

tests were two sided, and values of $p < 0.05$ were considered to indicate statistical significance.

RESULTS

Patient population

Between January 1999 and December 2009, 2,183 patients with lung cancer from 2 institutions were enrolled in this study. The clinical characteristics of all 2,183 patients are listed in Table 1. One thousand five hundred ninety-one (73%) patients were male and 592 (27%) patients were female. Median age was 70 years, with a range of 15-93 years. Seventy-six percent of all 2,183 patients had smoking history. Causes for detection of lung cancer were as follows ; medical screening (21%), symptom related to lung cancer (55%), examination during follow-up of other

diseases (24%). Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) constituted 87% and 13% of all lung cancer patients, respectively. With regard to NSCLC, the predominant histological type was adenocarcinoma (51%) followed by squamous cell carcinoma (25%), large cell carcinoma (3%) and others (8%).

As shown in Table 2, 471 (22%), 213 (10%), 24 (1%), 116 (5%), 238 (11%), 370 (17%) and 678 (31%) patients had stage IA, IB, IIA, IIB, IIIA, IIIB and IV lung cancer, respectively. In NSCLC, 783 (41%) and 1,060 (56%) patients had early (IA, IB, IIA and IIB) and advanced (IIIA, IIIB and IV) stages, respectively. In SCLC, 133 (48%) patients had distant metastasis, which was consistent with previous report (13). Among all 2,183 patients, 1,481 (68%) and 702 (32%) belonged to younger and elderly population, respectively. There was no significant difference in stage distribution between younger and elderly population in NSCLC, SCLC and total lung cancer.

Table 1. Patient characteristics

No.	2,183
Age (Year)	
Median (Range)	70 (15-93)
Gender	
Male	1,591 (73%)
Female	592 (27%)
Histology	
Non-small cell lung cancer	1,905 (87%)
Adenocarcinoma	1,112 (51%)
Squamous cell carcinoma	545 (25%)
Large cell carcinoma	69 (3%)
Others	179 (8%)
Small cell lung cancer	278 (13%)

Treatment modalities and patient survival

Next, we analyzed the first-line treatment modalities in 1,763 patients who were initially treated in two participating institutions. Five hundred sixteen (29%), 191 (11%), 58 (3%), 755 (43%) and 216 (12%) patients were treated with chemotherapy, chemo-radiotherapy, thoracic radiotherapy, operation and best supportive care, respectively. As expected, there was remarkable difference of initial treatment among histological subtypes of lung cancer, namely, operation was undergone in almost half (48%) of NSCLC patients, while SCLC patients were predominantly treated with chemotherapy (60%).

Table 2. Comparison of clinical stages for lung cancer between younger and elderly patients.

Stage	Number of patients (%)								
	NSCLC			SCLC			Total		
	Younger	Elderly	Total	Younger	Elderly	Total	Younger	Elderly	Total
IA	303 (23)	146 (24)	449 (24)	11 (6)	11 (12)	22 (8)	314 (21)	157 (22)	471 (22)
IB	125 (10)	82 (13)	207 (11)	4 (2)	2 (2)	6 (2)	129 (9)	84 (12)	213 (10)
IIA	16 (1)	7 (1)	23 (1)	1 (1)	0 (0)	1 (0)	17 (1)	7 (1)	24 (1)
IIB	62 (5)	42 (7)	104 (5)	8 (4)	4 (4)	12 (4)	70 (5)	46 (7)	116 (5)
IIIA	142 (11)	66 (11)	208 (11)	21 (11)	9 (10)	30 (11)	163 (11)	75 (11)	238 (11)
IIIB	202 (16)	105 (17)	307 (16)	43 (23)	20 (22)	63 (23)	245 (17)	125 (18)	370 (17)
IV	408 (32)	137 (22)	545 (29)	94 (51)	39 (42)	133 (48)	502 (34)	176 (25)	678 (31)
unstaged	37 (3)	25 (4)	62 (3)	4 (2)	7 (8)	11 (4)	41 (3)	32 (5)	73 (3)
Total	1,295	610	1,905	186	92	278	1,481	702	2,183

NSCLC : non-small cell lung cancer, SCLC : small cell lung cancer
 younger : 74 years or younger patients, elderly : 75 years or older patients

Among 1,763 patients, 1,218 (69%) and 545 (31%) belonged to younger and elderly population, respectively. We compared the initial treatment modalities for lung cancer among these populations. The proportions of NSCLC patients treated with operation and that of SCLC patients treated with chemotherapy were comparable between younger and elderly population (48% in younger, 47% in elderly and 59% in younger, 61% in elderly, respectively). On the other hand, the proportion of patients treated with chemo-radiotherapy tended to be lower in elderly than younger population (5% vs. 11% in NSCLC ($p=0.13$), 11% vs. 27% in SCLC ($p=0.06$) and 6% vs. 13% in total lung cancer ($p=0.12$), respectively), and that of patients treated with best supportive care tended to be higher in elderly than younger population (25% vs. 8% in NSCLC ($p=0.08$), 13% vs. 3% in SCLC ($p=0.18$) and 23% vs. 7% in total lung cancer ($p=0.09$), respectively), while these differences were not significant (Table 3).

Finally, we evaluated the survival of the subgroup underwent chemotherapy in Tokushima University Hospital. Between January 1999 and December 2009, 204 and 84 patients with NSCLC were treated with chemotherapy, chemo-radiotherapy, respectively. The median TTP and the median survival time (MST) of patients treated with chemotherapy and chemo-radiotherapy were 3.5 months, 13.0 months and 7.0 months, 18.0 months, respectively (Fig. 1 and 2). Among 204 NSCLC patients treated with chemotherapy, 33 (16%) belonged to elderly population. The median TTP and the MST of 33 elderly patients treated with chemotherapy were 3.3 months and 18.0 months, respectively (Fig. 3), which were comparable with those of total population.

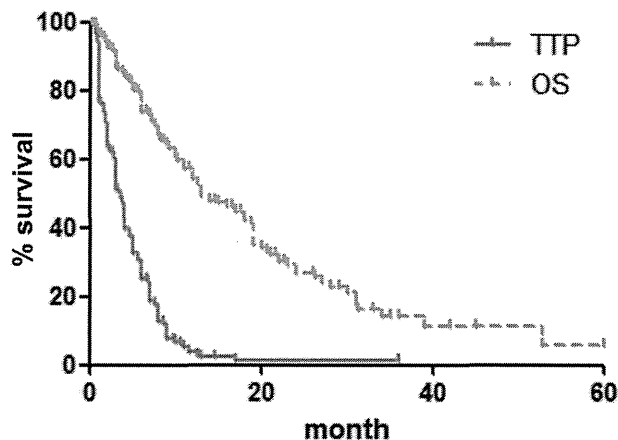


Figure 1. The survival of NSCLC patients treated with chemotherapy.

The survival of 204 NSCLC patients treated with chemotherapy was calculated according to the Kaplan-Meier method. The median TTP and MST were 3.5 months and 13.0 months, respectively.

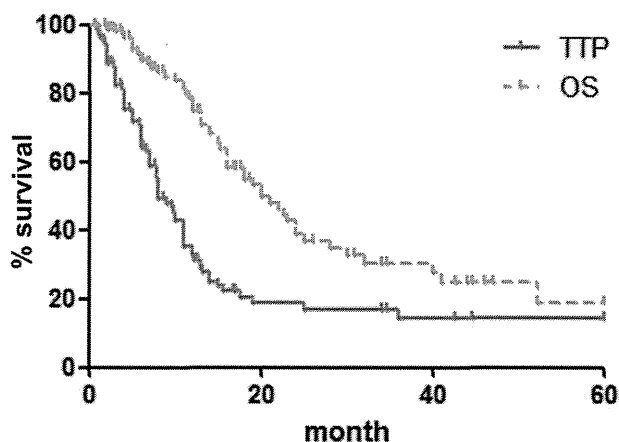


Figure 2. The survival of NSCLC patients treated with chemo-radiotherapy.

The survival of 84 NSCLC patients treated with chemo-radiotherapy was calculated according to the Kaplan-Meier method. The median TTP and MST were 7.0 months and 18.0 months, respectively.

Table 3. Comparison of treatment modalities for lung cancer between younger and elderly patients.

Treatment	Number of patients (%)								
	NSCLC			SCLC			Total		
	Younger	Elderly	Total	Younger	Elderly	Total	Younger	Elderly	Total
Chemotherapy	311 (29)	71 (15)	382 (25)	90 (59)	44 (61)	134 (60)	401 (33)	115 (21)	516 (29)
Chemo-radiotherapy	116 (11)	26 (5)	142 (9)	41 (27)	8 (11)	49 (22)	157 (13)	34 (6)	191 (11)
Thoracic radiotherapy	17 (2)	36 (8)	53 (3)	2 (1)	3 (4)	5 (2)	19 (2)	39 (7)	58 (3)
Operation	513 (48)	221 (47)	734 (48)	14 (9)	7 (10)	21 (9)	527 (43)	228 (42)	755 (43)
Best supportive care	87 (8)	116 (25)	203 (13)	4 (3)	9 (13)	13 (6)	91 (7)	125 (23)	216 (12)
Others	22 (2)	3 (1)	25 (2)	1 (1)	1 (1)	2 (1)	23 (2)	4 (1)	27 (2)
Total	1,066	473	1,539	152	72	224	1,218	545	1,763

NSCLC : non-small cell lung cancer, SCLC : small cell lung cancer
 younger : 74 years or younger patients, elderly : 75 years or older patients

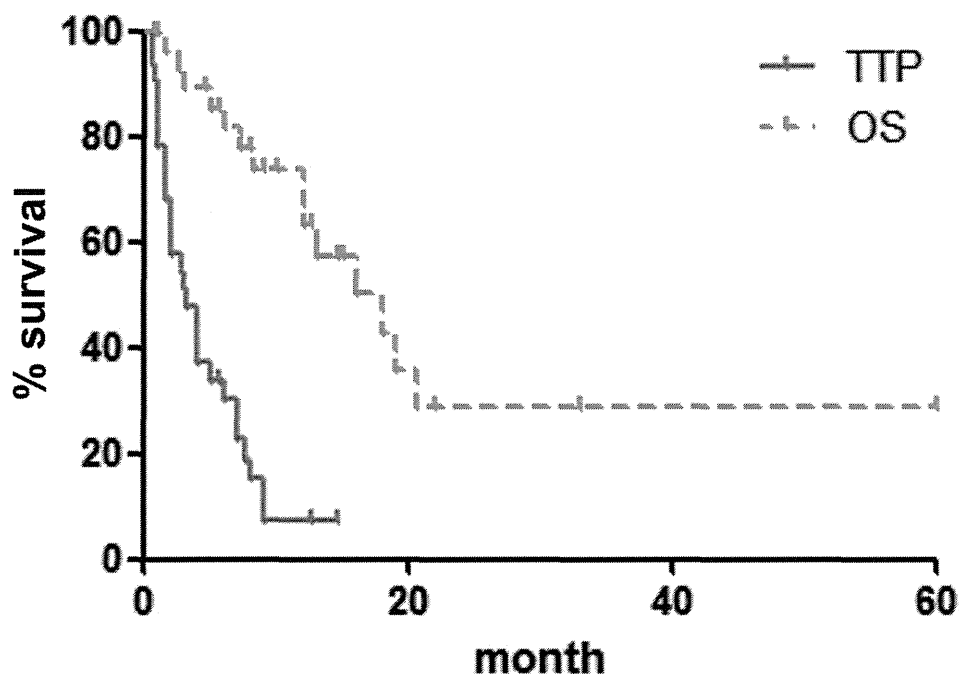


Figure 3. The survival of elderly NSCLC patients treated with chemotherapy.

The survival of 33 elderly NSCLC patients treated with chemotherapy was calculated according to the Kaplan-Meier method. The median TTP and MST were 3.3 months and 18.0 months, respectively.

DISCUSSION

Lung cancer has been the most common cancer in the world since 1985 (14), and by 2002, there were 1.35 million new cases, representing 12.4% of all new cancers. It was also the most common cause of death of cancer, with 1.18 million deaths, or 17.6% of the world total. The prognosis for people diagnosed with lung cancer remains poor worldwide, with 5-year relative survival typically between 6 to 14% among males and 7 to 18% among females (15).

The incidence and mortality of lung cancer are very much influenced by past exposure to tobacco smoking (16). For the year 2000, an estimated 85% of lung cancer in men and 47% of lung cancer in women is the consequence of tobacco smoking. A higher proportion of lung cancer deaths were attributable to smoking than for any other disease (17). It has been estimated that nearly three quarters (71%) of lung cancer deaths worldwide were caused by smoking in 2000 (17). By 2015, this would result in a projected 1.18 million smoking related lung cancer deaths per year (7). In this study, 76% of lung cancer patients had smoking history, indicating that the high prevalence of tobacco smoking in Tokushima Prefecture was closely related to the occurrence and morbidity of lung cancer in consistent with previous reports.

Lung cancer is, to a major extent, a disease of the elderly (8). The prevalence and societal burden of this disease will increase as more people survive into old age. Given that the population older than 65 years constitutes the fastest-growing segment and is projected to double by the year 2030 (18), elderly lung cancer patients are anticipated to be going to increase hereafter. Worldwide during 2002, 5% of lung cancer cases were diagnosed among people aged 0 to 44 years, 14% in the 45 to 54 age group, 25% in the 55 to 64 age group, and 55% among those aged 65 years and over (19). In all parts of Japan and Tokushima Prefecture, 33,165 and 265 elderly (75 years or older) patients died of lung cancer in 2005, respectively. The proportions of elderly patients in all parts of Japan and Tokushima Prefecture constituted 53% and 58% of all lung cancer-related death, respectively (12), suggesting that around half of lung cancer patients belonged to elderly population in Japan. In the present study, 32% (702/2,183) of lung cancer patients was elderly population. As Tokushima University Hospital and Tokushima Prefectural Central Hospital are highly specified hospitals to the intensive cancer treatment, advanced lung cancer patients who are considered to be intolerable to intensive treatment owing to poor performance status and/or high age tend to be avoided for referral to our two institutions by primary doctors. Therefore, the discrepancy of the

proportion of elderly patient population between previous reports and our study might be explained by the under-estimation of the number of elderly lung cancer patients in the present study.

According to cancer registry data in National Cancer Center, Japan (12), age-standardized lung cancer mortality rates (per 100,000) in 2005 were comparable between all parts of Japan (15.63) and Tokushima Prefecture (15.57). But Tokushima Prefecture had a tendency to have more elderly patients who died of lung cancer than all parts of Japan. The proportions of elderly patients in total population, male and female were 53% and 58%, 51% and 55%, and 60% and 67% in all parts of Japan and Tokushima Prefecture, respectively (Table 4). These epidemiologic data might reflect the trend that elderly population in Tokushima Prefecture had been growing faster than cross-sectional population of Japan.

In the present study, there was no difference in the proportion of NSCLC patients initially treated with operation between younger and elderly population (48% and 47%, respectively), suggesting that operation could be actively performed even in elderly lung cancer patients after consideration for its indication. Similarly, the proportion of SCLC patients treated with chemotherapy was also comparable between younger and elderly population (59% and 61%, respectively), reflecting the fact that SCLC is characterized by significant sensitivity to initial chemotherapy (20). On the other hand, the proportion of patients initially treated with chemo-radiotherapy tended to be lower in elderly than younger population (5% vs. 11% in NSCLC, 11% vs. 27% in SCLC

and 6% vs. 13% in total lung cancer, respectively), and that of patients initially treated with best supportive care tended to be higher in elderly than younger population (25% vs. 8% in NSCLC, 13% vs. 3% in SCLC and 23% vs. 7% in total lung cancer, respectively), while these differences were not significant (Table 3). Elderly lung cancer patients commonly have poor performance status and higher incidence of smoking-related comorbidities, such as cardiovascular diseases, chronic pulmonary emphysema, pulmonary fibrosis and so on. These factors might lead to the physician's discretion to avoid aggressive anti-cancer treatment in these patients.

A number of randomized clinical trials support the conclusion that the combined modality of chemotherapy and radiotherapy improve survival compared with either chemotherapy or radiotherapy alone for locally advanced NSCLC (21, 22). The response rate (RR) and MST in the phase III trials that use chemo-radiotherapies have been reported to be 49-84% and 14.5-16.5 months, respectively (23, 24). In this study, the MST of 84 patients treated with chemo-radiotherapy was 18.0 months, which was comparable with previous phase III studies.

The standard chemotherapy regimen for advanced NSCLC is considered to be two-drug combination chemotherapy with platinum agents and new-generation non-platinum antitumor agents, such as paclitaxel, docetaxel, gemcitabine, and vinorelbine. The RR, TTP and MST in the phase III trials that use these combination chemotherapies have been reported to be 17-28%, 3-4 months and 7-9 months, respectively (25-27). In this study, the

Table 4. Comparison of epidemiologic trends of lung cancer between all parts of Japan and Tokushima Prefecture in 2005.

Population	Lung cancer-related death			ASR
	Younger No. (%)	Elderly No. (%)	Total No. (%)	
Total				
Japan	28,898 (47)	33,165 (53)	62,063 (100)	15.63
Tokushima	194 (42)	265 (58)	459 (100)	15.57
Male				
Japan	22,196 (49)	22,993 (51)	45,189 (100)	24.97
Tokushima	155 (45)	187 (55)	342 (100)	26.49
Female				
Japan	6,702 (40)	10,172 (60)	16,874 (100)	7.13
Tokushima	39 (33)	78 (67)	117 (100)	5.88

cited from reference 12

younger : 74 years or younger patients, elderly : 75 years or older patients

ASR : age-standardized lung cancer mortality rate (per 100,000)

median TTP and the MST of patients treated with chemotherapy were 3.5 months, 13.0 months, respectively, in consistent with previous phase III studies. Moreover, it is noteworthy that the median TTP and the MST of 33 elderly patients treated with chemotherapy were shown to be 3.3 months, 18.0 months, respectively, which were comparable with those of total population. These results indicated the significant findings that chemotherapy had survival benefit even in elderly patients if they were properly selected for the indication of chemotherapy. Further studies should be warranted to elucidate the therapeutic efficacy and safety of chemotherapy in elderly patients with advanced NSCLC.

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BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo

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Summary

Members of the bone morphogenetic protein (BMP) family have been implicated in the development and maintenance of vascular systems. Whereas members of the BMP-2/4 and osteogenic protein-1 groups signal via activin receptor-like kinase (ALK)-2, ALK-3 and ALK-6, BMP-9 and BMP-10 have been reported to bind to ALK-1 in endothelial cells. However, the roles of BMP-9–ALK-1 signaling in the regulation of endothelial cells have not yet been fully elucidated. Here, using various systems, we examined the effects of BMP-9 on the proliferation of endothelial cells. Vascular-tube formation from ex vivo allantoic explants of mouse embryos was promoted by BMP-9. BMP-9, as well as BMP-4 and BMP-6, also induced the proliferation of in-vitro-cultured mouse embryonic-stem-cell-derived endothelial cells (MESECs) by inducing the expression of vascular endothelial growth factor receptor 2 and Tie2, a receptor for angiopoietin-1. A decrease in ALK-1 expression or expression of constitutively active ALK-1 in MESECs abrogated and mimicked the effects of BMP-9 on the proliferation of MESECs, respectively, suggesting that BMP-9 promotes the proliferation of these cells via ALK-1. Furthermore, in vivo angiogenesis was promoted by BMP-9 in a Matrigel plug assay and in a BxPC3 xenograft model of human pancreatic cancer. Consistent with these in vivo findings, BMP-9 enhanced the proliferation of in-vitro-cultured normal endothelial cells from dermal tissues of adult mice and of tumor-associated endothelial cells isolated from tumor xenografts in host mice. These findings suggest that BMP-9 signaling activates the endothelium tested in the present study via ALK-1.

Key words: Embryonic stem cell, Allantois, Tumor angiogenesis, Pancreatic cancer, VEGFR2, Tie2

Introduction

Blood vessels consist of a lining of endothelial cells surrounded by mural cells (pericytes and vascular smooth muscle cells), and play essential roles in the maintenance of tissue homeostasis by carrying oxygen and nutrients to distant organs (Carmeliet, 2005). Defects in vascular systems thus result in many types of human disease. Hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Weber-Rendu disease, is characterized by telangiectases in the nose, oral cavity and gastrointestinal tract, as well as arteriovenous malformations in multiple organs, including lung, liver and brain, which can result in severe ischemic injury or stroke (Shovlin et al., 2000). HHT is an autosomal dominant vascular disease, the majority of cases of which are caused by mutations in the gene for either activin receptor-like kinase (ALK)-1 (*ACVRL1*) or endoglin (*ENG*), which are a type-I receptor and co-receptor for members of the transforming growth factor (TGF) β family, respectively.

The TGF β family consists of structurally related and multifunctional proteins, including TGF β s, activins, Nodal and bone morphogenetic proteins (BMPs) (Feng and Derynck, 2005). Members of the TGF β family signal via heteromeric complexes of type II and type I serine/threonine kinase receptors. Upon ligand binding, the constitutively active type II receptor kinase phosphorylates the type I receptor, which in turn activates downstream signal transduction cascades, including Smad pathways. Activins and TGF β s bind to type I receptors known as ALK-4 and ALK-5, respectively. BMPs bind three BMP type I receptors (ALK-2, ALK-3 and ALK-6). The activated type I receptors phosphorylate receptor-regulated Smad proteins (R-

Smads). Smad2 and Smad3 transduce signals for TGF β s, activins and Nodal, whereas Smad1, Smad5 and Smad8 are specific to signaling of BMPs (Miyazono et al., 2010). An exception to this is ALK-1, which is preferentially expressed in endothelial cells. ALK-1 binds TGF β and activates the Smad1 and Smad5 pathways (Oh et al., 2000). Goumans and colleagues showed that TGF β activates ALK-5–Smad2/3 and ALK-1–Smad1/5/8 pathways, leading to inhibition and activation of endothelial cell migration and proliferation, respectively (Goumans et al., 2002).

BMP-family members are subdivided into separate subgroups on the basis of similarity of primary amino acid sequence. They include the BMP-2/4 group (BMP-2 and BMP-4), osteogenic protein-1 (OP-1) group (BMP-5, BMP-6, BMP-7 and BMP-8) and BMP-9/10 group (BMP-9 and BMP-10) (Miyazono et al., 2010). BMP-9 has been shown to be highly expressed in fetal liver (Celeste et al., 1994) and in the liver of adult mice (Miller et al., 2000). BMP-9 exhibits growth-stimulatory effects on primary rat hepatocytes (Song et al., 1995), and has also been implicated in osteogenesis, chondrogenesis (Majumdar et al., 2001), glucose and fatty-acid metabolism (Chen et al., 2003), iron homeostasis (Truksa et al., 2006) and the differentiation of cholinergic neurons (López-Coviella et al., 2000).

Recent studies have shown that BMP-9 binds with high affinity to ALK-1 and endoglin in endothelial cells, and induces phosphorylation of Smad1 and/or Smad5 (Brown et al., 2005). This signaling inhibits basic fibroblast growth factor (FGF)-stimulated proliferation and migration of bovine aortic endothelial cells and vascular endothelial growth factor (VEGF)-induced

angiogenesis (Scharpfenecker et al., 2007). BMP-9 also inhibits the migration and growth of human dermal microvascular endothelial cells (HMVEC-ds) (David et al., 2007) and the DNA synthesis of human aortic endothelial cells (Upton et al., 2009) via ALK-1. Furthermore, BMP-9 is present in the circulating blood to maintain the maturation of blood vessels (David et al., 2008). These findings suggest that BMP-9 has inhibitory effects on endothelial cells, a conclusion that is not consistent with previous reports that TGF β -mediated ALK-1 signaling activates endothelium (Goumans et al., 2002).

The development of the vascular system includes two distinct processes: vasculogenesis and angiogenesis (Coults et al., 2005). During mouse embryogenesis, the initial development of vascular endothelium, termed vasculogenesis, occurs in the mesodermal layer of the yolk sac on embryonic day 7 (E7.0), yielding structures termed blood islands (E7.5-E8.5). The blood islands consist of endothelial progenitors termed angioblasts, which express VEGF receptor-2 (VEGFR2), which is also known as Flk1 (Kennedy et al., 1997; Nishikawa et al., 1998). In angiogenesis, new vessels sprout from the pre-existing vessels and are further remodeled to form mature blood vessels. In embryos, angiogenesis contributes to the establishment of hierarchical vascular trees after endothelial capillary networks have been formed by vasculogenesis. In adults, angiogenesis is essential for the repair and remodeling of tissues during wound healing and ischemia, and for the physiological female reproductive cycle. Neovascularization also plays a pivotal role in pathological processes such as tumor growth, chronic inflammation and diabetic vasculopathy.

Embryonic vascular differentiation has been recapitulated in an in-vitro-differentiation system from embryonic stem cells (ESCs) (Vittet et al., 1996; Hirashima et al., 1999; Yamashita et al., 2000). When VEGFR2-expressing (VEGFR2⁺) endothelial progenitors isolated from differentiation cultures are re-differentiated in the presence of VEGF, various endothelial markers are sequentially upregulated in a pattern similar to that observed in early embryos (Vittet et al., 1996). Using this system, we have previously shown that BMP-4 stimulates the proliferation and migration of endothelial cells via activation of VEGF-VEGFR2 and angiopoietin-1 (Ang-1)-Tie2 signaling in mouse embryonic-stem-cell-derived endothelial cells (MESECs) (Suzuki et al., 2008), whereas Kiyono and Shibuya (Kiyono and Shibuya, 2003) reported that BMP-4 induces apoptosis of capillary endothelial cells during rat pupillary-membrane regression. It is thus possible that BMP-9 has various effects depending on the cellular context.

In the present study, we examined the roles of BMP-9 in embryonic vascular development and adult angiogenesis. We found that the addition of BMP-9 to culture significantly promoted vasculogenesis of the allantois and induced proliferation of MESECs. Furthermore, we showed that BMP-9-induced proliferation was transduced via ALK-1. Finally, BMP-9 enhanced angiogenesis in Matrigel plug assays and pancreatic carcinoma xenografts in adult mice. These findings suggest that BMP-9 is capable of activating endothelium via ALK-1 and inducing vessel formation in vivo.

Results

Expression of components of the BMP-9 signaling pathway in E8.25 mouse embryos

We first examined whether the components of the BMP-9-ALK-1 signaling pathways are present in mouse embryos. The development of vascular systems in mouse embryos begins at E7.5, when

VEGFR2⁺ endothelial progenitors initiate endothelial differentiation. At E8.5, the murine allantois, which lays down the umbilical vasculature that is critical for fetal survival and development, is formed and vascularizes intrinsically (Downs et al., 2001), rather than by angiogenesis involving the yolk sac or fetus. This tissue has therefore been used to study embryonic vessel formation.

E8.25 embryos were excised to obtain the allantois, which was subjected to semi-quantitative reverse transcriptase (RT)-PCR analyses. Endothelial and mural cells that differentiated from mouse ESCs (Yamashita et al., 2000; Watabe et al., 2003) were also examined for the expression of various BMP-9 signaling components. As shown in Fig. 1, expression of BMP-9 ligand was detected in allantoic, endothelial and mural cells. Furthermore, expression of type I receptors (ALK-2, ALK-3 and ALK-6) was also observed in all three samples (Fig. 1), whereas that of ALK-1 was restricted to the allantoic and endothelial cells, confirming endothelium-specific expression of ALK-1. Taken together with the findings that other BMP-related signaling components, including type II receptors (BMPRII), R-Smads (Smad1, Smad5 and Smad8) and Co-Smad (Smad4), are expressed in the allantois (data not shown), these results suggest that signals mediated by BMP-9 are activated in the allantois of E8.25 embryos.

Induction of vessel formation by BMP-9 in ex vivo allantoic explants

Next, to examine the roles of BMP-9 in vasculogenesis and angiogenesis, we used an isolated allantois model (Downs et al., 2001). Excised allantoises from mouse E8.25 embryos were individually cultured in suspension for 2 days. When BMP-9 was added to the ex vivo culture, the PECAM1-positive area of allantoic explants was increased (Fig. 2A,B). However, the vessels formed in the presence of BMP-9 seemed disorganized and disconnected compared with the control (Fig. 2A). These findings suggest that BMP-9 promotes irregular formation of embryonic vascular vessels.

BMP-9 increases the number of MESECs

Because we observed pro-vasculogenic and -angiogenic effects of BMP-9 in an ex vivo culture system, we next examined the effects of BMP-9 signaling on embryonic endothelial cells with an in vitro

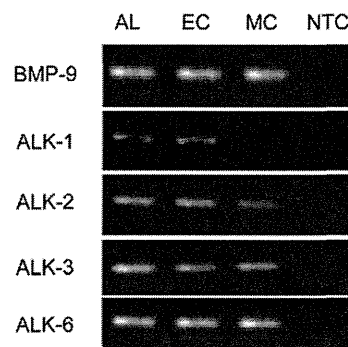


Fig. 1. Expression of BMP-related signaling components in allantoic explants. Allantoises (AL) were excised from E8.25 mouse embryos and subjected to semi-quantitative RT-PCR analyses for BMP-9, ALK-1, ALK-2, ALK-3 and ALK-6. Endothelial cells (EC) and mural cells (MC) were obtained by in vitro differentiation of CCE mouse ESCs and were also subjected to RT-PCR analysis for comparison of expression with that in allantois. NTC, no template control.

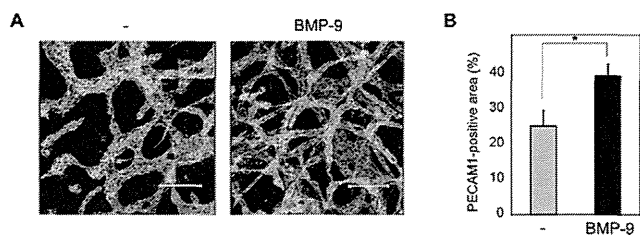


Fig. 2. Effects of BMP-9 on vessel formation from allantoic explants. (A) Allantoises were excised from E8.25 embryos and cultured in the absence (-) or presence of BMP-9 (4 ng/ml), followed by immunostaining for PECAM1 (green). Scale bars: 100 μ m. (B) Percent PECAM1-positive area. Each value represents the mean of seven fields. Error bars, s.d. * P <0.00004.

vascular differentiation system from mouse ESCs (Yamashita et al., 2000; Watabe et al., 2003). VEGFR2⁺ endothelial progenitor cells derived from ESCs differentiate predominantly into endothelial cells when cultured in serum-free SFO medium in the presence of VEGF. When BMP-9 was added to culture of VEGFR2⁺ cells, the number of endothelial cells obtained was significantly increased and their sheet formation was significantly inhibited (Fig. 3A,B), which is similar to our previous finding that BMP-4, a member of the BMP-2/4 group, significantly increases the number of MESECs (Suzuki et al., 2008) (Fig. 3B).

When BMP-6, a member of the OP-1 group, was tested in this proliferation assay, it also increased the number of MESECs (Fig. 3B). These findings suggest that members of all three BMP subfamilies are capable of promoting the proliferation of embryonic endothelial cells during differentiation from VEGFR2⁺ progenitor cells.

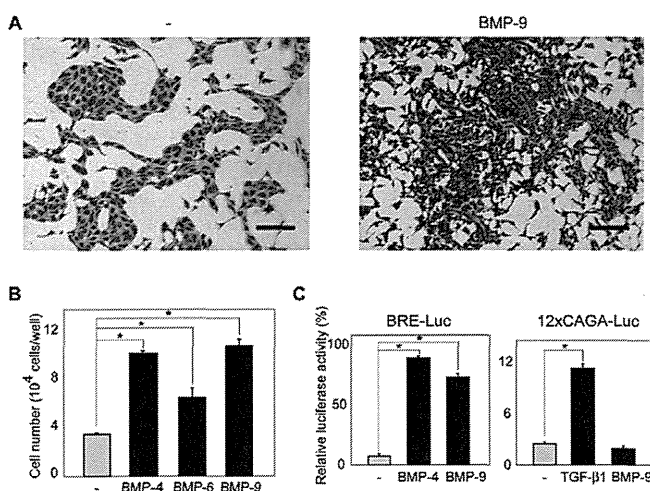


Fig. 3. Effects of BMP-9 on the number of MESECs. (A,B) VEGFR2⁺ cells (5×10^4 /well) derived from CCE cells were cultured in serum-free SFO medium containing 30 ng/ml of VEGF in the absence (-) or presence of BMP-4 (60 ng/ml), BMP-6 (360 ng/ml) or BMP-9 (1 ng/ml) for 2 days, followed by photography (A; '-' and BMP-9) and determination of cell numbers (B). Scale bars: 200 μ m. (C) Luciferase promoter assays were carried out using MESECs to determine whether BMP-9 activates BMP-related (BRE-Luc) or TGF β -related (12x CAGA-Luc) intracellular signaling pathways. BMP-4 (60 ng/ml) and TGF β 1 (1 ng/ml) were used as controls for BRE-Luc and 12x CAGA-Luc, respectively. Error bars, s.d. * P <0.006.

BMPs activate the Smad1/5/8 signaling axis, whereas TGF β , activins and Nodal activate the Smad2/3 signaling axis. To determine which intracellular Smad signaling pathways are activated by BMP-9 in MESECs, we carried out luciferase reporter assays using promoter reporter constructs that specifically respond to each signal. As shown in Fig. 3C, BMP-9 activated BMP-Smad1/5/8-specific (BRE-Luc) but not TGF β -Smad2/3-specific (12x CAGA) reporter constructs, which were activated by BMP-4 and TGF β , respectively. We also examined whether the concentrations of BMPs used in the proliferation assay activate the Smad1/5/8 signaling pathways at comparable levels. Activation of Smad1/5/8 signaling pathways can also be monitored by the expression level of Id1, a faithful target of BMP signaling (Valdimarsdottir et al., 2002). As shown in supplementary material Fig. S1, BMP-4, BMP-6 and BMP-9 induced Id1 expression to a similar relative extent as they enhanced the proliferation of MESECs (Fig. 3B). These findings suggest that activation of BMP-9 increases the number of MESECs by activating Smad1/5/8 signaling pathways.

Specific inhibition of the effects of BMP-9 on MESEC proliferation by soluble ALK-1

Although all members of the BMP family exhibit similar effects on endothelial cell proliferation, BMP-9 and BMP-10 have been reported to utilize ALK-1, whereas BMP-4 and BMP-6 bind ALK-2, ALK-3 and ALK-6 (David et al., 2007; Scharpfenecker et al., 2007). To examine whether the BMP-9 used in the assay preferentially binds to ALK-1 on MESECs, we used soluble chimeric receptors containing extracellular domains of ALK-1 and ALK-3 (ALK-1-Fc and ALK-3-Fc, respectively). As shown in Fig. 4A, ALK-1-Fc inhibited the BMP-9-mediated increase in number of MESECs, whereas control-Fc or ALK-3-Fc did not. These findings suggest that BMP-9 binds to ALK-1 with higher affinity than to ALK-3 in MESECs.

Increase of ALK-1 expression during endothelial differentiation

Although ALK-1 has been shown to be preferentially expressed in endothelial cells, its expression during endothelial differentiation from ESC-derived vascular progenitors has not been elucidated. We therefore examined the expression of ALK-1, ALK-3 and vascular markers over time during in vitro differentiation of ESC-derived VEGFR2⁺ cells. The expression of PECAM1, an endothelial marker, began to increase from 12 hours after stimulation with VEGF in the absence of serum (Fig. 4B) (Kawasaki et al., 2008). The level of expression of ALK-1 in VEGF-stimulated ESC-derived progenitor cells was similar to that of PECAM1, whereas that of endoglin increased earlier than those of PECAM1 and ALK-1. By contrast, ALK-3 expression was detected in the VEGFR2⁺ endothelial progenitors and decreased during endothelial differentiation. Although these findings need to be confirmed at protein levels, they suggest that ALK-1 signals mediated by BMP-9 are activated after endothelial cells are differentiated from ESC-derived VEGFR2⁺ cells, whereas ALK-3 signals mediated by BMP-4 can be activated in VEGFR2⁺ progenitor cells.

Essential role of ALK-1 in the BMP-9-mediated increase in number of MESECs

Although BMP-9 was shown to bind preferentially to ALK-1, it remains unclear whether the BMP-9-induced increase in the number

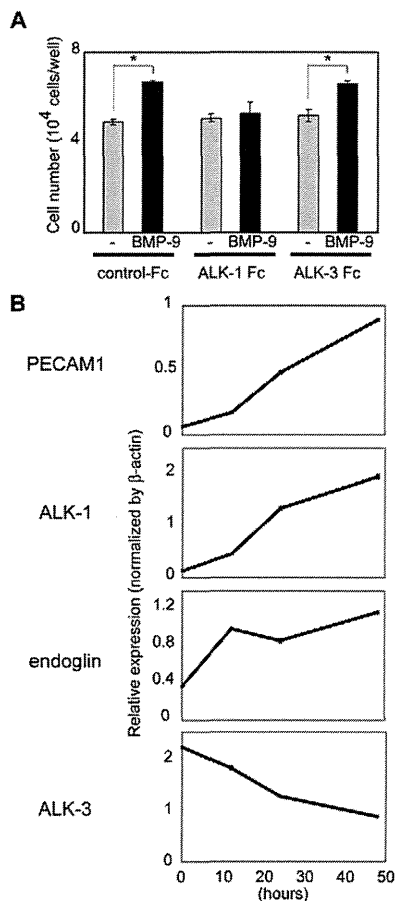


Fig. 4. Involvement of ALK-1 in the BMP-9-mediated increase in number of MESECs. (A) VEGFR2+ cells (5×10^4 /well) derived from CCE cells were cultured in serum-free SFO medium containing 30 ng/ml of VEGF in the absence (–) or presence of BMP-9 (1 ng/ml) in combination with control-Fc (30 ng/ml), ALK-1-Fc (30 ng/ml) or ALK-3-Fc (30 ng/ml) for 2 days, followed by cell-proliferation analysis. Each value represents the mean of three determinations; error bars, s.d. * $P < 0.004$. (B) Expression of vascular markers and ALKs during in vitro differentiation of ESC-derived VEGFR2+ cells. Quantitative RT-PCR analysis of PECAM1, ALK-1, endoglin and ALK-3 of VEGFR2+ cells stimulated with 30 ng/ml of VEGF in the absence of serum. Error bars, s.d.

of MESECs is mediated by ALK-1. We therefore knocked down ALK-1 expression in ESC-derived VEGFR2+ cells using siRNA during endothelial cell differentiation in the presence of serum. As shown in Fig. 5A, the level of transcripts encoding ALK-1 was significantly decreased by its specific siRNA.

Although the number of MESECs was not significantly altered by knocking down ALK-1 expression, BMP-9 failed to increase the number of MESECs only when ALK-1 expression was knocked down (Fig. 5B). These findings suggest that ALK-1 is required for BMP-9 to increase the number of MESECs.

Expression of constitutively active ALK-1 increases the number of MESECs

We next examined whether the expression of constitutively active ALK-1 (caALK-1) regulates the proliferation of MESECs in a fashion similar to the addition of BMP-9. Because we wished to induce the expression of caALK-1 in differentiating endothelial

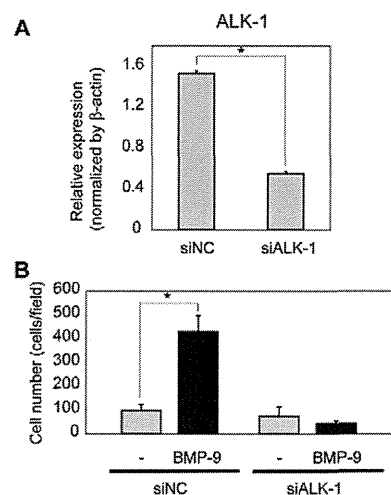


Fig. 5. Effects of decreased ALK-1 expression on MESEC proliferation. MGZ5 ESC-derived VEGFR2+ cells were transfected with siRNA for *ALK-1* (siALK-1) or negative control siRNA (siNC), and cultured in the absence (–) or presence of BMP-9 (1 ng/ml) in combination with VEGF (30 ng/ml) and serum for 4 days. (A) Expression of endogenous level of *ALK-1* was determined by quantitative RT-PCR analyses. Each value represents the mean of three determinations; error bars, s.d. * $P < 0.002$. (B) Roles of ALK-1 on the proliferation of MESECs. In order to determine the number of endothelial cells, ESC-derived vascular cells containing both endothelial and mural cells were stained for PECAM1 and SMA, followed by counting the numbers of endothelial cells in each field. Each value represents the mean of five determinations; error bars, s.d. * $P < 0.00002$.

progenitor cells instead of undifferentiated ESCs, we established ESC lines carrying a tetracycline (Tc)-regulatable caALK-1 transgene (Tc-caALK-1) or no transgene (Tc-empty) (Masui et al., 2005; Mishima et al., 2007).

We differentiated the Tc-empty and Tc-caALK-1 ESCs into VEGFR2+ vascular progenitor cells in the presence of Tc, so that no transgene expression would be induced. Sorted VEGFR2+ cells were re-differentiated in the presence or absence of Tc. As shown in Fig. 6A, in endothelial cells derived from Tc-caALK-1 ESCs, expression of the caALK-1 transgene was induced only in the absence of Tc. When caALK-1 was expressed, the number of MESECs was significantly increased (Fig. 6B), consistent with the effects of addition of BMP-9. When caALK-1 transgene was expressed in MESECs, Id1 expression was significantly increased (Fig. 6C), a finding also observed when BMP-9 was added to culture of MESECs (Fig. 7). These findings suggest that activation of Smad1/5/8 signaling by expression of caALK-1 mimics the signaling induced by BMP-9.

Induction of endothelial cell proliferation by BMP-9-ALK-1 signaling via stimulation of VEGF-VEGFR2 and Ang-1-Tie2 signaling

We next attempted to identify the targets of BMP-9-ALK-1 signaling that induce endothelial cell proliferation. VEGF-VEGFR2 signaling pathways are known to stimulate endothelial proliferation, survival and migration. Ang-1 is an agonist for the Tie2 tyrosine kinase receptor, which inhibits apoptosis of endothelial cells (Maisonpierre et al., 1997; Holash et al., 1999). We previously showed that BMP-4 induces the expression of transcripts for

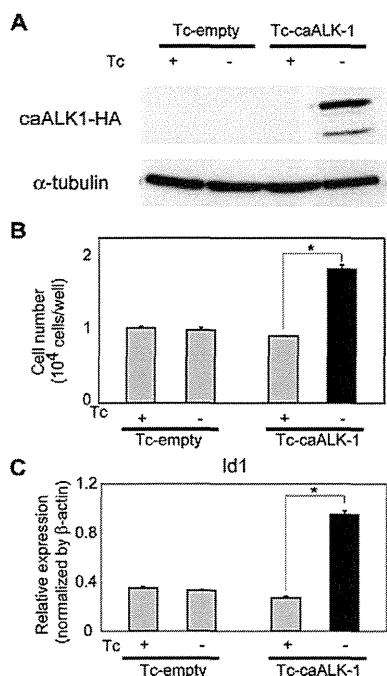


Fig. 6. Effects of constitutively active ALK-1 expression on MESEC proliferation. VEGFR2+ vascular progenitor cells derived from ESCs carrying a tetracycline (Tc)-regulated transgene encoding hemagglutinin (HA)-tagged constitutively active ALK-1 (Tc-caALK-1) or control transgene (Tc-empty) were cultured in the presence (+) or absence (-) of Tc. After 2 days of culture in the absence of serum, the MESECs obtained were subjected to immunoblotting for HA-epitope to determine the level of expression of *ALK-1* transgene (A), to cell-proliferation assay (B) or to quantitative RT-PCR analysis for *Id1* expression (C). Error bars, s.d. * $P < 0.003$.

VEGFR2 and Tie2, suggesting that activation of VEGF-VEGFR2 and Ang-1-Tie2 signaling by BMP-4 leads to the activation of MESECs (Suzuki et al., 2008).

In addition to increasing *Id1* levels, BMP-9 increased the levels of transcripts for VEGFR2 and Tie2 (Fig. 7). Furthermore, we identified VEGF as a putative target of BMP-9-ALK-1 signaling in MESECs, suggesting that the BMP-9-mediated increase in the number of MESECs is promoted by the activation of VEGF-VEGFR2 and Ang-1-Tie2 signaling. In order to examine the roles of VEGF-VEGFR2 signals in the BMP-9-mediated promotion of angiogenesis, we treated allantoic explants with SU1498, a specific inhibitor of VEGFR2 kinase. As shown in supplementary material Fig. S2, the addition of SU1498 reduced the formation of blood vessels from allantoic explants, showing that blockade of VEGF signaling inhibits angiogenesis. Although BMP-9 enhanced blood-vessel formation in control explants, this augmentation was completely abrogated by SU1498. These results further suggested that VEGF-VEGFR2 signals play important roles in the promotion of angiogenesis by BMP-9.

Interestingly, expression of *ALK-1* transcripts increased upon the addition of BMP-9 to culture of MESECs, suggesting that BMP-9-ALK-1 signaling is self-activating as a result of a positive-feedback mechanisms. In addition, we found that the expression of endoglin is upregulated by BMP-9; this might modulate BMP-9-ALK-1 signaling.

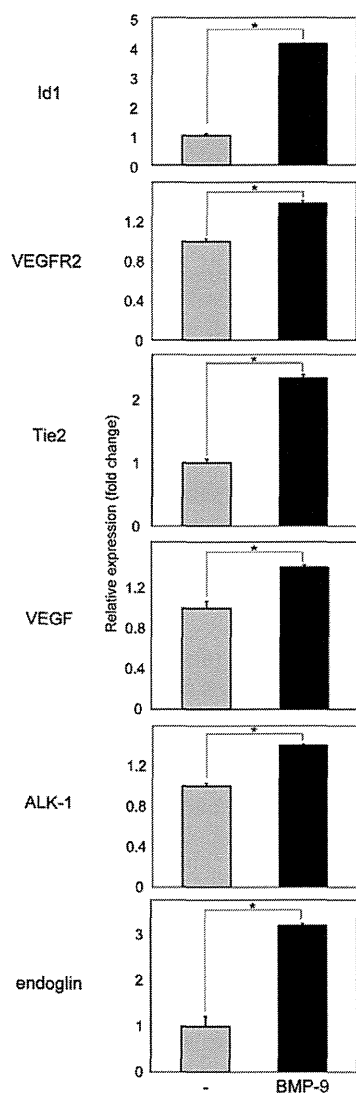


Fig. 7. Effects of BMP-9 on the expression of various angiogenesis-related factors. VEGFR2+ cells derived from CCE cells were cultured in serum-free SFO medium containing 30 ng/ml of VEGF in the absence (-) or presence of 1 ng/ml of BMP-9 for 2 days, and subjected to quantitative RT-PCR analyses for the expression of *Id1*, VEGFR2, Tie2, VEGF, ALK-1 and endoglin. Quantified mRNA values were normalized to the amount of β -actin mRNA, and results are given as fold change. Error bars, s.d. * $P < 0.006$.

BMP-9 enhances in vivo angiogenesis in Matrigel plug assays

We next attempted to examine whether present ex vivo and in vitro findings that BMP-9 activates the proliferation of endothelial cells can be applied to angiogenesis in adults. Adult angiogenesis includes not only neo-angiogenesis, which involves the proliferation of existing endothelial cells, but also vasculogenesis, which involves the differentiation of endothelial progenitor cells circulating in peripheral blood (Asahara and Kawamoto, 2004). As a model of angiogenesis in adults, we chose a Matrigel plug assay.

Matrigels mixed with FGF-2 in combination with BMP-9 were subcutaneously injected to Balb/c mice. One week later, Matrigel plugs were harvested, and resulting vasculature was examined by staining for PECAM1. As shown in Fig. 8A and B, a significantly

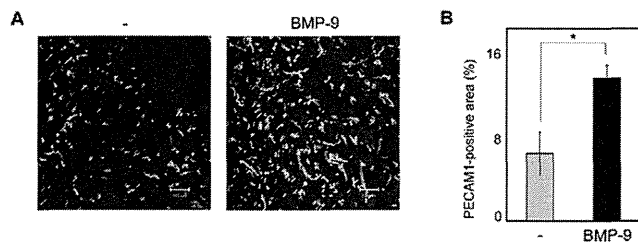


Fig. 8. Effects of BMP-9 on angiogenesis in a Matrigel plug assay in vivo. Matrigel plugs mixed with 1 $\mu\text{g/ml}$ FGF-2 in the absence (-) or presence of 10 ng/ml of BMP-9 were subcutaneously injected in BALB/c mice. After 1 week, Matrigel plugs were harvested and examined for vascular density. (A) Immunostaining for PECAM1 (green) of sections obtained from both types of Matrigel plugs. Scale bars: 100 μm . (B) Percent PECAM1-positive area. Each value represents the mean of ten sections; error bars, s.d. * $P < 0.005$.

higher density of microvessels was observed in the Matrigels that contained BMP-9 than in the controls. These findings suggest that BMP-9 promotes in vivo adult angiogenesis.

Promotion of tumor angiogenesis by BMP-9 in a human pancreatic-cancer xenograft model

We next tried to extend our finding that BMP-9 enhances angiogenesis in Matrigel plugs to another model of angiogenesis. Because previous studies showed that BMP-2 induced tumor angiogenesis in human lung- and breast-cancer xenograft models (Langenfeld and Langenfeld, 2004; Raida et al., 2005), we examined whether BMP-9 also plays any roles in tumor angiogenesis.

In order to avoid effects on tumor cells themselves of BMP-9 signaling, we established BxPC3 pancreatic adenocarcinoma cells that express green fluorescence protein (GFP; control) or *BMP-9* transgenes. Because BxPC3 cells exhibit a homozygous deletion of the *SMAD4* gene (Kleeff et al., 1999), BMP-9 secreted from BxPC3-BMP-9 tumor cells cannot activate the Smad pathways by itself. We confirmed that BMP-9 expression does not affect the rate of growth of tumor cells in vitro (data not shown). When conditioned medium prepared from BxPC3-BMP-9 cells was added to MDA-MB-231 cells, the expression of Id1 was significantly induced compared with when medium from BxPC3-GFP cells was added (data not shown).

Both types of BxPC3 cells were subcutaneously grafted to immunocompromised Balb/c nude mice to obtain tumors. Tumor vasculature was examined by staining for PECAM1. As shown in Fig. 9A and B, a significantly higher density of microvessels was observed in the tumors derived from BxPC3-BMP-9 cells than in the controls. These findings suggest that BMP-9 also promotes in vivo tumor angiogenesis.

BMP-9 promotes the proliferation of primary cultured normal and tumor-associated endothelial cells derived from adult mice

Present findings that BMP-9 promotes angiogenesis in Matrigels and tumors transplanted to mice prompted us to investigate whether BMP-9 induces the proliferation of endothelial cells directly or indirectly via activation of surrounding cells, including inflammatory cells. Recent findings have revealed that tumor-associated endothelial cells (TECs) are different from normal endothelial cells (NECs) in many respects (Hida et al., 2004; Hida

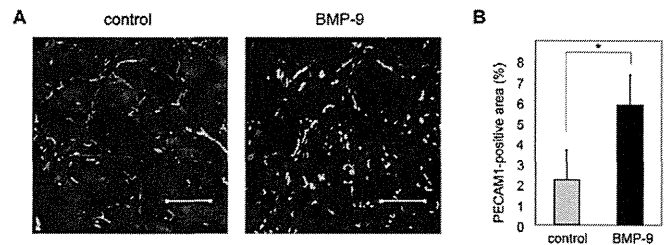


Fig. 9. Effects of BMP-9 on tumor angiogenesis in a mouse xenograft model of human pancreatic cancer. BxPC3 human pancreatic adenocarcinoma cells that were infected with lentiviruses encoding GFP (control) or BMP-9 were subcutaneously inoculated in BALB/c nude mice. After 5 weeks, tumors were excised and examined for vascular density. (A) Immunostaining for PECAM1 (green) of sections obtained from both types of tumors. Scale bars: 100 μm . (B) Percent PECAM1-positive area. Each value represents the mean of 15 sections; error bars, s.d. * $P < 0.0000003$.

et al., 2008; Akino et al., 2009). In order to examine the effects of BMP-9 on the proliferation of both types of primary cultured endothelial cells, we prepared TECs from renal carcinoma xenografts (OSRC-2) in nude mice and NECs from dermal tissues as a normal counterpart of TECs (see Materials and Methods). When NECs and TECs were cultured in the absence or presence of BMP-9, proliferation of both types of cell was significantly promoted by BMP-9 (Fig. 10). These results suggest that BMP-9 induces angiogenesis via direct activation of endothelial cells in normal and cancerous conditions.

Discussion

In the present study, we demonstrated for the first time that BMP-9 induces ex vivo allantois vascular formation, proliferation of in vitro-cultured MESECs, and in vivo angiogenesis. Using in vitro systems, we showed that ALK-1 is necessary and sufficient for the BMP-9-induced proliferation of MESECs, and that BMP-9 induces the expression of various targets, including VEGFR2 and Tie2, involved in the proliferation of endothelial cells.

We previously reported that BMP-4 induces the proliferation of MESECs via activation of VEGFR2 and Tie2 signaling (Suzuki et al., 2008). Although in the present study we showed that members of all three BMP subfamilies (BMP-2/4, OP-1 and BMP-9/10) induce the proliferation of MESECs (Fig. 3B), their effects on MESECs seem to differ, given the differential onset of receptor expression and induction of other target genes. Because the

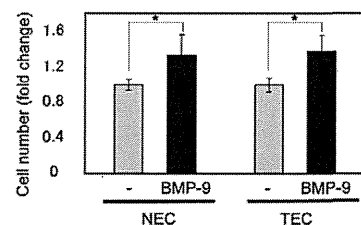


Fig. 10. Effects of BMP-9 on the number of normal and tumor-associated endothelial cells isolated from adult mice. Normal endothelial cells (NECs) prepared from dermal tissues and tumor-associated endothelial cells (TECs) prepared from renal carcinoma xenografts in nude mice were cultured in the absence (-) or presence of BMP-9 (5 ng/ml) for 3 days, followed by determination of cell numbers by MST assay. Each value represents the mean of five determinations; error bars, s.d. * $P < 0.003$.

expression of ALK-3, a receptor for BMP-4, begins before mesoderm induction, BMP-4–ALK-3 signaling is involved in mesoderm induction (Lawson et al., 1999; Winnier et al., 1995; Dunn et al., 1997; Mishina et al., 1995; Ahn et al., 2001) and formation of VEGFR2+ angioblasts (Park et al., 2004). By contrast, ALK-1 expression parallels that of PECAM1, further confirming that ALK-1 is an endothelial marker. These findings highlight the difference between the effects of BMP-4 and BMP-9: BMP-4 signaling occurs in the early stages during differentiation of progenitor cells, whereas BMP-9 signaling occurs only in fully differentiated endothelial cells.

In addition, we identified ALK-1 as a target of BMP-9 signaling. The BMP-9-mediated positive-feedback mechanisms involved in the regulation of ALK-1 expression seem to play important roles during embryonic vascular formation, because BMP-9–ALK-1 signaling enhances the proliferation of endothelial cells. We also found that endoglin expression is induced by BMP-9 in MESECs, consistent with our previous finding that adenovirally transduced caALK-1 induced the expression of endoglin in human umbilical-vein endothelial cells (Ota et al., 2002). Endoglin serves as a co-receptor for TGF β -family signals (Yamashita et al., 1994). In vitro studies have shown that endoglin protein is able to form complexes with ALK-1 and to enhance the effects of ALK-1 in endothelial cells (Blanco et al., 2005; Goumans et al., 2002; Lebrin et al., 2004). The phenotype of endoglin-deficient mice strongly resembles that of ALK-1-deficient mice, suggesting that endoglin also plays a role in ALK-1 signaling in angiogenesis (Li et al., 1999). Although both ALK-1 and endoglin are involved in the pathogenesis of HHT, the roles of endoglin in MESECs remain to be elucidated.

BMP-9 has been reported to inhibit the proliferation and migration of endothelial cells (David et al., 2007; Scharpfenecker et al., 2007), a finding that is inconsistent with our results that BMP-9 inhibits the proliferation of three kinds of endothelial cells: MESECs, NECs and TECs. However, as previously reported (David et al., 2007), we also found that BMP-9 inhibits proliferation of HMVEC-ds (data not shown), suggesting that the effects of BMP-9 on endothelial cell proliferation are cell-type dependent. Identification of the components that determine whether BMP-9 induces or inhibits endothelial proliferation is of great interest.

Consistent with our in vitro data, we found that BMP-9 enhanced the angiogenesis in ex vivo culture of allantois and in vivo Matrigel plug assay. We also found that BMP-9 secreted by BxPC3 carcinoma cells affected the tumor microenvironment and promoted angiogenesis by directly activating endothelial cells, which is crucial for tumor growth. However, the tumors derived from BMP-9-expressing BxPC3 cells were smaller than control tumors (data not shown). Because Smad4 is defective in BxPC3 cells, the BMP-9 secreted from BxPC3-BMP-9 cells is unable to activate Smad signaling in the tumor cells, although we cannot exclude the possibility that BMP-9 activated Smad-independent signaling in tumor cells and thereby attenuated tumor growth, which we were not able to observe in vitro (data not shown). It is also noteworthy that the newly formed vasculature in BxPC3-BMP-9 tumor (Fig. 9) seemed to be immature and non-functional compared with that in the controls. We observed that pericyte coverage of the newly formed vessels was decreased in the tumors derived from BxPC3-BMP-9 cells (data not shown). These observations suggest that the in vivo growth of BxPC3 cells does not principally depend on newly formed vasculature, which typically is the case for pancreatic tumors. It will be of great interest to examine the effects of BMP-9 on the growth of other types of tumor cells.

Angiogenesis plays crucial roles in numerous pathological conditions such as inflammation and tumorigenesis. In addition, regenerative medicine for vascular systems has attracted much attention as a potential means of eliminating the vascular-system defects accompanying diabetes and other diseases. The present findings are thus useful in suggesting BMP-9 as a putative target or other type of resource in the treatment of inflammation, cancer and vascular dysfunction.

Materials and Methods

Cells and cell culture

The CCE ESC line was obtained from Michael J. Evans (University of Cambridge, UK). MGZ5 and MGZRTcH ESC lines were obtained from Hitoshi Niwa (RIKEN CDB, Kobe, Japan). Maintenance, differentiation, culture and cell sorting of ESCs were performed as previously described (Yamashita et al., 2000; Suzuki et al., 2008; Kokudo et al., 2008). Establishment of Tc-inducible ESC lines from parental MGZ5TcH2 cells was as described (Masui et al., 2005; Mishima et al., 2007). VEGF (30 ng/ml), BMP-4 (60 ng/ml), BMP-6 (360 ng/ml), BMP-9 (1 ng/ml), TGF β 1 (1 ng/ml), control-Fc (30 ng/ml), ALK-1-Fc (30 ng/ml) and ALK-3-Fc (30 ng/ml) were purchased from R&D Systems. The BxPC3 human pancreatic adenocarcinoma cell line was obtained from American Type Culture Collection (Manassas, VA). BxPC3 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS).

Isolation and culture of TECs and NECs

Endothelial cells were isolated as previously described (Ohga et al., 2009). In brief, TECs were isolated from renal carcinoma xenografts (OSRC-2) in nude mice aged 8–12 weeks (Sankyo Laboratory, Tokyo, Japan). NECs were isolated from the dermal tissue as a control. Both types of endothelial cells were isolated using a magnetic cell-sorting system (Miltenyi Biotec) according to the manufacturer's instructions using fluorescein isothiocyanate (FITC)-anti-PECAM1 antibody. PECAM1-positive cells were sorted and plated onto 1.5% gelatin-coated culture plates and grown in EGM-2 MV (Clonetics) and 15% FBS. Diphtheria toxin (500 ng/ml; Calbiochem) was added to TEC subcultures to kill any remaining human tumor cells and to NECs to ensure technical consistency. The isolated endothelial cells were purified by a second round of purification, using FITC–Bandeirea Simplicifolia lectin 1-B4, and purity was determined by flow cytometry. Growth of TECs and NECs was quantified after 3 days of the culture in the absence or presence of BMP-9 by MTS assay using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocol.

Cell-proliferation assays

The number of MESECs was counted in different ways depending on the conditions of differentiation from ESC-derived VEGFR2+ cells. When ESC-derived VEGFR2+ cells were re-differentiated in the absence of serum, we obtained only endothelial cells (Yamashita et al., 2000). Therefore, cell numbers were counted using a Coulter Counter (Yamato Kagaku). When ESC-derived VEGFR2+ cells were re-differentiated in the presence of serum, we obtained both endothelial cells and mural cells. Such mixed cell populations were immunostained for PECAM1, an endothelial cell marker, and smooth muscle α -actin (SMA), a mural cell marker, and subjected to manual counting of endothelial cells in the fields of photographs.

Luciferase assay

The transcriptional activation induced by BMPs and TGF β 1 was measured using BRE-Luc and 12 \times CAGA-Luc, which are luciferase reporter constructs containing BMP-responsive and TGF β -responsive elements, respectively (Korchynskiy and ten Dijke, 2002; Dennler et al., 1998). ESC-derived VEGFR2+ vascular progenitor cells were seeded in 24-well plates and then transiently transfected with promoter-reporter constructs, followed by stimulation with ligands. Cell lysates were then prepared, and luciferase activities in the lysates were measured with the Dual-Luciferase reporter system (Promega) using a luminometer (MicroLumat Plus, Berthold). Values were normalized to *Renilla*-luciferase activity under control of cytomegalovirus promoter.

RNA interference

siRNAs for mouse *ALK-1* (Stealth RNAi Oligo ID MSS235821) and negative control (Stealth RNAi Negative Control Med GC) were purchased from Invitrogen, and were introduced into cells using HiPerFect reagent (QIAGEN) according to the manufacturer's instructions.

Ex vivo allantois assay

Explant culture of mouse allantois was carried out as previously described (Downs et al., 2001). Briefly, allantoises were excised from concepti of ICR mice at E8.25 in phosphate-buffered saline. Individual allantoises were cultured on eight-well culture slides in 250 μ l of minimum essential medium (Invitrogen) supplemented with 10% FBS and β -mercaptoethanol. After 2 days of culture at 37°C in a 5% CO₂

incubator, allantois explants were subject to immunohistochemical examination. When SU1498 (Cosmobio) was added to the culture, dimethyl sulfoxide (DMSO) was used as vehicle control.

Matrigel plug assay

Balb/c male mice aged 5-6 weeks were obtained from CLEA Japan (Tokyo, Japan) and Sankyo Laboratory (Tokyo, Japan). All animal experimental protocols were performed in accordance with the policies of the Animal Ethics Committee of the University of Tokyo. Matrigel plug assays were carried out as previously described (Kano et al., 2005). Briefly, regular Matrigel (BD Biosciences) was mixed with FGF-2 (R&D Systems) and heparin (Aventis Pharma) in combination with 10 ng/ml BMP-9, and injected subcutaneously into the abdominal region of mice ($n > 5$ mice per group). Matrigels were harvested 1 week after injection and were subject to immunohistochemistry for PECAM1.

Lentivirus production and infection

A lentiviral expression system was used to establish BMP-9-expressing BxPC3 cells (Shibuya et al., 2003). cDNA encoding human BMP-9 was cloned from liver cDNA of Multiple Tissue cDNA Panels (Clontech) using the following primers: forward, 5'-TTCCTCAGAGCAAACAGCA-3' and reverse, 5'-GTTGTGCTCAAATCCCATT-3'. BMP-9 cDNA was subcloned into the pENTR vector and subsequently transferred into the pCS-EF-Rfa lentiviral expression vector by the LR recombination reaction (Invitrogen). For production of lentiviral vectors, 293FT cells (Invitrogen) were transfected using Lipofectamine 2000 (Invitrogen) with three plasmids: vector construct, VSV-G- and Rev-expressing construct (pCMV-VSV-G-RSV-Rev) and packaging construct (pCAG-HIVgp). The culture supernatants were collected and viral particles were concentrated by centrifugation. For lentiviral infection, 1×10^5 BxPC3 cells were infected with lentivirus vectors in suspension and plated in six-well culture plates.

Balb/c nude mouse model of human pancreatic cancer

Balb/c nude male mice aged 5-6 weeks were obtained from CLEA Japan (Tokyo, Japan) and Sankyo Laboratory (Tokyo, Japan). A total of 5×10^6 BxPC3 tumor cells in 100 μ l phosphate-buffered saline ($n > 5$ mice per group) were injected subcutaneously into the left flank of each mouse and allowed to grow for 5 weeks, at which point the major axis of tumors was approximately 10-mm long.

Immunohistochemistry and immunoblot analysis

Staining of cultured cells was performed as previously described (Watabe et al., 2003; Kokudo et al., 2008). Excised mouse tissue samples from Balb/c mice grafted with BxPC3 cells were snap-frozen in a dry-ice acetone bath for immunohistochemistry. Frozen samples were further sectioned at 10- μ m thickness in a cryostat and subsequently incubated with primary and secondary antibodies. Monoclonal antibodies to PECAM1 (Mec13.3) and SMA (1A4) for immunohistochemistry were purchased from BD Pharmingen and Sigma, respectively. Stained specimens were examined using a phase-contrast microscope (Model IX70; Olympus) or an LSM 510 META confocal microscope (Carl Zeiss). All images were imported into Adobe Photoshop as JPEGs or TIFFs for figure assembly. Images were processed using ImageJ (NIH) to quantify PECAM1-positive areas. Immunoblot analysis was performed as described (Watabe et al., 2003). Antibodies to HA and α -tubulin for immunoblot analysis and immunohistochemistry were obtained from Sigma. The bound antibody was detected using a chemiluminescent substrate (ECL; Amersham) and a LAS-4000 Luminescent image analyzer (Fuji Photo Film).

RNA isolation and RT-PCR analysis

Total RNAs were extracted using the RNeasy Mini Kit (Qiagen). RNAs were reverse-transcribed by random hexamer priming using Superscript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR analysis was performed using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) and Power SYBR Green (Applied Biosystems). All expression data were normalized to those for β -actin. The primer sequences are shown in supplementary material Table S1.

Statistical analysis

Results were statistically examined using the two-sided Student's *t*-test. Differences were considered significant at $P < 0.05$.

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Review

Angiopoietin-1/Tie2 receptor signaling in vascular quiescence and angiogenesis

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Summary. Angiopoietin (Ang) 1 is a ligand for endothelium-specific receptor tyrosine kinase Tie-2. In adult vasculature, Ang1/Tie2 signaling is thought to regulate both maintenance of vascular quiescence and promotion of angiogenesis. However, it has been unknown how Tie2 signal regulates these distinct biological functions. Recently, we and Alitalo's group have clarified that Ang1 assembles distinct Tie2 signaling complexes in either presence or absence of endothelial cell-cell adhesions. Ang1 induces *trans*-association of Tie2 at cell-cell contacts, whereas Tie2 is anchored to the extracellular matrix (ECM) by Ang1 at the cell-substratum interface. *Trans*-associated Tie2 and ECM-anchored Tie2 activate distinct signaling pathways. In this review, we discuss how Ang1/Tie2 signal regulates both maintenance of vascular quiescence and promotion of angiogenesis, especially focusing on the roles of *trans*-associated Tie2 and ECM-anchored Tie2.

Key words: Angiopoietin-1, Tie2, Quiescence, Angiogenesis, KLF2

Introduction

During embryogenesis, vascular blood vessels form through both vasculogenesis and angiogenesis (Yancopoulos et al., 2000). After birth, most blood vessels in the body become quiescent. However, angiogenesis occurs in some specific tissues, such as the cycling ovary and the placenta during pregnancy, and is promoted during wound healing and repair (Peters et al.,

2004; Adams and Alitalo, 2007). Therefore, angiogenesis is implicated in the pathogenesis of various diseases, such as cancer, arthritis, diabetic retinopathy and macular degeneration (Folkman, 2001; Bergers and Benjamin, 2003; Peters et al., 2004). In the quiescent vessels, the endothelial cells lining vessels tightly contact with each other and do not proliferate and migrate (Dejana, 2004; Dejana et al., 2008, 2009). On the other hand, during angiogenesis, the cells that lose cell-cell contacts are allowed to proliferate and migrate, thereby resulting in sprouting and branching of new capillaries from the pre-existing vessels (Shibuya and Claesson-Welsh, 2006; Wallez et al., 2006; Adams and Alitalo, 2007; Lamalice et al., 2007).

Whether endothelial cells remain quiescent or undergo angiogenesis is determined by the balance between angiostatic and angiogenic signals. Angiogenic signals are mainly induced by endothelial-specific receptor tyrosine kinases (RTKs) and their ligands; vascular endothelial growth factor (VEGF)-VEGF-receptor (VEGFR), ephrin-Eph receptor, and fibroblast growth factor (FGF)-FGF receptor (Yancopoulos et al., 2000; Jones et al., 2001; Shibuya and Claesson-Welsh, 2006; Adams and Alitalo, 2007; Kuijper et al., 2007). On the other hand, angiogenesis is negatively regulated by the signals evoked by angiostatic factors such as thrombospondin, angiostatin, endostatin and vasohibin (Dawson et al., 1999; Rege et al., 2005; Sato and Sonoda, 2007; Tabruyn and Griffioen, 2007). Interestingly, angiopoietin (Ang) 1/Tie2 receptor signal is known to regulate both vascular quiescence and angiogenesis (Jones et al., 2001; Peters et al., 2004; Brindle et al., 2006; Eklund et al., 2006; Fiedler and Augustin, 2006; Augustin et al., 2009; Fukuhara et al., 2009). However, it was unknown how Ang1/Tie2 signal regulates these paradoxical actions. Recently, we and Alitalo's group have clarified that Ang1 and Tie2 form distinct signaling complexes at cell-cell and cell-

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extracellular matrix (ECM) contacts in the presence or absence of inter-endothelial cell adhesions, respectively (Fukuhara et al., 2008; Saharinen et al., 2008). In this review, we further discuss the significance of these distinct signaling complexes in vascular quiescence and angiogenesis by referring to the new findings thereafter.

Ang-Tie receptor system

Tie1 and Tie2 belong to a family of Tie RTKs found to be expressed in endothelial cells. The Ang family consists of Ang1, Ang2, Ang3 and Ang4. Of them, Ang1 and Ang2 are well characterized. Ang1 binds to Tie2 and stimulates its kinase activity, whereas Ang2 is known to act as a context-dependent agonist or antagonist for Tie2 (Augustin et al., 2009). Mice lacking either *Ang1* or *Tie2* exhibit embryonic lethality due to defective vascular development (Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996). The vessels of both mutant animals show fewer number of endothelial cells and a defect in the adhesions between endothelial cells and the underlying mural cells. Therefore, Ang1/Tie2 signal is thought to play a crucial role in the maturation and stabilization of the embryonic vasculature. Tie1-deficient embryos also exhibit compromised vascular maintenance owing to impaired endothelial integrity (Puri et al., 1995, 1999; Sato et al., 1995). Although none of the Ang family members directly bind to Tie1, it has been reported that Tie1 can interact with Tie2 and signal in a heterodimeric complex in vitro (Saharinen et al., 2005). However, it has been also reported that Tie1 negatively regulates Tie2-driven signaling and endothelial cell survival (Marron et al., 2007; Yuan et al., 2007). Thus, how Tie1 functionally interacts with Tie2 and is involved in developmental vascular formation remains unclear.

Role of Ang1/Tie2 signal in vasculature quiescence

Tie2 is expressed and tyrosine-phosphorylated in endothelium of most adult tissues (Schlaeger et al., 1997; Wong et al., 1997). Ang1 is produced primarily by peri-endothelial support cells such as pericytes and vascular smooth muscle cells (Davis et al., 1996; Stratmann et al., 1998; Sugimachi et al., 2003). Therefore, it is believed that activation of Tie2 by Ang1 secreted from peri-endothelial cells contributes to the maintenance of vascular quiescence.

The most important cellular function of Ang1/Tie2 signal is its ability to promote cell survival, which is essential for the maintenance of vascular quiescence. Consistently, cartilage oligomeric matrix protein (COMP)-Ang1, a potent and stable Ang1 variant, protects against radiation-induced endothelial cell apoptosis in vivo (Cho et al., 2004). Ang1 stimulates phosphatidylinositol 3-kinase (PI3K) activity through recruitment of its regulatory p85 subunit into the phosphorylated tyrosine residue 1102 in the cytoplasmic domain of Tie2, which subsequently leads to activation

of cell survival kinase AKT (Kontos et al., 1998; Jones et al., 1999; Kim et al., 2000b; Papapetropoulos et al., 2000). Ang1/Tie2 signal-induced AKT activation results in phosphorylation and inhibition of the forkhead transcription factor Foxo1 (Daly et al., 2004). Since Foxo1 up-regulates expression of genes associated with vascular destabilization/remodeling and endothelial cell apoptosis, inhibition of Foxo1 by Ang1 may contribute to endothelial cell survival and blood vessel stability. In addition, Ang1-induced PI3K/AKT pathway induces up-regulation of the apoptosis inhibitor, survivin, in endothelial cells (Papapetropoulos et al., 2000). Furthermore, Tadros et al. have also suggested the role of A20 binding inhibitor of nuclear factor- κ B (NF- κ B) activation-2 (ABIN-2) in Ang1-induced cell survival (Tadros et al., 2003).

Ang1 potentiates endothelial barrier function by strengthening inter-endothelial cell adhesions. Mice over-expressing Ang1 develop vessels resistant to inflammatory agent-induced vascular leakage (Thurston et al., 1999, 2000). Consistently, Ang1 augments inter-endothelial cell adhesions regulated by vascular endothelial (VE)-cadherin, leading to the decreased permeability of cultured endothelial cell monolayer (Gamble et al., 2000). VEGF weakens VE-cadherin-dependent cell adhesions through the Src tyrosine kinase pathway (Paul et al., 2001; Weis et al., 2004; Gavard and Gutkind, 2006). Recently, Gavard et al. reported that Ang1/Tie2 signal counteracts Src activation by VEGF, thereby inhibiting VEGF-induced endothelial barrier dysfunction (Gavard et al., 2008). Moreover, it has been reported that Ang1 inhibits VEGF-induced Ca^{2+} influx through the transient receptor potential canonical-1 to preserve endothelial barrier function (Jho et al., 2005). Two members of Rho family small GTPases, Rac and Rho, have opposite effects on endothelial barrier function; the former increasing barrier function and the latter reducing barrier function. Mammoto et al. have reported that Rac-mediated inhibition of Rho through p190RhoGAP is critical for the protective effect of Ang1 against endotoxin-induced vascular leakage (Mammoto et al., 2007). In addition, it has been suggested that sphingosine kinase-1, a key enzyme catalyzing the formation of sphingosine-1-phosphate, is responsible for the vessel-stabilizing effect of Ang1 (Li et al., 2008).

Ang1/Tie2 signal has been shown to exert potent anti-inflammatory effects. Ang1 attenuates VEGF-induced adhesion of leukocytes to endothelial cells by inhibiting expression of cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Kim et al., 2001). In addition, tumor necrosis factor- α (TNF- α)- and VEGF-induced expression of tissue factor is also reduced by Ang1 stimulation in human umbilical vein endothelial cells (Kim et al., 2002). It has been suggested that these anti-inflammatory effects of Ang1 are mediated by the PI3K/AKT signaling pathway (Kim et al., 2001, 2002). In addition, Ang1 induces recruitment of ABIN-2 to Tie2, which in turn inhibits

NF- κ B, a transcription factor involved in inflammatory gene expression (Hughes et al., 2003).

Role of Ang1/Tie2 signal in angiogenesis

In addition to maintenance of vascular quiescence, Tie2 signaling is involved in physiological and pathological angiogenesis in adult vasculature. Tie2 is highly expressed in the endothelium of adult tissues undergoing angiogenesis such as ovary and uterus, and up-regulated in the neovessels during wound healing (Wong et al., 1997; Peters et al., 2004). In addition, Tie2 expression is elevated in the endothelium of angiogenic tumor vessels, suggesting the role of Tie2 in tumor angiogenesis (Peters et al., 2004). Consistently, it has been reported that soluble Tie2 receptor that is capable of blocking Tie2 activation by Ang1 reduced tumor growth and metastasis by inhibiting tumor angiogenesis *in vivo* (Lin et al., 1997, 1998).

COMP-Ang1 is shown to promote angiogenesis in the mouse corneal micropocket assay (Cho et al., 2004). Another report also indicates that VEGF-induced angiogenesis is potentiated by Ang1 *in vivo* (Asahara et al., 1998). However, Nambu et al. reported that increased expression of Ang1 in the retina significantly suppresses ischemia- or VEGF-induced renal neovascularization (Nambu et al., 2004, 2005). These results imply that Ang1/Tie2 signal may exert both angiogenic and angiostatic functions in a context-dependent manner.

Angiogenesis requires proliferation and migration of vascular endothelial cells. Although Ang1 induces activation of extracellular signal-regulated kinase (ERK) 1/2, a key regulator of cell proliferation (Harfouche et al., 2003; Brindle et al., 2006), the mitogenic effect of Ang1/Tie2 signal is still controversial (Witzenbichler et al., 1998; Kanda et al., 2005; Brindle et al., 2006). In contrast, Ang1/Tie2 signal potently promotes endothelial cell migration (Witzenbichler et al., 1998; Fujikawa et al., 1999; Cascone et al., 2003; Chen et al., 2004). Activated Tie2 associates with the adaptor protein, Dok-R (also known as Dok-2), which in turn establishes binding sites for Nck and p21-activated kinase (PAK). The Dok-R/Nck/PAK pathway is involved in Ang1-induced endothelial cell migration (Jones and Dumont, 1998; Master et al., 2001; Jones et al., 2003). Adaptor protein ShcA is also recruited to the activated Tie2 to enhance endothelial cell migration (Audero et al., 2004). Activation of PI3K by Ang1/Tie2 signal is involved not only in cell survival, but also in cell motility. Endothelial cell migration induced by Ang1 may be mediated by PI3K-dependent activation of focal adhesion kinase (FAK) and Rho family small GTPases Rho and Rac (Kim et al., 2000a; Cascone et al., 2003). In addition, the production of nitric oxide and reactive oxygen species is also suggested to be involved in Ang1-induced cell migration (Babaei et al., 2003; Chen et al., 2004, 2006; Kim et al., 2006).

Distinct signaling pathways induced by *trans*-associated Tie2 and extracellular matrix-anchored Tie2

Endothelial cell-cell adhesions are essential for maintenance of vascular quiescence. On the other hand, the endothelial cells undergoing angiogenesis weaken or lose the cell-cell contacts. Recently, we and Alitalo's group have clarified that Ang1-activated Tie2 forms distinct signaling complexes at cell-cell and cell-ECM contacts in the presence or absence of inter-endothelial cell adhesions, respectively (Fukuhara et al., 2008; Saharinen et al., 2008). In confluent endothelial cells, Tie2 is recruited to cell-cell contacts in response to Ang1. At the cell-cell contacts, oligomerized Ang1 bridges Tie2 expressed in adjoining cells, resulting in the formation of homophilic Tie2 *trans*-association. In clear contrast, in the absence of cell-cell adhesions, Tie2 is anchored to the cell-ECM interface through Ang1 binding to the ECM.

To further investigate the biological significance of distinct Tie2 localizations, we examined the downstream signaling pathways induced by Ang1 in the presence or absence of cell-cell contacts. Among Tie2-mediated signaling molecules, AKT and ERK1/2 are important for cell survival and cell migration/proliferation, respectively. AKT is preferentially activated by Ang1 in the presence of cell-cell contacts (Fukuhara et al., 2008). Consistently, Alitalo's group has also reported that phosphorylation of endothelial nitric oxide synthase (eNOS), a substrate for AKT, is induced only in cell-cell junctions, but not in motile cells without cell-cell contacts (Saharinen et al., 2008). In contrast, Ang1 preferentially activates ERK1/2 signaling pathway in the absence of cell-cell contacts (Fukuhara et al., 2008). In the cell lacking cell-cell adhesions, Ang1 induces integrin-dependent focal complex formation, leading to activation of FAK. FAK is involved in integrin-mediated ERK1/2 activation. Thus, the preferential activation of ERK1/2 by Ang1 occurs partly through integrin-dependent FAK activation. These findings imply that *trans*-associated Tie2 at cell-cell contacts and ECM-anchored Tie2 at cell-substratum contacts induce preferential activation of AKT and ERK1/2 pathways, respectively.

Vascular quiescence regulated by *trans*-associated Tie2

The preferential activation of AKT by *trans*-associated Tie2 may contribute to the vessel-stabilizing effect of Ang1 (Fig. 1). AKT phosphorylates the forkhead transcription factor Foxo1 and eNOS, which play important roles in endothelial functions (Daly et al., 2004; Chen et al., 2005). Previous work has suggested that AKT-Foxo1 pathway is involved in Ang1-induced endothelial cell survival and blood vessel stability (Daly et al., 2004). In addition, it has been reported that AKT-

eNOS pathway is required for vascular maturation (Chen et al., 2005). Since Ang1 preferentially induces phosphorylation of Foxo1 and eNOS in the presence of cell-cell contacts, *trans*-associated Tie2 may mediate vessel stabilization through the AKT-Foxo1 and AKT-eNOS signaling pathways.

Tie2 is known to interact with vascular endothelial protein tyrosine phosphatase (VE-PTP), which plays an important role in maintenance of vascular stability (Fachinger et al., 1999). VE-PTP also associates with VE-cadherin and reduces the tyrosine phosphorylation of VE-cadherin, thereby restricting vascular permeability (Nawroth et al., 2002; Nottebaum et al., 2008). Analysis of VE-PTP-deficient mice also reveals the requirement of VE-PTP for developmental vascular formation (Dominguez et al., 2007). Alitalo's group has reported that VE-PTP co-localizes with Tie2 at cell-cell contacts, but not at cell-ECM contacts (Fig. 1) (Saharinen et al., 2008). Furthermore, it has been suggested that Tie2 and VE-PTP cooperatively reduce the permeability of endothelial cell monolayer (Saharinen et al., 2008). Thus, *trans*-associated Tie2 may form a complex with VE-PTP at cell-cell contacts, thereby maintaining vascular quiescence.

Angiogenesis regulated by extracellular matrix-anchored Tie2

In contrast to the effect of *trans*-associated Tie2 on vascular quiescence, ECM-anchored Tie2 may promote angiogenesis (Fig. 2). Alitalo's group noticed that in migrating endothelial cells lacking the cell-cell

adhesions, Dok-R is phosphorylated by Ang1 and colocalized with Tie2 at the cell-ECM contacts in the rear of the motile cells, suggesting the involvement of Dok-R in endothelial cell migration upon Ang1 stimulation (Saharinen et al., 2008). We also found that Ang1-induced endothelial cell migration is prevented by inhibition of the ERK1/2 pathway, suggesting that ECM-anchored Tie2 enhances endothelial motility through the ERK1/2 signaling pathway (Fukuhara et al., 2008). Our results are consistent with the previous report that the ERK 1/2 pathway is involved in endothelial cell migration and angiogenesis (Eliceiri et al., 1998).

Differential gene regulation by *trans*-associated Tie2 and extracellular matrix-anchored Tie2

We employed DNA microarray analyses to identify the genes regulated by Ang1/Tie2 signal in the presence or absence of cell-cell contacts. Ang1 regulates distinct sets of genes in the presence or absence of cell-cell contacts (Fukuhara et al., 2008). Interestingly, in the presence of cell-cell contacts, Ang1 up-regulates the genes involved in vascular stability, which include *Krüppel-like factor 2* (KLF2), *zinc finger protein 36*, *C3H type-like 2* (*TIS11d*), *connexin 40* (*Cx40*), *delta-like 1* (*Dll1*) and *delta-like 4* (*Dll4*). KLF2 is known to maintain vascular quiescence by negatively regulating inflammation and angiogenesis (SenBanerjee et al., 2004; Bhattacharya et al., 2005; Dekker et al., 2006; Lin et al., 2006). (We discuss KLF2 later in this manuscript). *TIS11d* interacts with 3'-untranslated region of VEGF mRNA and decreases its stability, leading to down-

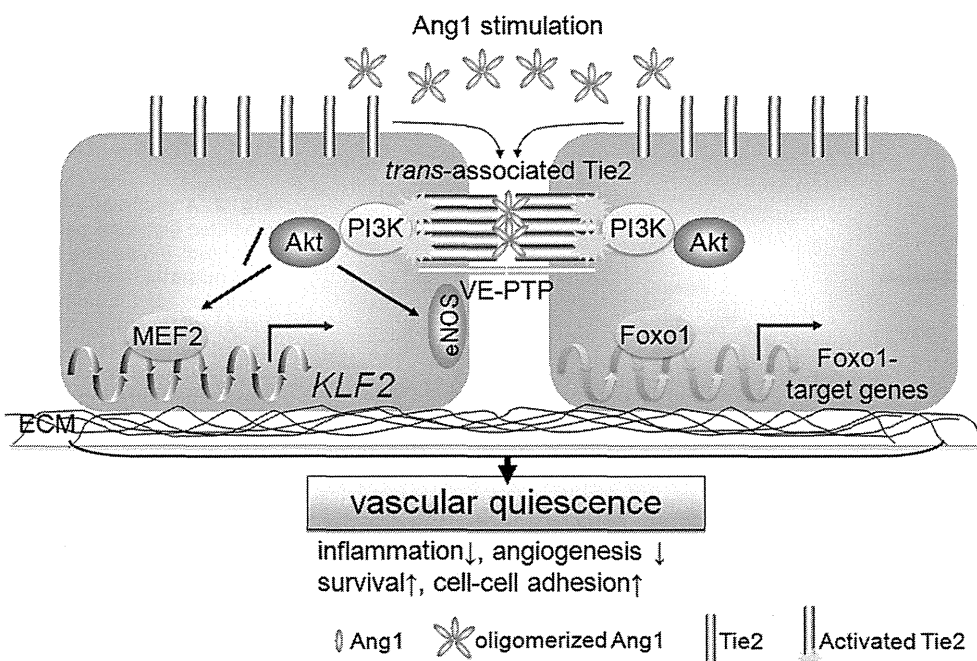


Fig. 1. Angiopoietin-1/Tie2-mediated signaling pathways involved in maintenance of vascular quiescence? Angiopoietin (Ang) 1 induces *trans*-association of Tie2 at cell-cell contacts, which leads to preferential activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. Activated AKT stimulates transcriptional activity of myocyte enhancer factor 2 (MEF2) to induce expression of *Krüppel-like factor 2* (KLF2), which in turn inhibits inflammation and angiogenesis. At the same time, the activated AKT phosphorylates and inhibits forkhead transcription factor Foxo1, thereby down-regulating the expression of Foxo1 target genes involved in endothelial cell apoptosis and vascular remodeling. AKT also activates eNOS, contributing to vascular maturation. *Trans*-associated Tie2 associates with vascular endothelial protein tyrosine phosphatase (VE-PTP), thereby enhancing endothelial cell-cell adhesions. ECM, extracellular matrix.

Angiostasis and angiogenesis by Ang1/Tie2

regulation of VEGF protein expression (Ciais et al., 2004). Since recent work suggests the role of endothelium-expressed VEGF in vascular homeostasis (Lee et al., 2007), the Ang1/Tie2 signal may regulate a cell-autonomous VEGF signaling pathway in endothelial cells through TIS11d. Cx40 is the predominant gap junction protein expressed in vascular endothelium and is required for inter-endothelial cell communication (Sohl and Willecke, 2004). Dll1 and Dll4, ligands for Notch receptor, have been reported to be involved in angiogenic sprouting (Noguera-Troise et al., 2006; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007; Phng and Gerhardt, 2009). Within angiogenic sprout, VEGF induces Dll4 expression in endothelial tip cells, which leads to Notch activation in adjacent stalk cells and the suppression of VEGF receptor expression, thereby establishing the adequate ratio between tip and stalk cells required for correct sprouting and branching patterns. Yana et al. previously found that Tie2 is expressed in endothelial stalk cells, but not in tip cells (Yana et al., 2007). Thus, Dll1 and Dll4 expression induced by trans-associated Tie2 may lead to inhibition of angiogenic sprouting in stalk cells through the Notch signaling pathway.

KLF2 induction by *trans*-associated Tie2

As described above, DNA microarray analyses revealed that Ang1 selectively induces KLF2 expression in the presence of cell-cell contacts. KLF2 belongs to a zinc-finger family of transcription factors and plays an important role in vascular stability (Atkins and Jain, 2007). In adult endothelium, KLF2 is induced by laminar shear stress and exerts atheroprotective effects (Dekker et al., 2002, 2005; Huddleson et al., 2004). KLF2 inhibits cytokine-induced expression of pro-inflammatory target genes such as VCAM-1 and E-selectin (SenBanerjee et al., 2004; Lin et al., 2006).

Furthermore, KLF2 down-regulates expression of VEGFR2, thereby inhibiting VEGF-induced angiogenesis and hyperpermeability (Bhattacharya et al., 2005; Dekker et al., 2006). Interestingly, Ang1 also acts as an anti-inflammatory and anti-permeability factor (Gamble et al., 2000; Kim et al., 2001; Brindle et al., 2006). Therefore, these findings prompted us to examine the involvement of KLF2 in Ang1/Tie2 signal-dependent vascular stabilization. Consistently, we found that induction of KLF2 by Ang1 is essential for inhibition of VEGF-mediated inflammation (Sako et al., 2009). Furthermore, we investigated the intracellular signaling pathways involved in Ang1-induced KLF2 expression (Sako et al., 2009). It has been previously reported that activation of myocyte enhancer factor 2 (MEF2) transcription factor by ERK5 MAP kinase is involved in shear stress-induced KLF2 expression (Kumar et al., 2005; Parmar et al., 2006). Similarly, our analysis showed that MEF2 is responsible for Ang1-induced KLF2 expression. However, siRNA knockdown experiments revealed the dispensability of ERK5 for Ang1-induced KLF2 expression. Instead, we found that PI3K/AKT signaling pathway is involved in transcriptional activation of MEF2 by Ang1 (Sako et al., 2009). Collectively, *trans*-associated Tie2 stimulates transcriptional activity of MEF2 through a PI3K/AKT pathway, thereby inducing KLF2 expression and contributing to the maintenance of vascular quiescence (Fig. 1).

How is the transition between distinct Tie2 signaling complexes regulated?

Ang1 maintains the vascular quiescence through *trans*-associated Tie2, whereas ECM-anchored Tie2 by Ang1 promotes angiogenesis (Fukuhara et al., 2008; Saharinen et al., 2008). However, it remains unknown how the transition between *trans*-associated Tie2 and

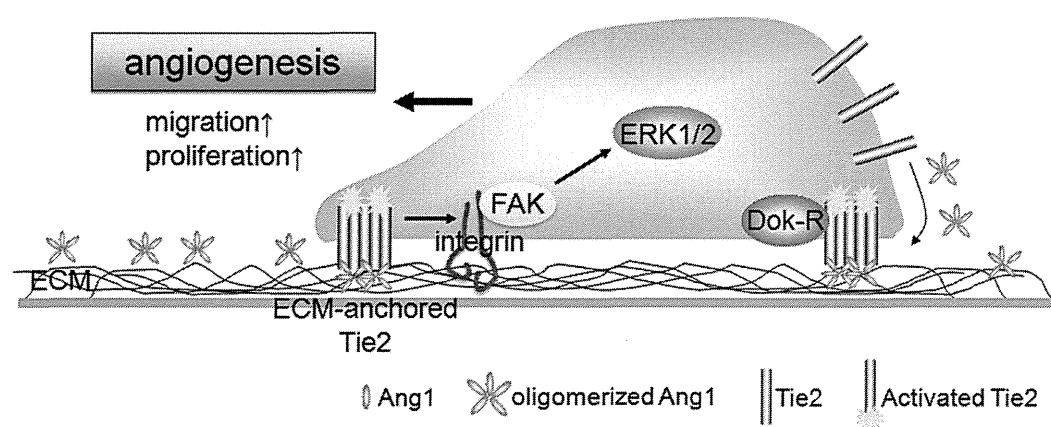


Fig. 2. Angiopoietin-1/Tie2-mediated signaling pathways involved in angiogenesis. Angiopoietin (Ang) 1 is capable of binding to extracellular matrix (ECM). Therefore, Tie2 is anchored by ECM-bound Ang1 to cell-substratum interface in the absence of cell-cell contacts. ECM-anchored Tie2 induces integrin-dependent focal complex formation, leading to activation of focal adhesion kinase (FAK). The activated FAK subsequently induces the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway

involved in endothelial cell migration and proliferation, thereby promoting angiogenesis. ECM-anchored Tie2 also regulates endothelial cell motility through an adaptor protein, Dok-R.