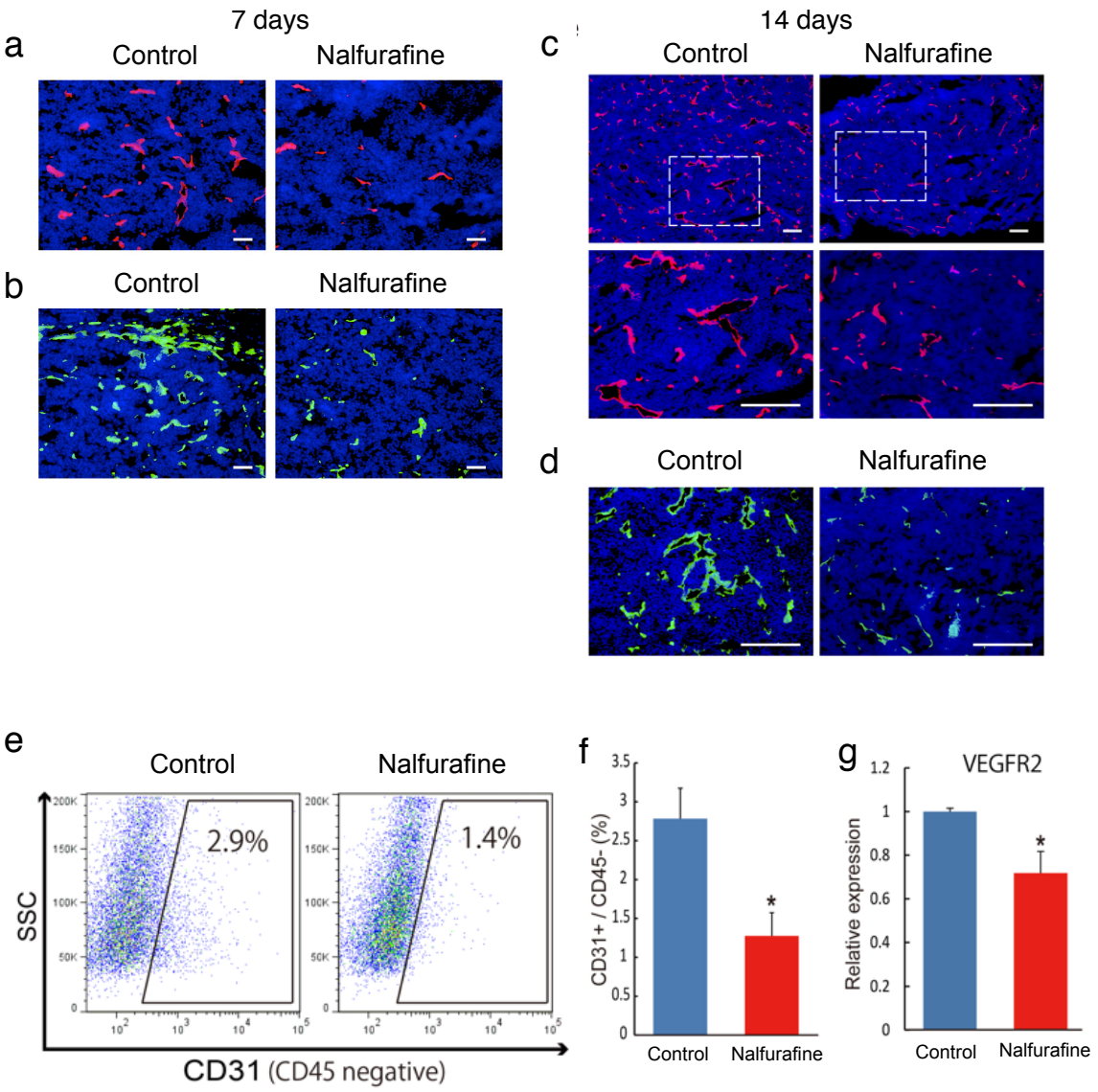


Figure 2-4. Suppression of tumor angiogenesis by nalfurafine in graft mice.
(a) Fluorescent staining for CD31 (red) at 7 days after tumor transplantation. Nuclei are stained with DAPI (blue). Left panel, PBS treated. Right panel, nalfurafine (1 μg/kg)-treated. Scale bars: 50 μm. (b) Fluorescent staining for VE-cadherin (red) at 7 days after tumor transplantation. Nuclei are stained with DAPI (blue). Left panel, PBS treated. Right panel, nalfurafine (1 μg/kg)-treated. Scale bars: 50 μm. (c) Fluorescent staining for CD31 (red) at 14 days after tumor transplantation. Nuclei are stained with DAPI (blue). Left panel, PBS treated. Right panel, nalfurafine (1 μg/kg)-treated. Scale bars: 200 μm. (d) Fluorescent staining for VE-cadherin (green) at 14 days after tumor transplantation. Nuclei are stained with DAPI (blue). Left panel, PBS treated. Right panel, nalfurafine (1 μg/kg)-treated. Scale bars: 200 μm. (e) Flow cytometry. X-axis: CD31 (CD45 negative), Y-axis: SSC. Percentages of $CD31^{+}/CD45^{-}$ ECs among tumors-dissociated cells are indicated. (f) Quantitative evaluation on $CD31^{+}/CD45^{-}$ EC population in tumors by FACS. Percentages of $CD31^{+}/CD45^{-}$ cell population among tumors-dissociated cells. Tumors transplanted PBS-treated (n = 4) and nalfurafine (1 mg/kg)-treated (n = 4) mice are shown (*$p<0.05$ vs. Control). (g) qPCR showing mRNA expression of VEGFR2 in purified ECs from B16 grafted PBS-treated (n = 4) and nalfurafine (1 μg/kg (n = 4))-treated mice at 14 days after tumor transplantation.

Discussion

Endogenous angiogenesis inhibitors physiologically modulate angiogenesis during tissue remodeling as well as tumor formation [38,84]. In this study, I first demonstrated that the κ opioid system acts the novel endogenous angiogenesis inhibitor in tumor. Zabrenetzky et al revealed that expression of thrombospondin1 (TSP1), which is the first protein to be recognized as an endogenous angiogenesis inhibitor is inversely correlated with malignant progression in melanoma, lung and breast carcinoma [45]. Suppression of TSP1 augmented tumor angiogenesis through matrix metalloprotease 9 production and enhancement of VEGFR2 signaling [45]. In contrast, TSP1 overexpression resulted in delayed tumor growth by inhibition tumor angiogenesis [46]. Present data showed similar results that a loss of KOR function, through the use of KO mice, remarkably increased tumor angiogenesis and tumor growth. Conversely, treatment with KOR agonists prevented tumor growth.

To date, approximately 30 endogenous angiogenesis inhibitors have been identified [43]. Many endogenous angiogenesis inhibitors including TSP1 are fragments of naturally occurring extracellular matrix and basement membrane proteins [44]. Although novel anti-angiogenesis drugs that target other molecules on tumors are needed to complement conventional therapies, it is difficult to accurately control their expression and to apply them in clinical medicine. In contrast, opioids have been used clinically as effective analgesics for a

long time. My findings showed that the KOR system could act directly on tumor angiogenesis to suppress VEGFR2 expression; to my knowledge, this is the first report of an endogenous angiogenesis inhibitor of a ligand-receptor system that suppresses the expression of VEGF receptor. Thus, the molecular regulation of KOR systems may be a potential therapeutic strategy for cancers.

Interestingly, I found that the KOR agonist nalfurafine, which has been clinically approved in Japan for use in hemodialysis-related uremic pruritus, could be useful for tumor therapy by suppressing tumor angiogenesis, and thus could offer therapeutic benefits beyond the relief of cancer pain. However, patients develop tolerance to opioid receptor agonists including nalfurafine through repeated use [90]. Although a low dose (0.1-10 mg/kg, *b.i.d.*) of nalfurafine, which is effective for managing itching and pain in mice, significantly inhibited tumor angiogenesis and tumor growth at 7 and 14 days, an extremely high dose (150 mg/kg) had no significant effect on tumor growth (data not shown). These results suggest that continuous treatment with KOR agonists might lead to the development of tolerance to their anti-angiogenic effects on tumors. Therefore, more precise and careful observations are required to establish tumor therapies with KOR agonists. Nevertheless, a better understanding of the antiangiogenic effects of κ opioids and the ability to manipulate the ligand-receptor system of opioids in tumor angiogenesis should greatly contribute to basic vascular biology as well as to applied cancer therapy beyond the relief of cancer pain.

Chapter 3

The κ-opioid receptor agonist nalfurafine enhances the chemotherapy-induced survival advantage in pancreatic cancer-bearing mice

Introduction

Numerous clinical observations have indicated that advanced pancreatic cancer with peritoneal dissemination is only slightly influenced by systemic chemotherapy, and its prognosis remains poor. Thus, the progression of peritoneal dissemination in pancreatic cancer patients is regarded as a terminal condition [91,92]. Accordingly, more effective therapies for pancreatic cancer are needed to improve therapeutic responses and to increase survival in pancreatic cancer patients.

Tumor angiogenesis enables cancer cells to intake nutrients and oxygen leads to the acquisition of mobility and invasiveness, which resulted in tumor growth and metastasis [2,35]. There is a growing body of evidence that the activation of several angiogenesis-related factors, such as VEGF, in the tumor microenvironment can cause tumor angiogenesis and aggravation, suggesting that anti-angiogenic therapies could be an important approach to cancer treatment [37,38]. In clinical medicine, it has been generally accepted that an anti-angiogenic agent (e.g., bevacizumab, a humanized monoclonal immunological sequestering VEGF-A) should be administered in combination with various chemotherapies to enhance their effects [39].

Opioid systems mainly consist of three different types of opioid receptors, μ, δ, and κ (MOR, DOR, and KOR), and the respective endogenous peptide. These opioid systems regulate a wide range of physiological functions such as pain, the emotional response and the

reward circuitry in neural tissues [47,48]. Clinically, opioid analgesics such as morphine, a MOR agonist, have been broadly applied to relieve pain associated with all types of cancer. However, independent studies have shown that morphine can either decrease or increase tumor growth in mice [74,75], and the effects of opioids on tumor growth are still unclear. In our recent studies, we first found that κ-opioid peptides acted as novel anti-angiogenic modulators by suppressing the expression of VEGF receptors during vascular differentiation in development [71] and tumor growth as shown in chapter 1 and 2. In addition, a novel KOR agonist nalfurafine, also known as nalfurafine, was recently synthesized in Japan [65] and has been clinically approved for use in hemodialysis-related uremic pruritus.

In this study, I demonstrated whether nalfurafine could enhance the chemotherapy-induced survival advantage in pancreatic cancer-bearing mice.

Materials and methods

Animals

In this study, the experiments were performed on male C57BL/6J mice (Tokyo Laboratory Animals Science), aged 6 weeks at the start of the experimental procedures. All animals were housed 5 per cage and kept in a room with an ambient temperature of 23 ± 1 °C and a 12 hr light-dark cycle (lights on 8:00 a.m. to 8:00 p.m.). Food and water were available ad libitum during the experimental period and behavioral testing occurred in the morning.

Pancreatic cancer cell inoculation

Pan02 cells, a murine pancreatic ductal adenocarcinoma (PDAC) cell line, were kind gifts from Dr. Aoki (National Cancer Center Research Institute, Tokyo, Japan). Pan02 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich Co.) supplemented with 10% FBS, 1% antibiotics and 1% L-glutamine (Thermo Fisher Scientific Inc.). The density of Pan02 cells was adjusted to 1 x 10^6 cells in 1 ml PBS. In the experimental group, the cell suspension was injected into the abdominal cavity. In the control group, PBS was injected into the abdominal cavity instead of Pan02 cells. Body weight and food consumption were measured 12 and 24 days after tumor implantation.

Hunching behavior was examined as previously described at Suzuki *et al* [93]. Mice were placed individually in the center of an open field arena and observed for 300 s. The hunching score was the total time (s) the mouse exhibited hunching behavior multiplied by the scoring factor, which was defined according to Sevcik *et al.* [94]: 0: normal coat luster, displays exploratory behavior; 1: mild rounded-back posture, normal coat luster, displays slightly reduced exploratory behavior; 2: severe rounded-back posture, displays considerably reduced exploratory behavior, piloerection, intermittent abdominal contractions. Behavioral testing was performed at 12 and 24 days after tumor inoculation.

Survival studies

Beginning 7 days after tumor inoculation, mice were injected intraperitoneally with saline, gemcitabine (GEM; 100 mg/kg, *bis. in 7d.*; Wako Pure Chemical Industries Ltd., Osaka, Japan), nalfurafine (NAL; 10 μg/kg, *b.i.d*) or GEM/NAL for 6 weeks. The survival time of each group was calculated from the date of enrollment to the date of death from tumor inoculation by the Kaplan-Meier method.

Cell viability assay

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT) assay. In the MTT assay, Pan02 cells (5 x 10^3 cells/well) were cultured in a 96-well plate and then treated with gemcitabine (0.01-1μM) or nalfurafine (0.01-1μM) for 24

hr. Forty-eight hr after drug treatments, 20 μL of MTT solution (5 mg/mL, Sigma-Aldrich Co.) was added to each well of the culture medium. After incubation for another 2 hr, the medium was removed, and 100μL of dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries Ltd.) was added to resolve formazan crystals. Optical density was measured using a luminometer (Glomax, Promega Co., Madison, WI, USA) at an absorption wavelength of 600 nm (test wavelength) and 750nm (reference wavelength). In each experiment, three replicates were prepared for each sample. The proportion of living cells was determined based on the difference in absorbance between samples and controls.

Tube formation assay

Tube formation assay was conducted as previously described in Chapter 1. HUVECs (1.5×10^4 cells/well) were cultured in a 24-well plate coated with 150 μl Matrigel Basement Membrane Matrix GFR (BD Biosciences). The tube formation assay was performed under treatment with 10 μM nalfurafine.

Statistical analysis

Data are expressed as the mean with SEM. No data points were removed from the statistical analysis except as specified. The data were subjected to an unpaired *t*-test or one-way ANOVA test followed by the Bonferroni multiple comparisons test as appropriate for the experimental design. The data with time-dependent changes were analyzed using

two-way RM ANOVA followed by the Bonferroni post-hoc test, where appropriate. Mortality data were compared using Kaplan-Meier plots and the log-rank test. All statistical analyses were performed with Prism version 5.0 (GraphPad Software).

Results

Characterization of pancreatic cancer-bearing mice

First, I generated a mouse model of pancreatic cancer with peritoneal metastasis by the intraperitoneal inoculation of murine pancreatic cancer Pan02 cells (Fig. 3-1a). To assess the effect of tumor inoculation, I observed the changes in body weight and food consumption in mice for 24 days after tumor inoculation. Both the body weight and food consumption in mice with tumor inoculation were significantly decreased compared to those in control mice (Fig. 3-1b, c). Next, I examined visceral pain-related behavior caused by intraperitoneal tumor inoculation in mice. Hunching behavior has been previously described as a measure of abdominal pain caused by pancreatic cancer in mice [93,94]. Here I demonstrated that marked spontaneous visceral pain-related behavior was observed in mice at 12 and 24 days after tumor inoculation compared to that in control mice (Fig. 3-1d, e; *P<0.05, ***P<0.001 vs. Control, unpaired *t*-test; Fig. 3-1d, e; $F_{(1,6)}$=33.31, P=0.0012 for interaction, Two-way RM ANOVA). Collectively, I hypothesized that these tumor-bearing mice showed peritoneal metastasis of pancreatic cancer.

Treatment with the KOR agonist nalfurafine enhanced the survival advantage of gemcitabine in pancreatic cancer-bearing mice

In general, gemcitabine is widely recommended as first-line chemotherapy against

pancreatic cancer. On the other hand, as shown in chapter 1 and 2, I previously found that KOR agonists may be able to inhibit tumor growth through inhibiting tumor angiogenesis. Therefore, to investigate the effect of the KOR agonist nalfurafine on the anti-tumor effect of gemcitabine, I examined whether combined treatment with gemcitabine and nalfurafine could prolong survival in a mouse model of pancreatic cancer with peritoneal metastasis according to Kaplan-Meier method (Fig. 3-2a). As a result, I first confirmed that peritoneal metastasis-model mice that were injected with gemcitabine (100mg/kg, *bis. in 7d.*) for 6 weeks from 7 days after tumor inoculation exhibited clearly prolonged survival compared with that in control mice (Fig. 3-2b, c; ***P<0.001 vs. Control; proportion of median survival, 2.232; 95% CI, 1.826 to 2.638; an improvement of 42.5 days). In contrast, the repeated-intraperitoneal administration of only nalfurafine at the dose of 10 μg/kg (*b.i.d.*), which has shown its pharmacological effects as shown in chapter 2, slightly prolonged survival in peritoneal metastasis-model mice (Fig. 3-2b, c; *P<0.05 vs. Control; proportion of median survival, 1.275; 95% CI, 0.859 to 1.692; an improvement of 9.5 days). Interestingly, I demonstrated that the survival time of mice that were treated with gemcitabine in combination with nalfurafine was significantly greater than that of mice treated with gemcitabine alone (Fig. 3-2b, c; $^{\#}$P<0.05 vs. GEM; proportion of median survival, 1.130; 95% CI, 0.774 to 1.486; an improvement of 10.0 days). Taken together, these results suggest that treatment with nalfurafine enhanced the survival advantage induced by treatment with gemcitabine in pancreatic cancer-bearing mice.

Treatment with nalfurafine suppresses tumor angiogenesis, but not cell growth, in tumor cells

To clarify the mechanisms that underlie the enhancement of the gemcitabine-induced survival advantage under combined treatment with nalfurafine in pancreatic cancer-bearing mice, I next investigated whether *in vitro* treatment with nalfurafine could directly suppress the growth of Pan02 cells using MTT assays. In this study, treatment with gemcitabine (0.01-1μM) significantly suppressed the growth of Pan02 cells in a concentration-dependent manner (Fig. 3-3a), whereas the growth of Pan02 cells was not affected by treatment with nalfurafine (0.01-1μM) (Fig. 3-3b).

I next examined whether *in vitro* treatment with nalfurafine could directly inhibit angiogenesis using HUVEC tube formation assays. As a result, treatment with 10μM nalfurafine dramatically suppressed HUVEC tube formation (Fig. 3-3c) as well as shown in chapter 1.

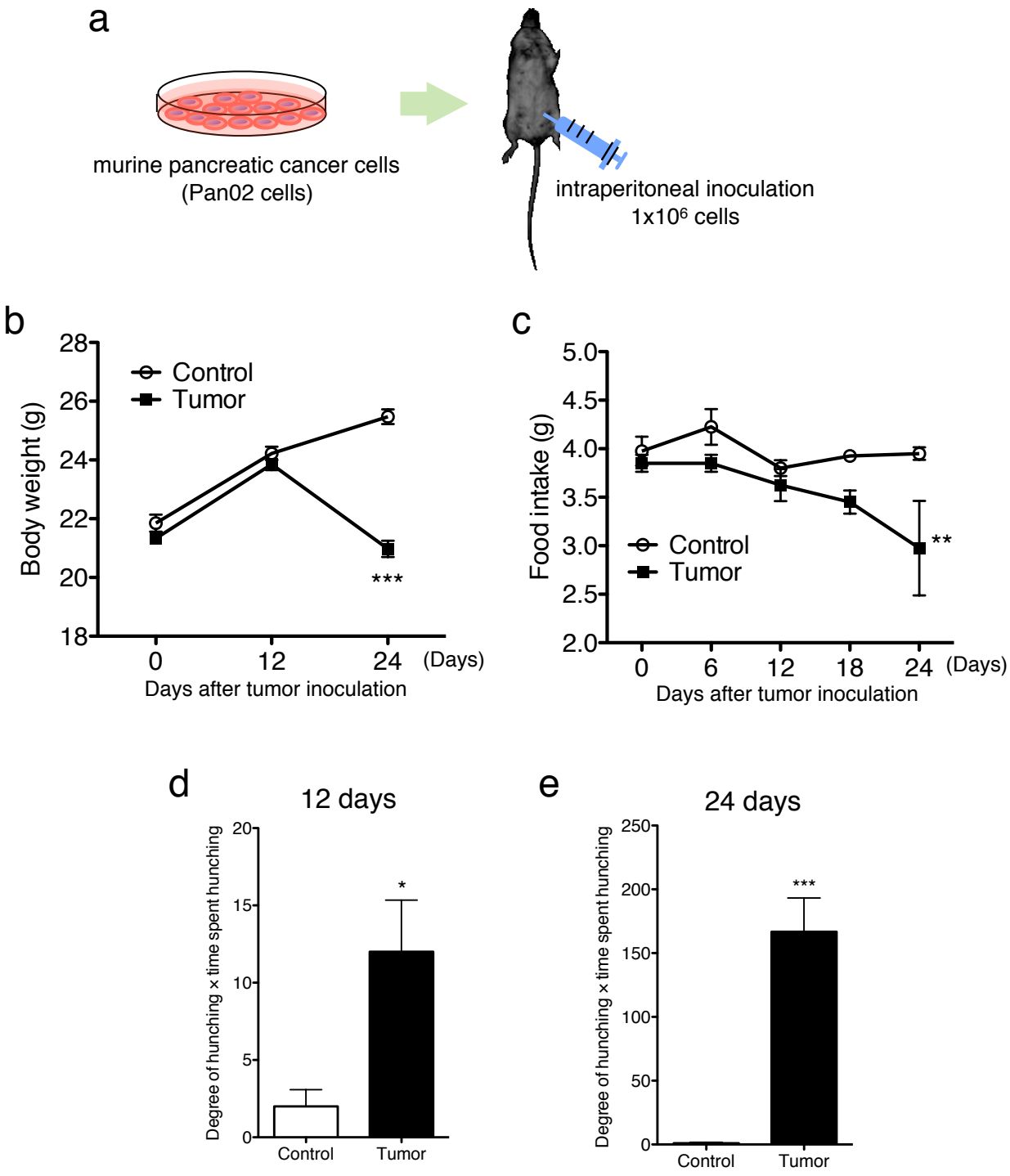
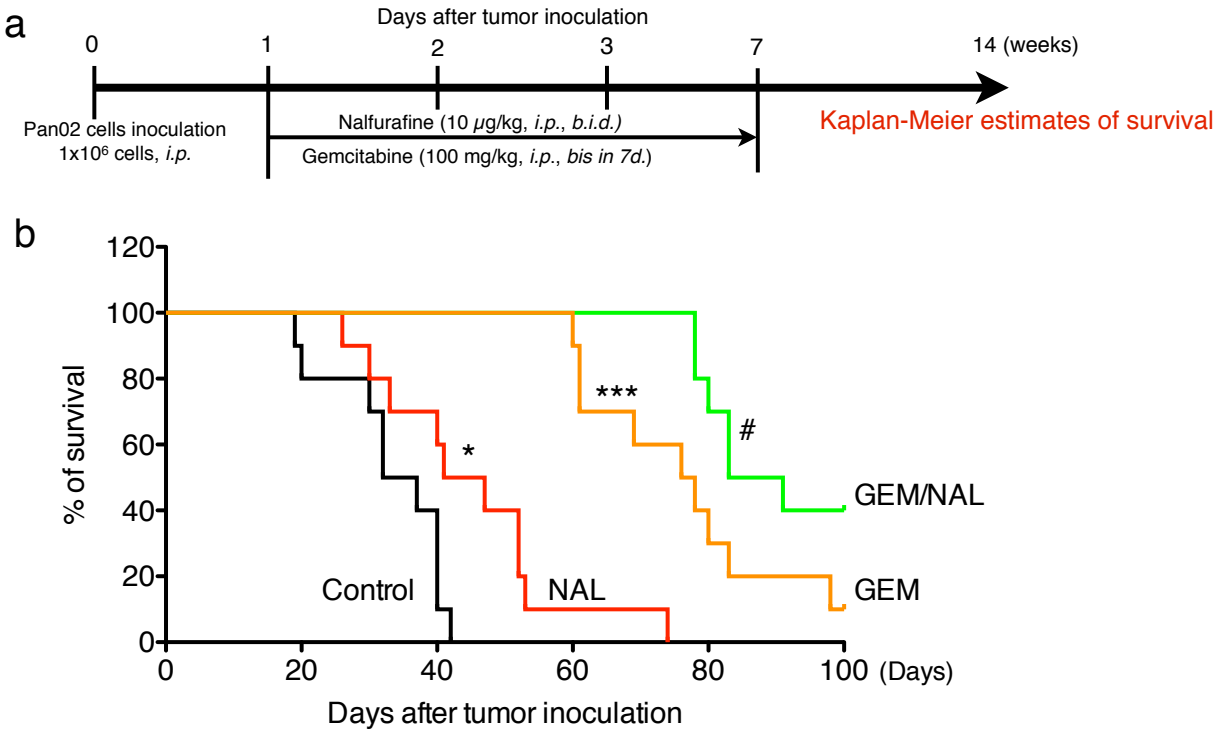


Figure 3-1. Characterization of pancreatic cancer-bearing mice.
(a) Schematic illustration of the intraperitoneal inoculation of Pan02 cells. (b, c) Time-course changes in body weight (b) and food consumption (c) of tumor-bearing mice after tumor inoculation. Each point represents the mean with SEM (n = 4, **p<0.01, ***p<0.001 vs. Control group). (d, e) Changes in visceral pain-related behavior of tumor-bearing mice in terms of the degree of hunching and time spent hunching (over 300 s) at 12 and 24 days after tumor inoculation. Each column represents the mean with SEM (n = 4, *p<0.05, ***p<0.001 vs. Control group).



c

	Median survival	Hazard ratio	95% CI
Control	34.5 days	-	-
NAL	44.0 days	0.259 (vs Cont)	0.084 to 0.793
GEM	77.0 days	0.041 (vs Cont)	0.010 to 0.162
GEM/NAL	87.0 days	0.314 (vs GEM)	0.103 to 0.957

Figure 3-2. Effects of the combined treatment with gemcitabine and nalfurafine on survival rates in pancreatic cancer-bearing mice.
(a) Protocol for the combined treatment with gemcitabine (GEM, 100mg/kg) and nalfurafine (NAL, 10 μg/kg) in pancreatic cancer-bearing mice. (b) Survival curves of pancreatic cancer-bearing mice treated with saline (Control), GEM, NAL and GEM/NAL (n = 10, Log-rank test: *p<0.05, ***p<0.001 vs. control group, #p<0.05 vs. GEM group). (c) The median survival and hazard ratio in Kaplan-Meier plots of pancreatic cancer-bearing mice treated with saline (Control), GEM, NAL or GEM/NAL.

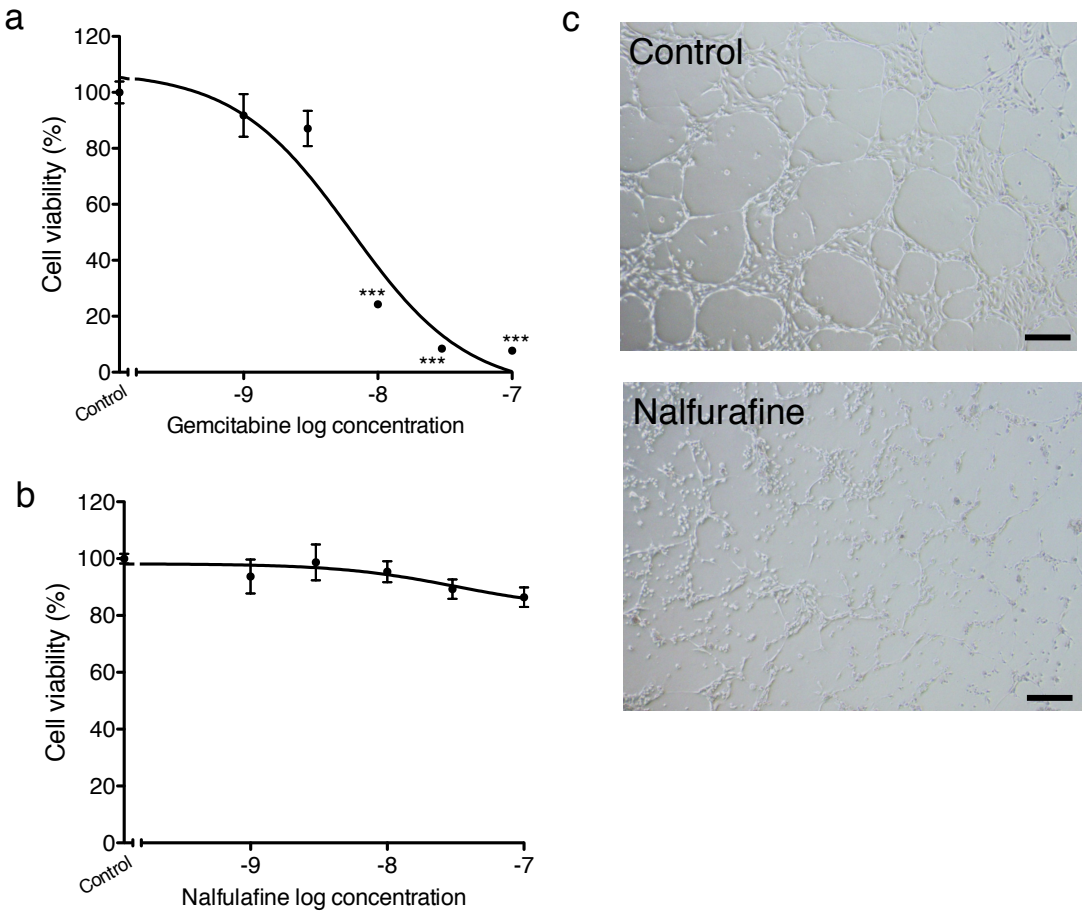


Figure 3-3. Nalfurafine inhibit angiogenesis, but not cancer cell growth.
(a,b) Changes in cell viability of Pan02 cells under treatment with gemcitabine (a, 0.01-1 μM) and nalfurafine (b, 0.01-1 μM). Each data point represents the mean with SEM (n = 5, ***p<0.001 vs. Control). (c) Representative photographs of vasculature in the HUVEC tube formation assay under treatment with 10 μM nalfurafine. Scale bars: 200 μm.

Discussion

PDAC is known to be a leading cause of cancer-related deaths with poor survival rates and a poor prognosis [91,92,95]. Thus, more effective therapies are needed. In this study, I demonstrated that the KOR agonist nalfurafine could have potential for facilitating the chemotherapy-induced survival advantage in pancreatic cancer-bearing mice, since I identified that nalfurafine was useful for anti-cancer therapy through inhibiting tumor angiogenesis, as shown in chapter 1 and 2.

A large number of patients with PDAC are incredibly refractory and show cachexia and systemic metastasis [96,97]. There has been little improvement in patient outcomes, even though considerable effort has been directed at optimizing the use of chemotherapy (e.g., gemcitabine) for pancreatic cancer [95,98,99]. First, I generated peritoneal metastasis-model mice by the intraperitoneal inoculation of Pan02 cells into their abdominal cavity. Next, I confirmed that these model mice exhibited weight loss, feeding suppression and spontaneous visceral pain-related behaviors induced by tumor inoculation, consistent with the description of clinical PDAC patients.

On the other hand, it has been widely accepted that tumor angiogenesis is crucial for tumor progression and metastasis, so that an anti-angiogenic agent is often included in the chemotherapy regimen in various cancer treatments [37,38]. Especially, there is a growing

body of evidence that VEGF, which is a critical factor in tumor angiogenesis, is highly expressed in the tumor microenvironment. As a result, it has been targeted for anti-cancer therapy using antibody drugs such as bevacizumab [39,89]. In our recent studies and as shown in chapter 1 and 2, we clarified that endogenous κ-opioid peptides acted as novel anti-angiogenic modulators by inhibiting VEGF signaling during vascular differentiation in development and tumor progression, which occurs through the suppression of VEGF receptor expression, VEGFR2 and NRP1, via the inhibition of cAMP/PKA signaling [71]. In addition, I demonstrated that tumor growth was dramatically suppressed by the repeated administration of KOR agonists in xenograft mice as shown in chapter 2. Therefore, in this study, I investigated whether the combined administration of gemcitabine and nalfurafine, which is a KOR agonist in widespread clinical use, could prolong survival in pancreatic cancer-bearing mice. I found that the administration of nalfurafine significantly enhanced the gemcitabine-induced survival advantage in pancreatic cancer-bearing mice.

To clarify the underlying mechanism of the effect of nalfurafine on the gemcitabine-induced survival advantage, I next investigated whether treatment with nalfurafine could directly inhibit the growth of Pan02 cells. Unexpectedly, I found that *in vitro* treatment with gemcitabine significantly suppressed the growth of Pan02 cells, while there were no changes with nalfurafine. Finally, I confirmed that *in vitro* treatment with nalfurafine dramatically inhibited HUVEC tube formation as well as shown in chapter 1. These results indicate that the suppression of tumor angiogenesis caused by the

administration of nalfurafine occurred via the inhibition of VEGF signaling along with the activation of KORs in host endothelial cells in the tumor.

Taken together, these findings suggest that nalfurafine suppressed tumor angiogenesis, but not cell growth in tumor, during tumor progression, leading to enhancement of the gemcitabine-induced survival advantage in pancreatic cancer-bearing mice. These findings may suggest a novel strategy for chemotherapy.

General Conclusion

The above findings led to the following conclusions:

In Chapter 1:

In the present study, I demonstrated a novel mechanism for the regulation of *de novo* angiogenesis through the opioid system. First, I found that KORs, but not MORs and DORs, were highly expressed in HUVECs and ECs purified from adult mice. Under these conditions, the treatment with selective KOR agonists, U50,488H and nalfurafine significantly inhibited EC migration and tube formation. Additionally, these inhibitory effects of KOR agonists were dramatically reversed by the treatment with nor-BNI, a selective KOR antagonist, and knock down of KOR expression using siRNA. These results indicate that KOR activation could directly suppress *de novo* angiogenesis. In the next study, to clarify the underlying mechanisms of the inhibitory effects on *de novo* angiogenesis via KOR activation, I investigated the changes in VEGF signaling, which is a key modulator of angiogenesis, by the treatment with KOR agonists. Interestingly, the results showed that KOR agonists significantly suppressed VEGFR2 expression.

These findings suggest that the KOR system may be an inhibitory regulator of *de novo* angiogenesis, inditcating that the κ opioid system is the first identified as endogenous inhibitory machinery to cAMP/PKA function of ECs in regulation of physiological and

pathogenic angiogenesis.

In Chapter 2:

I investigated whether κ opioid agonists could act as anti-cancer drugs through the inhibition of VEGF signaling. Interestingly, I found that Lewis lung carcinoma (LLC) or B16 melanoma grafted in KOR-null and PDYN-null mice showed increased proliferation of tumor compared with those in wild-type mice. In addition, the deletion of endogenous KOR system markedly enhanced tumor angiogenesis through the high expression of VEGFR2 in the tumor vascular endothelial cells. In contrast, repeated intraperitoneal injection of nalfurafine significantly inhibited tumor growth by suppressing tumor angiogenesis. Interestingly, nalfurafine had no inhibitory effects in B16-grafted in KOR-KO mice, indicating that nalfurafine specifically induced the inhibition of tumor angiogenesis and tumor growth through KOR activation.

These results indicate that the endogenous KOR system could act as a negatively modulator of tumor angiogenesis and progression. Interestingly, these findings show that the KOR agonist nalfurefine, which has already been clinically approved in Japan, can be a novel anti-cancer therapy for tumor angiogenesis inhibitor.

In Chapter 3:

I examined whether the administration of KOR agonist nalfurafine could enhance the

chemotherapy-induced survival advantage in pancreatic cancer-bearing mice. I confirmed that the pancreatic cancer-bearing mice exhibited clinical PDAC patient-like behaviors, such as weight loss, feeding suppression and spontaneous visceral pain. Under these conditions, we demonstrated that the administration of nalfurafine significantly enhanced the gemcitabine-induced survival advantage in pancreatic cancer-bearing mice. In addition, I confirmed that *in vitro* treatment with nalfurafine suppressed angiogenesis, but not the growth of cancer cells.

Taken together, these findings suggest that nalfurafine enhanced gemcitabine-induced survival advantage in pancreatic cancer-bearing mice through suppressing tumor angiogenesis.

In conclusion, the present study demonstrated that KOR system could act as an inhibitory modulator of *de novo* angiogenesis, such as tumor angiogenesis via suppressing VEGF signaling (Fig. 4) [72]. Thus, this novel knowledge provides significant progress for the fields of vascular biological researches and anti-cancer therapy. Furthermore, this study first demonstrated that nalfurafine has a potential as an anti-angiogenic agent, leading to a novel strategy for chemotherapy.

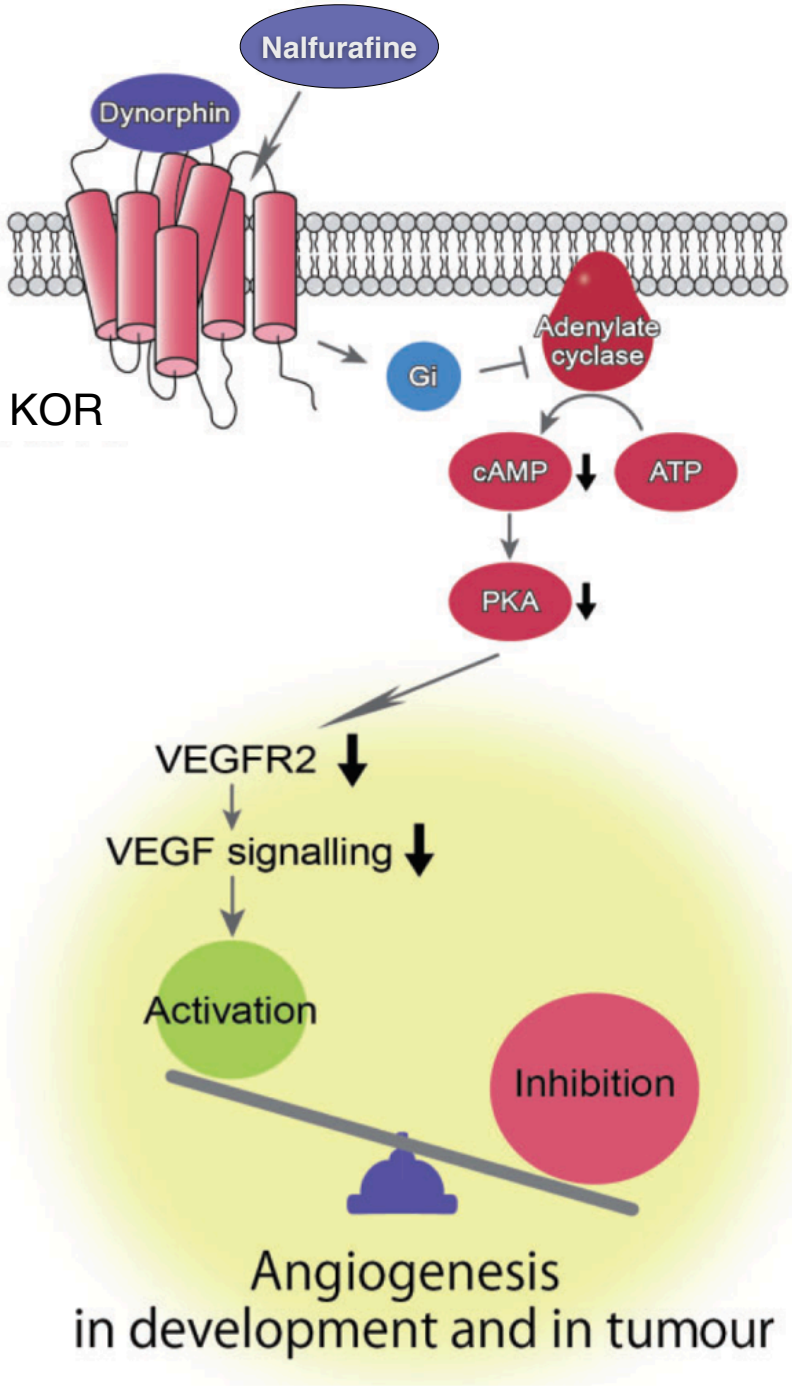


Figure 4. Molecular mechanisms of inhibition of physiological and tumor angiogenesis, and pathfinding by KOR signaling activation.
Signalling through κ opioid receptors (KOR) induced by dynorphin or nalfurafine regulates VEGF signalling, especially VEGFR2 expression, by activating the cAMP/PKA pathway in ECs. The balance between the expression of activators and inhibitors of angiogenesis controls angiogenesis in development and in tumors (adapted from Yamamizu and Hamada *et al.*, 2015) [72].

List of Publications

1. Yusuke Hamada, Yoshihiko Tasaki, Kana Morita, Kohei Yamamizu, Michiko Narita, Fukiko Matsuyama, Masami Suzuki, Daigo Ikegami, Kazuhiko Arakawa, Yasuyuki Nagumo, Miho Kawata, Yasuhito Uezono, Hiroshi Nagase, Kazunori Aoki, Jun K Yamashita, Naoko Kuzumaki and Minoru Narita: The κ-opioid receptor agonist nalfurafine enhances the chemotherapy-induced survival advantage in pancreatic cancer-bearing mice. *Jpn J Pharm Palliat Care Sci* (*in press*): **Chapter 3**

2. Kohei Yamamizu, Sadayoshi Furuta, Yusuke Hamada**,** Akira Yamashita, Naoko Kuzumaki, Michiko Narita, Kento Doi, Shiori Katayama, Hiroshi Nagase, Jun K Yamashita, Minoru Narita: κ Opioids inhibit tumor angiogenesis by suppressing VEGF signaling. *Sci Rep,* 3, 3213 (2013): **Chapter 1, 2**

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