TABLE I. Tumor Incidence and Distribution in Aged PSCA Heterozygous and Knockout Mice

Genotype	# Mice	Age at analysis (months)	Pathology (# affected animals)
PSCA +/+	13	16–21	Lung tumor and MD prostate adenoCA, probably two distinct primaries (1) Prostate epithelial hyperplasia (2) Prostate with PIN (4) Lymphoid aggregates in lung, salivary gland (2) Lung adenoCa (1) Normal (3)
PSCA +/−	10	18–21	WD prostate adenoCa, lymphoid aggregates in lung, salivary gland, kidney, prostate (1) Lymphoma in lung, spleen, liver, kidney (1) Prostate epithelial hyperplasia, and lymphoid aggregates in lung, salivary gland, spleen, kidney, prostate (1) Prostate with PIN (3) Normal (4)
PSCA −/−	10	9–21[a]	Prostate epithelial hyperplasia (3) Prostate with PIN (2) Microinvasive prostate adenoCa (1) Lymphoid aggregates in lung, salivary gland, kidney, prostate (1) Normal (3)

[a]One animal was killed at 9 months of age due to incurable ulcerative dermatitis.

Split-Dose Sublethal Gamma Irradiation of PSCA +/− and PSCA −/− Mice Does Not Alter Cancer Incidence or Organ Distribution

We tested whether the loss of PSCA was a "second hit" in the process of carcinogenesis, and would cause malignancy in conjunction with another mutagenic signal [33]. Mice of all three PSCA genotypes were treated with 4.5 and 6 Gy of gamma irradiation at 2 and 6 months of age respectively, to promote tumor formation [34]. We observed an increased incidence of PIN and prostate adenocarcinoma in PSCA +/− and PSCA −/− mice compared to PSCA +/+ mice (Table II) that was not statistically significant (P-value = 0.08). Several mice developed more than one malignancy, when compared to aging mice of the same background (Table II). Surprisingly, however, we did not observe any malignancies in kidney, stomach, pancreas, bladder, or the gastrointestinal tract, where PSCA is also expressed. These observations suggest that deletion of PSCA does not initiate or promote the development of primary epithelial tumors.

PSCA Deletion Enhances Metastatic Frequency of TRAMP Primary Prostate Tumors

Despite anatomic differences between mouse and human prostate, mouse models of prostate cancer recapitulate many aspects of the human disease [30] allowing investigation of the effects of deletion or ectopic overexpression of genes of interest. The TRAMP transgenic mouse model of prostate cancer expresses SV40 large T antigen behind the prostate-restricted probasin promoter. Animals develop PIN by 8 weeks and adenocarcinoma by 16 weeks of age [28], with infrequent metastasis to lymph nodes or other organs. TRAMP prostate tumors also overexpress PSCA [8,9]. TRAMP+ male mice of all three PSCA genotypes were collected and housed until 40 weeks of age, or until they became ill. TRAMP+ PSCA +/+ became ill at 28.6 (+/− 6.32) weeks, TRAMP+ PSCA +/− mice at 34.4 (+/− 5.32) weeks and TRAMP+ PSCA −/− mice at 31.4 (+/− 7.29) weeks.

PSCA +/+, +/−, and −/− mice displayed enlarged prostate lobes and seminal vesicles at autopsy (Fig. 2A). Prostatic intraepithelial neoplasia, well differentiated, moderately differentiated, and poorly differentiated adenocarcinomas were observed in all three genotypes of mice (Fig. 2B). Only one clear diagnosis of phylloides cancer was made in a TRAMP+, PSCA +/− mouse. 5/12 of the primary tumors of PSCA +/+ mice were poorly differentiated, while 3/31 of PSCA +/− and 2/16 of PSCA −/− tumors were poorly differentiated (Fig. 2B). This difference was not statistically significant, and is similar to previous reports of tumor histology in TRAMP mice [29,35,36]. Figure 2C shows H&E stained sections of representative tumors from each genotype.

TABLE II. Low-Level Gamma Irradiation Does Not ChangeTumor Distribution in PSCA +/− and −/− Mice

Genotype	# Mice	Age at analysis (months)	Pathology (# affected animals)
PSCA +/+	9	11–20	No abnormality (1) Myeloid infiltration of spleen, CML (1) Lymphoma (2) Bacterial liver infection (1) PIN (4[a])
PSCA +/−	12	11–20	No abnormality (1) Lymphoid aggregates in various tissues (1) Adenocarcinoma in lung (1) Sarcoma (1) Prostatic hyperplasia (1) PIN (7[ab])
PSCA −/−	17	11–20	No abnormality (3) Lymphoid aggregates (1) Lymphoma (2) AdenoCa of lung (1) PIN (8)[cd] Prostate adenoCa (2)[e]

[a]One animal also had lymphoid aggregates.
[b]One animal also had lymphoma.
[c]Five animals had pathology in addition (lung adenoCa (1), prostate adenoCa (2), leukemia (1), lymphoma (1)).
[d]One animal also had lymphoma.
[e]One animal also had leukemia.

Macro-metastases to the liver, kidney, lung and lymph nodes were detected in 61.3% of TRAMP+PSCA +/− and 56.25% of TRAMP+PSCA −/− mice (Fig. 2D). In contrast, only 33% of TRAMP+PSCA +/+ mice developed metastases. The frequency of metastasis in heterozygous compared to wild-type mice ($P = 0.0001$) and knockout compared to wild-type mice was statistically significant ($P = 0.007$) while heterozygous and knockout mice were similar to each other (P-value not significant). Representative H&E stained sections of metastases in liver, kidney, and lung are shown in Figure 2E. Histology showed that 1/12 TRAMP+, PSCA +/+ mice, 3/31 TRAMP+, PSCA +/− mice and 1/16 TRAMP+, PSCA −/− mice had adenocarcinoma in the bone marrow, although this was not a significant difference. It was surprising that the frequency of metastasis was the same in PSCA heterozygous and knockout mice, suggesting either that a 50% reduction in the level of PSCA is sufficient to increase metastasis, or that there is selection for complete loss of PSCA during tumor progression.

No tumors were observed in T antigen-negative PSCA-expressing organs indicating that increased tumor formation was a result of T antigen activity. These studies were conducted in TRAMP mice on a C57BL/6 background. We had similar findings in a smaller study with TRAMP mice on a mixed FVB × C57BL/6 background (data not shown) indicating that background gene variation did not affect metastatic frequency.

Frequent Loss of PSCA Expression in Metastases of TRAMP+PSCA +/− Mice

We evaluated PSCA message levels in primary tumors and metastases of TRAMP+PSCA +/+ and TRAMP+ PSCA +/− mice by RT-PCR. Message levels in the primary tumors varied considerably (Fig. 3A), while there was a dramatic loss of PSCA message in the metastases. We also conducted quantitative RT-PCR to quantify mPSCA message levels in primary tumors and metastases. Consistent with human tumors, more than half of wild-type and heterozygous primary tumors had increased PSCA message (Fig. 3B). In contrast, the majority of metastases had minimal to undetectable message levels. Together, these results suggest that there is a selection against PSCA expression during tumor progression, although it is unclear whether PSCA loss is a cause of metastasis or an effect of tumor progression. Our previously published mPSCA-specific monoclonal antibody is difficult to use for IHC [9]. While high mPSCA levels are detected consistently,

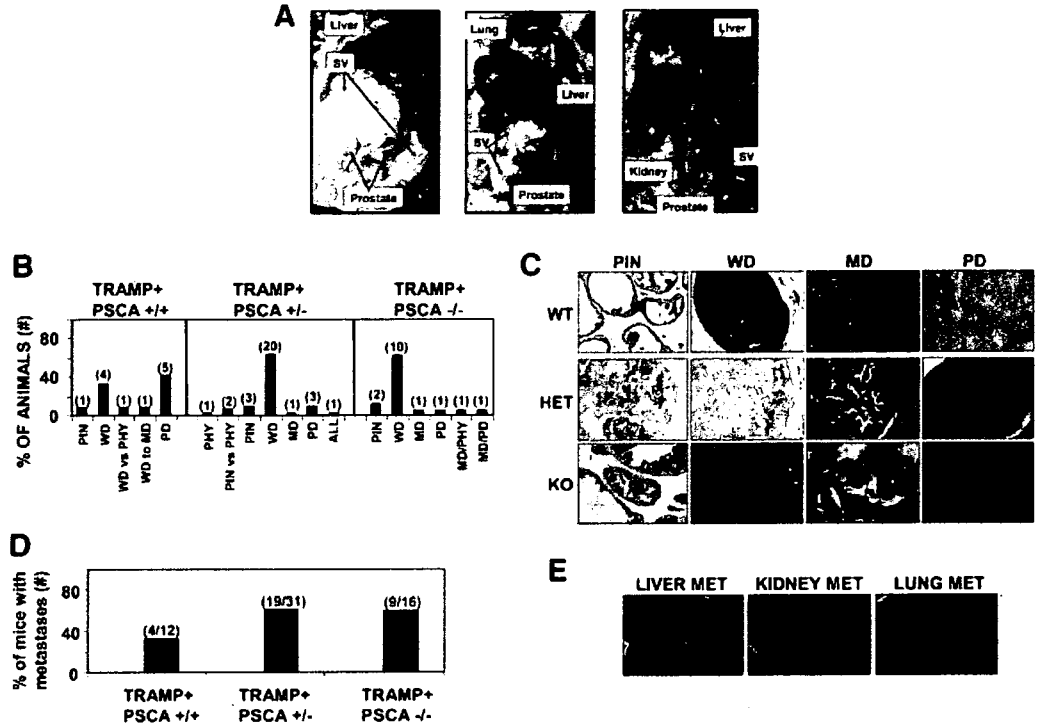


Fig. 2. Deletion of PSCA increases prostate cancer metastasis. **A**: Gross evaluation of mice upon autopsy shows macro-metastases to various soft tissues in male TRAMP+ PSCA +/− and TRAMP+ PSCA −/− mice (**left panel**, TRAMP+ PSCA +/+, **middle panel**, TRAMP+ PSCA +/−, **right panel**, TRAMP+ PSCA −/− animal). **B**: Histological evaluation of primary tumor confirms presence of primary prostate cancer in all genotypes. PIN, prostatic intraepithelial neoplasia; WD, well-differentiated adenocarcinoma; MD, moderately differentiated adenocarcinoma; PD, poorly differentiated adenocarcinoma; PHY, phylloides cancer. **C**: H&E sections from representative tumors of each genotype. 200× magnification. **D**: TRAMP+ PSCA +/− and TRAMP+ PSCA −/− mice have a higher frequency of metastasis than TRAMP+ PSCA +/− mice. **E**: H&E stained sections of representative liver, kidney and lung metastases from TRAMP+ PSCA heterozygous mice are shown.

results are variable when low amounts of protein are present (data not shown). Therefore, we quantified mPSCA by RT-PCR.

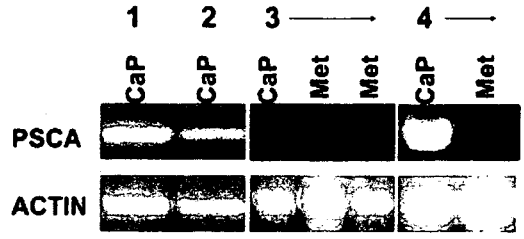
PSCA Deletion Does Not Alter Proliferation, Apoptosis, and Angiogenesis of TRAMP-Induced Primary Prostate Tumors

IHC analysis showed that primary tumors and metastases were T-Ag+ indicating their origin in the prostate (Fig. 4A, left panel). Ki67 staining (Fig. 4A, right panel) detected similar proportions of proliferating cells in primary tumors from all three genotypes, suggesting that the higher frequency of metastasis in TRAMP+ PSCA +/− and TRAMP+ PSCA −/− mice was not a direct result of increased cell growth.

Most of the primary tumors expressed AR while the metastases were low for AR (Fig. 4A, middle panel). This observation is consistent with recent studies reporting decreased AR message in poorly differentiated primary tumors [35] and decreased AR protein expression in primary tumors and most macro-metastases of TRAMP mice [36]. The PSCA promoter is androgen-responsive [32], suggesting that loss of PSCA expression in the metastases may be due to reduced AR expression.

Cleaved caspase-3 staining detected very few apoptotic cells, with no detectable difference between genotypes (Fig. 4B). Quantitation of the number of positive cells for each of the markers did not indicate a significant difference between genotypes for expression of AR, Ki67, or cleaved caspase-3 (Fig. 4C). A correlation between increased intraductual vascular density and tumor progression was reported in the TRAMP model [37]. We stained primary tumors with α-CD31 antibody to detect blood vessel formation (Fig. 4B, right panel). There appears to be a reduction in the number of blood vessels formed in TRAMP+ PSCA +/− primary tumors compared to WT or KO tumors (Fig. 4C). However, since the frequency of metastasis was similar in TRAMP+ PSCA heterozygous and knockout mice, the significance of this observation is unclear. These results suggested that the increased metastasis from TRAMP+ PSCA +/− and TRAMP+ PSCA −/− tumors was not a direct

A



B

	INCREASE			DECREASE		
	< 5-fold	5-10 fold	> 10-fold	< 5-fold	5-10 fold	> 10-fold
WT						
Primary	1	2	2	4		
Met					2	2
HET						
Primary	6	7	10	4		4
Met	2			1	2	20

Fig. 3. **A**: Semi-quantitative RT-PCR analysis shows varied mPSCA message levels in primary prostate tumors of TRAMP+ PSCA +/+ and TRAMP+ PSCA +/− mice. Murine beta actin message was amplified as quality control for the cDNA. 1 and 2: Primary prostate tumors from TRAMP+, PSCA +/+ mice. 3 and 4: Primary tumor (CaP) and metastases (Met) from a TRAMP+PSCA +/− mouse. **B**: Quantitative RT-PCR analysis was performed using first-strand cDNA. mPSCA and murine beta actin were amplified using Taqman Gene Expression Assays from Applied Biosystems. Relative abundance of mPSCA message was normalized to the amount of beta actin message. Numbers of primary tumor and metastasis samples tested are shown. For some animals, metastases from more than one location were tested for PSCA expression. For tumors from PSCA +/+ mice, fold increase or decrease in PSCA message was compared to the amount of message detected in an 11-month-old wild-type male animal. Fold change in PSCA message was compared to the message level in a 10-month-old PSCA +/− male animal for PSCA +/− animals.

result of increased angiogenesis, or decreased apoptotic cell death.

Aberrant Cellular Localization of Aurora-B Kinase and Survivin in PSCA +/− and −/− TRAMP Primary Tumors

Function of Aurora kinase family members is crucial to mitosis, meiosis and spindle checkpoint control. Overexpression of Aurora-A and Aurora-B kinases is correlated with tumor invasion and progression in a variety of tumor types including prostate cancer [31,38]. Cytoplasmic localization of Aurora-A was noted previously in TRAMP tumors and human prostate cancers [31], and correlated with increased seminal vesicle invasion, although the functional significance of this observation is unclear. Nuclear and cytoplasmic localization of Aurora-A was observed in primary tumors of all three genotypes (Fig. 5A). Quantification of the number of cells with nuclear versus cytoplasmic expression of Aurora-A in each tissue indicated significantly higher cytoplasmic expression in primary tumors of TRAMP+ PSCA +/− and TRAMP+ PSCA −/− mice (Fig. 5B). These data suggest that increased metastatic frequency is correlated with cytoplasmic localization of Aurora-A.

We observed Aurora-B localized predominantly to the nucleus in PSCA WT primary tumors, and predominantly cytoplasmic in PSCA heterozygous and knockout tumors (Fig. 5A, middle panel). However, the error bars were large and the difference was not statistically significant (Fig. 5B). Metastases from heterozygous and knockout mice showed nuclear Aurora-B staining (data not shown). Aurora-B is a chromosomal passenger protein and requires co-localization of the inhibitor of apoptosis protein, Survivin, for its function [39]. The complex of Aurora-B, Survivin, and INCENP in the nucleus directs appropriate mitotic progression. Similar numbers of cells expressing Survivin in the nucleus and cytoplasm of primary

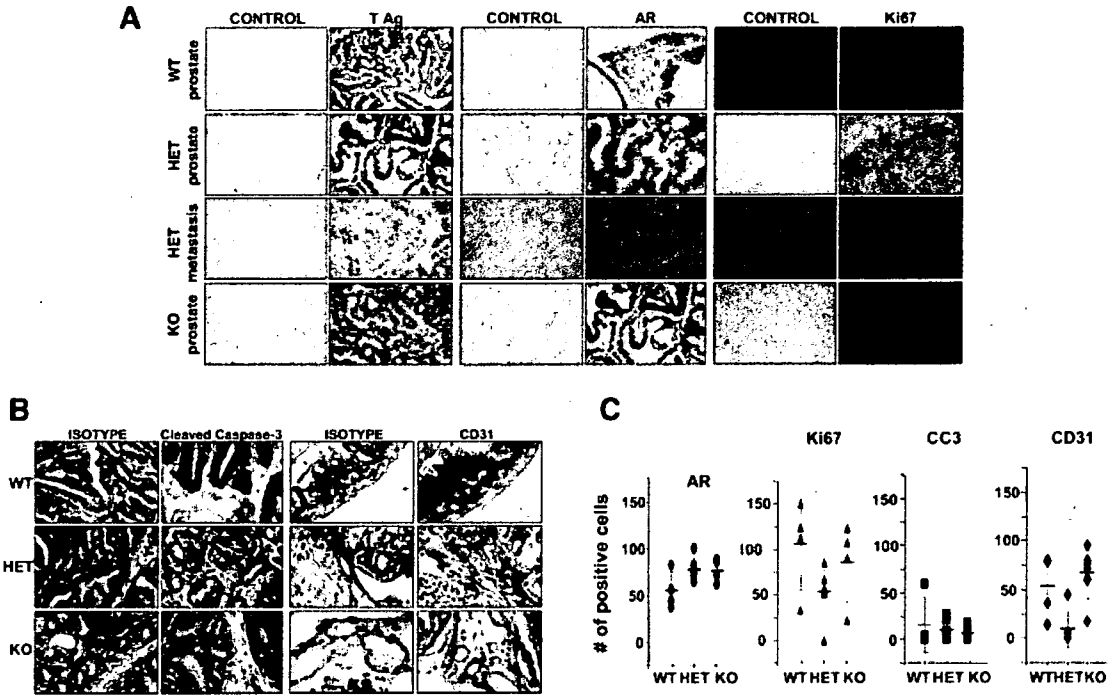


Fig. 4. Immunohistochemical analysis of primary tumor and metastasis. **A**: Representative well-differentiated primary tumor from each genotype and a representative metastasis from the liver of a TRAMP+ PSCA+/− animal are shown. **Left panel**: Tantigen staining. **Middle panel**: AR staining. **Right panel**: Ki67 staining. Sections were counter-stained with methyl green. **B**: Representative primary tumors from each genotype stained with α-cleaved caspase-3 antibody (upper panels) and α-CD31 antibody (lower panels) shows rare apoptotic cells and limited angiogenesis. 400× magnification. A serial section stained with isotype control for each antibody is shown on the left and the specific primary antibody is shown on the right. **C**: Quantification of the number of positive cells for each marker in primary tumors of all three genotypes are shown, and was performed as described in Materials and Methods Section.

tumors of all three genotypes were observed (Fig. 5A right panel, and 5B). However, the cytoplasmic localization of both Aurora-B and Survivin suggests aberrant mitotic activity in some PSCA heterozygous and knockout primary tumors.

DISCUSSION

We investigated the biological function of PSCA in epithelial cells. Our results show that PSCA is not involved in normal epithelial development. Malignant transformation requires two damaging events [40]. We hypothesized that if deletion of PSCA renders the cell unstable, then gamma irradiation would provide the second hit, resulting in increased primary tumor formation. Our observation that irradiation did not accentuate tumor development in PSCA +/− and −/− mice indicates that PSCA deletion is not an initiating or promoting event in primary tumor formation. On the other hand, Ly6 family members Sca-1 and Sca-2 are also expressed in normal prostate [24], and Sca-1 expression is elevated in PTEN knockout-induced prostate cancer [11]. Thus, such proteins may have overlapping function that compensates for PSCA in our knockout mice.

Tumors develop in TRAMP transgenic mice by SV40 T antigen activity which transforms cells and inactivates the tumor suppressor p53 [41]. Thus, this strong oncogene provides both an initiating and promoting event for cancer formation. Primary tumor formation was similar in TRAMP+ mice of all three genotypes. If PSCA could affect tumor initiation or promotion this cooperation may be more apparent in concert with a gene mutation that has a milder phenotype. For example, mice with targeted gene deletion of the homeobox protein Nkx3.1 develop PIN but do not progress to invasive cancer. As a result, synergistic effects of other genes such as p27kip1 in tumor progression can be investigated on this background [42].

Approximately half of the prostate tumors in TRAMP+ PSCA +/+ and TRAMP+ PSCA +/− mice expressed PSCA, in accordance with previous work from our group and others [8,9]. There was a dramatic

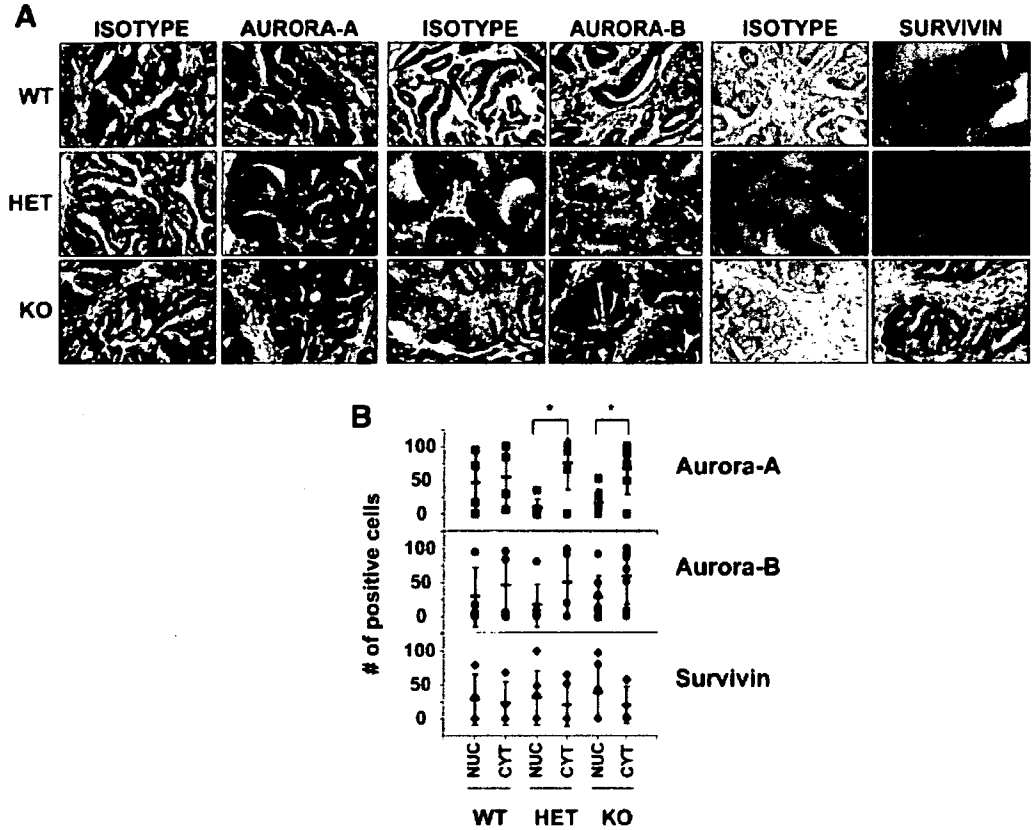


Fig. 5. Altered cellular localization of Aurora kinases in TRAMP+ PSCA +/− and TRAMP+ PSCA −/− tumors. **A**: Representative primary tumors stained with α-Aurora-A antibody (**left panel**) α-Aurora-B antibody (**middle panel**) and Survivin (**right panel**). 400× magnification. Isotype control for each antibody is shown to the left for each marker. **B**: The number of cells with positive nuclei versus the number of cells with positive cytoplasm in 150 representative cells were counted as described in Materials and Methods Section.

loss of PSCA expression in almost all of the metastases. Q-PCR analysis of laser capture microdissected PSCA +/− tumor DNA showed retention of the wild-type copy of the PSCA gene (data not shown) indicating that lack of PSCA expression in tumors of PSCA +/− mice was not due to gene loss.

Consistent with other studies [35,36] we also observed decreased AR expression in the metastases. However, this correlation was not absolute and other undefined epigenetic or signaling changes may also down-regulate PSCA expression.

Loss of PSCA expression may be enhanced when PSCA dosage is reduced from the outset, such as in PSCA +/− mice. Human bladder, stomach, and esophagus cancers showed abundant PSCA message in differentiated cancers in these tissues, but reduction of PSCA message in de-differentiated and thus more malignant cancers [43]. High PSCA message was found in normal mucosa, but was significantly reduced in head-and-neck squamous cell carcinoma [44]. Similarly, loss of Ly6 family member E48 is correlated with squamous cell carcinoma progression [45]. Together, these observations suggest that there may be selection against PSCA expression during cancer progression.

Overexpression of the Aurora kinase family is implicated in tumor progression. In normal tissues, Aurora-A is in the nucleus, and its kinase function is important for spindle checkpoint control. Aberrant localization to the cytoplasm in malignant tissues implicates this kinase in aneuploidy and carcinogenesis [46]. As previously reported for TRAMP tumors [31], we found Aurora-A kinase expression in prostate tumors from all three genotypes. Predominant cytoplasmic localization of Aurora-A in TRAMP+ PSCA +/− and TRAMP+ PSCA −/− tumors suggests a role for this kinase in metastasis in this model. It will be interesting to test whether blocking Aurora-A expression or cytoplasmic localization in TRAMP+ PSCA heterozygous or knockout cell lines affects the frequency of metastasis from these primary tumors.

Aurora-B regulates chromosome segregation, and overexpression of this kinase induces development of

multinucleated cells. The proteins Aurora-B, Survivin, Borealin, and Crm1 form the chromosomal passenger complex, necessary for proper chromosomal segregation. Survivin is a mitotic regulator and inhibitor of apoptosis in normal tissues. Cytoplasmic export of Survivin appears critical for its cytoprotective effect [47]. Nuclear localization of Survivin, and by extension, Aurora-B, is correlated with a better prognosis for cancer patients. Cytoplasmic Aurora-B is correlated with seminal vesicle invasion in prostate cancer. Cytoplasmic localization of both Aurora-B and Survivin in a portion of TRAMP+ PSCA +/− and −/− primary tumors, suggested that the increased metastasis from these tumors may be a result of greater chromosomal instability and/or increased resistance to cell death.

PSCA may be among a class of genes with paradoxical context-dependent functions. One example of such genes is the transcription factor Yin Yang 1 (YY1), a positive and negative regulator of transcription whose activity is associated with various phases of the cell cycle. YY1 expression is elevated predominantly in PIN and also in intermediate and high-grade tumors, suggesting a possible role for YY1 in prostate cancer development [48]. Conversely, tumors with regions of low YY1 expression correlated with poorer outcome and a higher chance of tumor recurrence, as measured by increases in serum PSA levels. Thus, decreased expression of YY1 may confer a survival advantage to metastatic prostate cancer cells [48]. The transforming growth factor-beta (TGFβ) family of proteins, particularly TGFβ is also an example of context-dependent function. TFGβ promotes embryonic tissue growth and morphogenesis, while activating cytostasis and cell death pathways in adult tissues [49]. Cancer cells can evade TGFβ growth control by inactivating the TGFβ receptor, or various signaling cofactors [49]. In transgenic mice expressing constitutively active type I TGFβ receptor, *Neu*-driven primary mammary tumors were delayed in growth, but formation of lung metastases was accelerated [50], highlighting the dual role of TGFβ in cancer development and progression.

CONCLUSIONS

We studied the function of PSCA in development and carcinogenesis. Our results demonstrate that deletion of PSCA increases cancer metastasis, while normal development and primary tumor formation are unaffected. Primary tumor formation and the development of metastases are distinct steps in the progression of cancer. Genetic and epigenetic changes are necessary for cell transformation, malignant growth, and metastasis [51,52]. A recent study shows that the combined activity of four "signature" genes increases breast cancer metastasis [52,53]. This activity is distinct from the transforming function of oncogenes that make a normal cell malignant. Our results suggest that PSCA may have a similar function in tumor progression. Further in vivo assays are needed to determine the ability of PSCA to prevent growth of experimental metastases.

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BMPs Promote Proliferation and Migration of Endothelial Cells via Stimulation of VEGF-A/VEGFR2 and Angiopoietin-1/Tie2 Signalling

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The differentiation, growth, and survival of endothelial cells (ECs) are regulated by multiple signalling pathways, such as vascular endothelial growth factors (VEGFs) and angiopoietins through their receptor tyrosine kinases, VEGF receptor (VEGFR) 2 and Tie2, respectively. Bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGF)-β family, have been implicated in the development and maintenance of vascular systems. However, their effects on EC proliferation remain to be elucidated. In the present study, we show that BMPs induce the proliferation and migration of mouse embryonic stem cell (ESC)-derived endothelial cells (MESECs) and human microvascular endothelial cells (HMECs). Addition of BMP-4 to culture induced significant proliferation and migration of both types of ECs. BMP-4 also increased the expression and phosphorylation of VEGFR2 and Tie2. These findings suggest that BMP signalling activates endothelium via activation of VEGF/VEGFR2 and Angiopoietin/Tie2 signalling.

Key words: embryonic stem (ES) cell, Flk1, human microvascular endothelial cell (HMEC), Tie2, VEGFR2.

Abbreviations: BMP, bone morphogenetic protein; ECs, endothelial cells; ESC, embryonic stem cell; HMEC, human microvascular endothelial cell; MCs, mural cells; MESEC, mouse embryonic stem cell-derived endothelial cell; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Blood vessels consist of a lining of endothelial cells (ECs) surrounded by mural cells (MCs; pericytes and vascular smooth muscle cells), carry oxygen and nutrients to distant organs and are crucial for organ growth in the embryo and repair of wounded tissues in the adult. Defects in the formation and maintenance of blood vessels thus contribute to the pathogenesis of numerous disorders (*1*). Development of the vascular system occurs in two distinct processes, vasculogenesis and angiogenesis (*2*).

During mouse embryogenesis, the initial development of vascular endothelium, termed vasculogenesis, occurs in the mesodermal layer of the yolk sac, yielding structures, termed blood islands. Blood islands consist of endothelial progenitors called angioblasts, which express vascular endothelial growth factor (VEGF) receptor-2 (VEGFR2), also known as Flk1 (*3*, *4*). This embryonic vascular differentiation has been recapitulated by an *in vitro* differentiation system from embryonic stem cells (ESCs) (*5–7*). When VEGFR2-expressing (VEGFR2+) endothelial progenitors, isolated from differentiation cultures, are re-differentiated in the presence of VEGF, various endothelial markers are sequentially up-regulated in a pattern that is similar to that observed in early embryos (*5*). Further analysis revealed that lineages of both ECs and MCs develop from a common ESC-derived endothelial progenitor cell type (*7*).

In angiogenesis, new vessels sprout from the pre-existing vasculature and are further remodelled 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 remodelling of tissues during wound healing and ischaemia, and for the physiological female reproductive cycle. Neovascularization also plays a pivotal role in pathological processes such as tumour growth, chronic inflammation and diabetic vasculopathy.

During vasculogenesis and angiogenesis, the differentiation, proliferation, and migration of ECs are regulated by a balance between positive and negative regulators. VEGF signalling through VEGFR tyrosine kinases has been implicated in regulation of the differentiation, growth, and integrity of ECs. VEGF stimulates endothelial differentiation of vascular progenitor cells derived from ESCs (*7*), and regulates the growth of differentiated ECs.

Angiopoietin (Ang)-1 binds and activates the Tie2 receptor tyrosine kinase, which is expressed almost exclusively on the surface of ECs (*8*). Ang-1 is required for maintenance of maximal interactions between ECs, MCs and the extracellular matrix (*9*). The Ang-1/Tie2 pathway plays important roles in embryonic

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development, physiological angiogenesis, maintenance of vascular integrity, inflammation and tumour growth (*10*). In the mature vasculature or cultured ECs, Ang-1 inhibits apoptosis (*11*) and promotes differentiation, sprouting and migration. In contrast, Ang-2 can act as either an agonist or an antagonist for Tie2, depending on the type of cell and the surrounding microenvironment (*12*). Ang-2 is expressed at sites of pericyte detachment and blood vessel remodelling in conjunction with VEGF, whereas in the absence of VEGF, Ang-2 activity leads to EC apoptosis. These findings strongly suggest that the members of the angiopoietin and VEGF families collaborate during different stages of angiogenesis (*13*).

In addition to VEGF/VEGFR2 and angiopoietins/Tie2 signalling, transforming growth factor (TGF)-β family proteins have been implicated in vascular development (*14*). The TGF-β family consists of structurally related and multifunctional proteins including TGF-βs, activins and bone morphogenetic proteins (BMPs). 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-βs bind to type I receptors known as activin receptor-like kinase (ALK)-4 and -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 3 transduce signals for TGF-βs and activins, while Smad1, 5 and 8 are specific for signalling of BMPs (*15*). An exception to this is ALK-1, which is preferentially expressed in ECs, also binds TGF-β and activates the Smad1/5 pathways (*16*). Recently, BMP-9 and BMP-10 were shown to bind to ALK-1 and to activate the Smad1/5 pathways (*17, 18*).

Perturbation of TGF-β family receptor signalling in humans leads to vascular disorders. Hereditary haemorrhagic telangiectasia is genetically linked to mutations of ALK-1 and endoglin, a co-receptor for TGF-β family members (*19*). Furthermore, loss-of-function mutations in the human BMP type II receptor (BMPR-II) are associated with the pathogenesis of familial primary pulmonary arterial hypertension (*20–22*). Studies of various mice knocked out for TGF-β family signalling components have also suggested their importance in vascular development (*23*). Mice lacking BMP-4 (*24*) or specific BMP receptors (*25, 26*) exhibit abnormal development of the heart and vasculature. Mice lacking Smad1 or Smad5 die at embryonic day 10.5–11.5 due to defects in vascular development, with enlarged blood vessels surrounded by decreased numbers of vascular smooth muscle cells (*27, 28*). The finding that mice deficient in BMPs, their receptors or their intracellular Smad effector proteins have impaired vascular development and the linkage of pulmonary arterial hypertension with mutations of BMPR-II suggest a role of the BMP/Smad signalling cascade in formation of the vascular system.

In vivo and *in vitro* gain-of-function analyses have also suggested a role of BMP signalling during vascular development. When CHO cells overexpressing BMP-4 were implanted in the avascular region of quail embryos, endothelial growth and capillary plexus formation were enhanced (*29*). Furthermore, *in vitro* experiments have shown that BMPs indeed modulate EC migration and capillary tube formation, an effect that can be antagonized by BMP antagonists (*29, 30*). However, the effects of BMPs on the proliferation of cultured ECs, especially embryonic ECs, have not been fully determined.

In the present study, we examined the effects of BMP signalling on the proliferation and migration of mouse ESC-derived endothelial cells (MESECs) and human microvascular endothelial cells (HMECs). Addition of BMP-4 to culture significantly induced proliferation and migration of these cells. The expression and phosphorylation of VEGFR2 and Tie2 were up-regulated by BMP-4 in both types of cells. These findings suggest that BMP signalling activates endothelium via activation of VEGF/VEGFR2 and Ang/Tie2 signalling.

MATERIALS AND METHODS

Cells and Cell Culture—Maintenance, differentiation, culture, and cell sorting of CCE and MGZ5 mouse ES cells (gifts from Drs M.J. Evans and H. Niwa, respectively) were as described (*7*). All experiments were carried out using both ES cell lines and yielded essentially similar results. HMEC, an immortalized human dermal microvascular EC line, was obtained from Dr T. Lawley, and was cultured in EGM-2 (Cambrex) containing 2% fetal bovine serum (FBS) and EC growth supplements (Clonetics). VEGF and BMP-4 were purchased from R&D. Concentrations of BMP-4 used in this study (60 and 10 ng/ml for MESECs and HMECs, respectively) were verified by studying its dose-dependent effects on their proliferation (data not shown).

Cell Growth and BrdU Incorporation Assays—Cell number was counted using a Coulter Counter (Yamato Kagaku). The nucleotide BrdU is incorporated during the S phase of the cell cycle. In order to quantify the ratio of DNA synthesis, BrdU incorporation assays were performed according to the manufacturer's protocol (Roche). Briefly, BrdU was added to culture for 2 h during incubation. Fixed cells were incubated with anti-BrdU antibodies, followed by detection of immune complexes with a fluorescein isothiocyanate (FITC)-labelled anti-mouse antibody and nuclear staining with propidium iodide (PI). Confocal images were obtained by laser scanning/confocal microscopy (LSM 510 META; Carl Zeiss). The proportion of BrdU-positive cells was determined among at least 50 cells counted in three different fields chosen randomly.

TUNEL Assay—For detection of apoptosis, TUNEL assays were performed according to the manufacturer's protocol (Roche). Briefly, permeabilized ECs were subjected to terminal deoxynucleotidyltransferase (TdT) reaction with FITC-12-dUTP followed by nuclear staining with PI and was observed under a laser scanning/confocal microscope. The proportion of TUNEL-positive cells was determined among at least 100 cells counted in three different fields chosen randomly.

Video Time-Lapse Microscopy—Time-lapse imaging of migrating cells was performed on a Leica DM IRB

microscope equipped with a hardware-controlled motor stage over 24 h in serum-free medium at 37°C/5% CO_2. Images of HMECs or MESECs were obtained with a LEICA DC 350F CCD camera every 15 or 20 min, respectively, and analyzed using Image J software (National Institutes of Health, USA). Migration of each cell was analyzed by measuring the distance travelled by a cell nucleus over the 24-h time period (*31*). Average migration speed was calculated by analyzing at least 10 cells/group.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (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 GeneAmp 5700 system (Applied Biosystems) and SYBR Green (Applied Biosystems). All expression data were normalized to those for β-actin. The primer sequences are available online as indicated in Table S1.

Immunohistochemistry and Western Blot Analysis—Monoclonal antibodies to PECAM1 (Mec13.3) and SMA (1A4) for immunohistochemistry were purchased from BD Pharmingen and SIGMA, respectively. Staining of cultured cells was performed as described (*32*). Stained cells were photographed using a phase-contrast microscope (Model IX70; Olympus) or confocal microscope. All images were imported into Adobe Photoshop as JPEGs or TIFFs for contrast manipulation and figure assembly. Antibodies to FLAG and α-tubulin for Western blot analysis and immunohistochemistry were obtained from SIGMA. Antibodies to mouse VEGFR2, Tie2, human VEGFR2 and phospho-tyrosine for Western blot analysis and immunohistochemistry were obtained from eBioscience, RDI, Santa Cruz and Chemicon, respectively. Western blot analysis was performed as described (*33*).

RESULTS

BMP-4 Increases Endothelial Cell Number—To study the effects of BMP signalling on embryonic ECs, we utilized an *in vitro* vascular differentiation system from mouse ESCs (*7*). VEGFR2+ endothelial progenitor cells derived from ESCs differentiate predominantly into ECs when cultured in serum-free SFO medium in the presence of VEGF (Fig. 1A). We have previously shown that these MESECs are capable of transducing BMP signals (*34*). When BMP-4 was added to culture, cell number was significantly increased (Fig. 1A).

We also examined whether this effect of BMP-4 can be observed in mature ECs. We chose HMECs because they are capable of transducing both BMP and TGF-β signals as shown by western blot analysis for phosphorylation of R-Smads (Fig. S1), luciferase assays (Fig. S2) and quantitative RT-PCR analyses for target genes of BMP and TGF-β (Fig. S3A, B, respectively). As shown in Fig. 1B, number of HMECs was also significantly increased by the addition of BMP-4. These findings suggest that BMP-4 is capable of inducing proliferation of both embryonic and mature ECs.

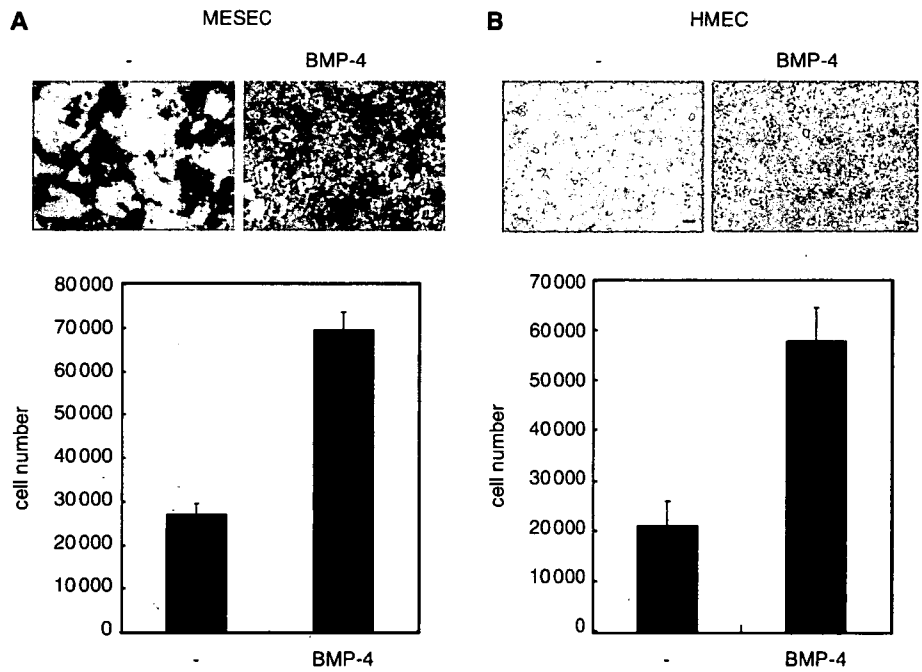

Fig. 1. **Effects of BMP-4 on number of ECs.** (A) VEGFR2+ cells (5×10^4/well) derived from CCE cells were cultured in serum-free SFO medium containing 30 ng/ml of VEGF in the absence or presence of 60 ng/ml of BMP-4, followed by PECAM1 (purple) immunostaining (top) and determination of cell number after 2 days (bottom). (B) HMECs (8×10^3/well) were cultured in serum-free EGM-2 medium in the absence or presence of 10 ng/ml of BMP-4, followed by photography (top) and determination of cell number after 6 days (bottom). Bars: 100 μm. Each value represents the mean of three determinations; Error bars, SD.

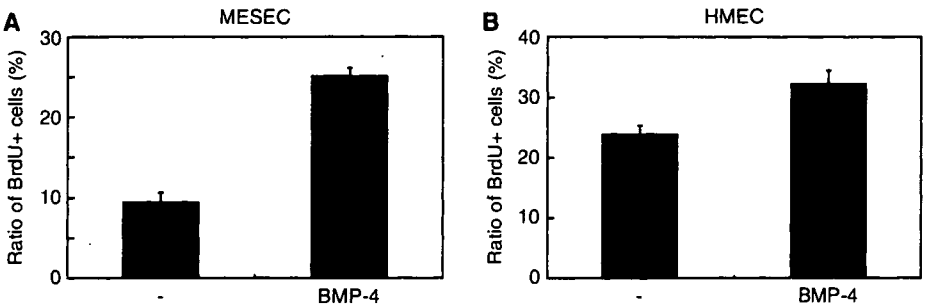

Fig. 2. **Effects of BMP-4 on DNA synthesis in ECs.** BrdU incorporation assays were carried out in MESECs (A) and HMECs (B) as described in MATERIALS AND METHODS. Each value represents the mean of eight determinations; Error bars, SE.

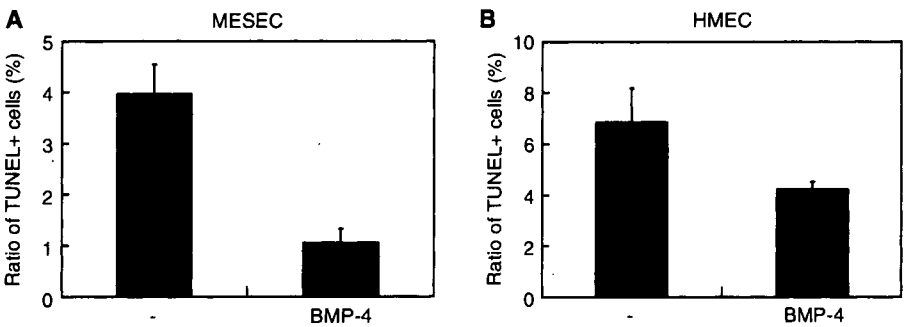

Fig. 3. **Effects of BMP-4 on apoptosis of ECs.** TUNEL assays were carried out in MESECs (A) and HMECs (B) as described in MATERIALS AND METHODS. Each value represents the mean of 3 (MESECs) and 10 (HMECs) determinations; Error bars, SE.

BMP-4 Promotes DNA Synthesis by ECs—Cell numbers are regulated by proliferation and apoptosis. To further examine the effects of BMP-4 on EC proliferation, we quantified the effects of BMP-4 on DNA synthesis in both types of ECs using BrdU incorporation assay. DNA synthesis was increased by BMP-4 in both types of ECs (Fig. 2), though to a more significant extent in MESECs.

BMP-4 Suppresses Apoptosis of ECs—We next examined the effect of BMP-4 on apoptosis of ECs by TUNEL assay. When BMP-4 was added, the ratio of TUNEL-positive cells was decreased in both MESECs and HMECs (Fig. 3). These findings suggest that BMP-4 increases EC numbers by induction of proliferation and inhibition of apoptosis.

BMP-4 Promotes Motility of ECs—Since induction of Id1 expression by BMP-6 is necessary and sufficient for BMP-induced activation of EC migration (*30*), we examined whether BMP-4 promotes the migration of ECs. Tracking of single ECs using video time-lapse microscopy showed that BMP-4 significantly increased the motility of MESECs (Fig. 4A) and HMECs (Fig. 4B). We further examined the effects of BMP-4 on Id1 expression in these ECs. As shown in Fig. 5A and B, BMP-4 significantly induced Id1 expression in MESECs and HMECs. These results provided further support for the previously reported finding that BMP-induced Id1 expression stimulates EC motility.

BMP-4 Induces the Expression and Phosphorylation of VEGFR2 Proteins—We next attempted to identify the mediators of BMP-4 inducing EC proliferation. Since VEGF/VEGFR2 signalling pathways stimulate the proliferation, survival and migration of ECs, we examined the effects of BMP-4 on the expression of VEGF and VEGFR2. As shown in Fig. 5C and D, BMP-4 induced the expression of transcripts for VEGFR2 in MESECs and HMECs. VEGFR2 is activated by phosphorylation of its tyrosine residues. We further examined the effects of BMP-4 on the synthesis and phosphorylation of VEGFR2 proteins by biochemical analyses. BMP-4 increased the levels of protein expression and phosphorylation of VEGFR2 in both MESECs and HMECs (Fig. 6A and B), suggesting that VEGFR2 signalling is activated when BMP-4 was added.

BMP-4 Induces the Expression and Phosphorylation of Tie2 Proteins—Ang-1 and -2 are agonist and antagonist, respectively, for the Tie2 tyrosine kinase receptor, which increases the integrity and inhibits apoptosis of ECs. We therefore studied if Ang/Tie2 signals are regulated by BMP signals in ECs. When BMP-4 was added to culture of both types of ECs, levels of transcripts for Tie2 (Fig. 5E and F) and Ang-2 (Fig. 5G and H) were increased and decreased, respectively, while those for Ang-1 were not altered (data not shown). Levels of Tie2 protein expression and phosphorylation of Tie2 were also increased in both types of ECs upon treatment with

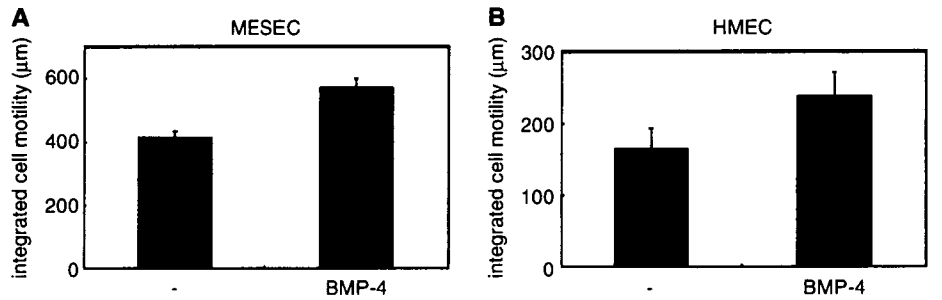

Fig. 4. **Effects of BMP-4 on endothelial cell migration.** Cell migration was measured by video-time lapse microscopy as described in MATERIALS AND METHODS. MESECs (A) and HMECs (B) were subjected to video microscopy for 24 h in the absence and presence of BMP-4. Results are integrated cell motility over 24 h. Each value represents the mean of 10 determinations; Error bars, SE.

BMP-4 (Fig. 6C and D), suggesting that Tie2 signalling is activated by BMP-4 stimuli.

DISCUSSION

While previous *in vitro* studies have shown that BMPs induce EC migration and capillary tube formation (*29*, *30*), the present finding that BMP-4 induces the proliferation of both embryonic and mature ECs (Fig. 1) reveals a novel mechanism by which BMP signalling contributes to vasculogenesis and angiogenesis.

We also found that BMP-4 induced the expression and phosphorylation of VEGFR2 tyrosine kinase (Figs. 5 and 6). When BMP-4-loaded beads are grafted into quail embryo lateral mesoderm, BMP-4 induces the expression of Quek1, the VEGFR2 homologue in quail (*35*), implicating similar molecular mechanisms in quail embryos. BMP-4 also induced the expression of VEGF in MESECs but not in HMECs (data not shown). Since VEGF/VEGFR2 signalling induces the proliferation and migration of ECs, the pro-angiogenic effects of BMP-4 appear to be mediated by activated VEGFR2 signals.

Furthermore, we found a novel relationship between BMP signalling and another pro-angiogenic signal. BMP-4 induced the phosphorylation of Tie2 receptor tyrosine kinase, possibly by induction of Tie2 expression and repression of Ang-2 expression in ECs (Figs. 5 and 6). While expression of Ang-1 was not altered in the ECs examined, BMP-4 induced Ang-1 expression in mouse ESC-derived MCs (data not shown), suggesting another role of BMPs in the activation of Tie2 signalling in blood vessels. Angiopoietin/Tie2 signalling elicits various effects including maturation of endothelial structures and survival of ECs. The roles of Tie2 activation during BMP-4-induced activation of ECs remain to be elucidated. In any case, since VEGF/VEGFR2 signalling is essential for the differentiation and proliferation of ECs, it is unlikely that BMP alone can induce proliferation of ECs in the absence of VEGF.

Vascular development during embryogenesis and adults requires the transition from the resolution to the activation phase and vice versa, which is determined by the balance between positive and negative regulators of ECs. Both pro- and anti-angiogenic properties have been ascribed to TGF-β by genetic studies of various knockout mice deficient for signalling components (*23*). Goumans and colleagues (*36*) showed that TGF-β can activate ALK-5/Smad2, 3 and ALK-1/Smad1, 5, 8 pathways, leading to inhibition and activation of cell migration and proliferation, respectively. We have also studied the roles of TGF-β signalling during endothelial differentiation from ESCs (*34*). TGF-β and activin inhibit EC proliferation, whereas inhibition of endogenous TGF-β and activin signalling by SB-431542, an inhibitor for ALK-4, 5, 7 kinases (*37*), facilitates their proliferation, suggesting that endogenous TGF-β and activin signalling predominantly suppresses embryonic endothelial growth. Taken together with the present finding, these results suggest that the transition between activation and resolution phases of ECs may be regulated by pro-angiogenic TGF-β, BMP/ALK-1, 2, 3, 6/Smad1, 5, 8 signalling and anti-angiogenic TGF-β/ALK-5/Smad2, 3 signalling.

In many types of cells, such as those of human breast and prostate carcinoma cell lines, BMPs induce the expression of $p21^{CIP1/WAF1}$, resulting in hypophosphorylation of pRb protein and growth arrest (*38–40*). During programmed capillary regression in the newborn rat eye, BMPs secreted by the lens induce apoptosis of ECs and inhibit endothelial tubulogenesis (*41*). These contradictory findings may be due to the characteristics of ECs (e.g. the profile of expression of BMP signalling components). Furthermore, other types of BMPs have also been shown to inhibit the growth of ECs. BMP-9 and 10 have been shown to inhibit the proliferation, migration, and tube formation of ECs via activation of ALK-1 (*17*, *18*). The effects of BMP-9 and 10 on the proliferation of MESECs and HMECs need to be elucidated in the future.

This growth-inhibitory effect of BMPs was previously found to participate in the pathogenesis of pulmonary arterial hypertension. Loss-of-function mutations in the BMPR-II gene have been found in patients with pulmonary arterial hypertension, which is characterized by abnormal intimal thickening and muscularization of pulmonary arterioles, which are believed to result from dysregulation of growth of endothelial and smooth muscle cells. While BMP signalling has been shown to inhibit the proliferation of smooth muscle cells (*42*), it has been shown to promote survival of pulmonary

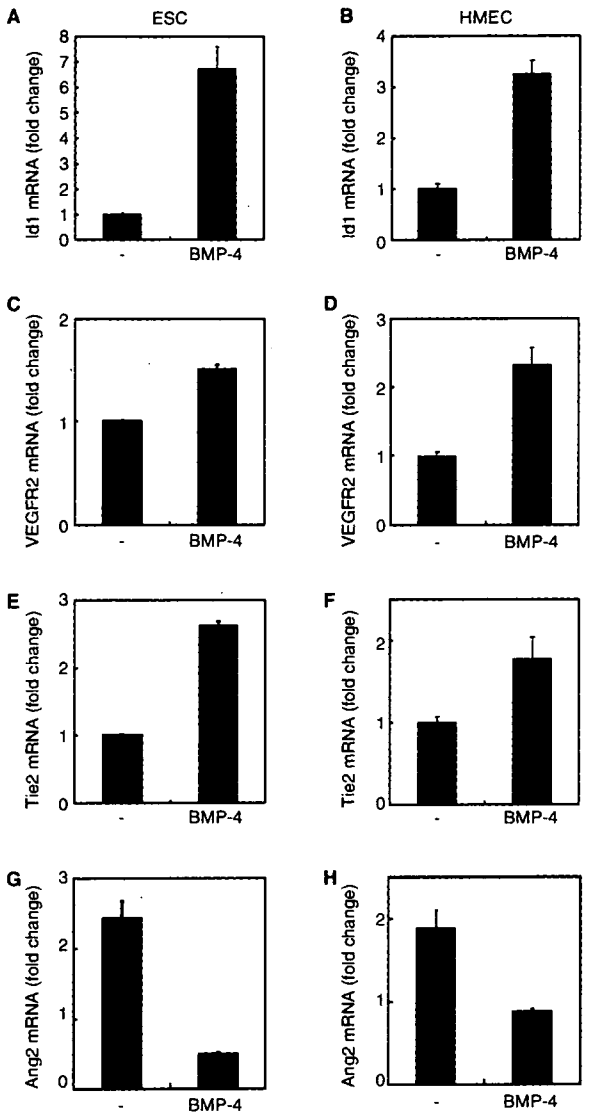

Fig. 5. **Effects of BMP-4 on expression of Id1, VEGFR2, Tie2, and Ang-2 in ECs.** MESECs (A, C, E, G) and HMECs (B, D, F, H) were cultured for 48 h in the absence or presence of BMP-4 and subjected to quantitative RT-PCR analyses for the expression of transcripts for Id1 (A, B), VEGFR2 (C, D), Tie2 (E, F) and Ang-2 (G, H). Quantitated mRNA values were normalized by the amounts of β-actin mRNA, and results are given as fold change. Each value represents the mean of three determinations; Error bars, SD.

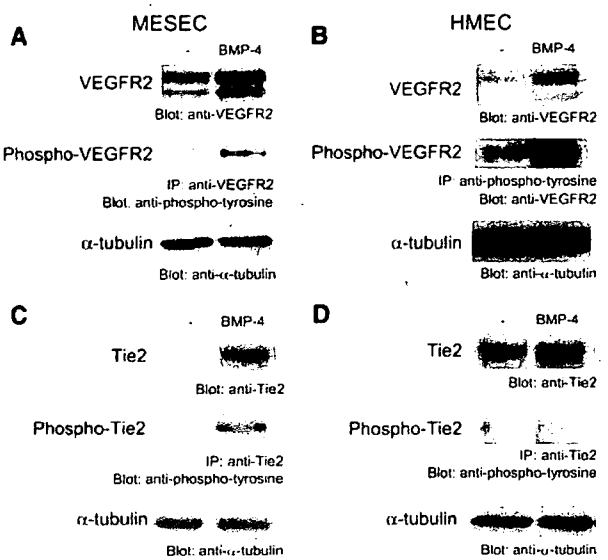

Fig. 6. **Effects of BMP-4 on expression and phosphorylation of VEGFR2 and Tie2 in ECs.** MESECs (A, C) and HMECs (B, D) were cultured for 48 and 24 h, respectively, in the absence or presence of BMP-4. Cell lysates were immunoblotted with anti-VEGFR2 (A, C) and anti-Tie2 (B, D) antibodies to examine the expression of VEGFR2 and Tie2 proteins, respectively (top panel). In order to determine levels of phosphorylation of VEGFR2 and Tie2 proteins, cell lysates were immunoprecipitated by anti-mouse VEGFR2 (A), anti-phospho-tyrosine (B), anti-mouse Tie2 (C) and anti-human Tie2 (D) antibodies, followed by immunoblotting by anti-phospho-tyrosine (A, C, D) and anti-human VEGFR2 (B) antibodies (middle panel). α-tubulin was used as a loading control (bottom panel).

artery ECs (*43*). Furthermore, the combination of apoptosis caused by inhibition of VEGFR2 with chronic hypoxia has been shown to result in selection of an apoptosis-resistant EC phenotype leading to increased proliferation of pulmonary artery ECs (*44*). Therefore, loss-of-BMPR-II-induced apoptosis in ECs may lead to the overproliferation of selected apoptosis-resistant cells, which may initiate the pathogenesis of pulmonary arterial hypertension.

Growth of solid tumours is highly dependent on sufficient blood supply by newly formed blood vessels. Many types of tumours including lung tumours and malignant melanomas express high levels of BMPs (*45*, *46*). In addition to their autocrine effects in promoting cell invasion and migration, a paracrine effect of BMPs on the vascular network has been reported. Under experimental conditions, recombinant BMP-2 produced large increases in size and number of tumour blood vessels, especially in the early phase of tumour growth (*45*, *46*). The findings of the present study suggest the possibility that targeting of BMP signalling may inhibit tumour angiogenesis and lead to tumour regression. Alternatively, activation of BMP signalling in endothelial and/or endothelial progenitor cells may be useful as a therapeutic strategy in regenerative medical treatment of ischaemia and vascular disorders.

Supplementary data are available at *JB* online.

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Activin-Nodal signaling is involved in propagation of mouse embryonic stem cells

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Summary

Embryonic stem (ES) cells are self-renewing cells that maintain pluripotency to differentiate into all types of cells. Because of their potential to provide a variety of tissues for use in regenerative medicine, there is great interest in the identification of growth factors that govern these unique properties of ES cells. However, the signaling pathways controlling ES cell proliferation remain largely unknown. Since transforming growth factor β (TGFβ) superfamily members have been implicated in the processes of early embryogenesis, we investigated their roles in ES cell self-renewal. Inhibition of activin-Nodal-TGFβ signaling by Smad7 or SB-431542 dramatically decreased ES cell proliferation without decreasing ES pluripotency. By contrast, inhibition of bone morphogenetic protein (BMP) signaling by Smad6 did not exhibit such effects, suggesting that activin-Nodal-TGFβ signaling, but not BMP signaling, is indispensable for ES cell propagation. In serum-free culture, supplementation of recombinant activin or Nodal, but not TGFβ or BMP, significantly enhanced ES cell propagation without affecting pluripotency. We also found that activin-Nodal signaling was constitutively activated in an autocrine fashion in serum-free cultured ES cells, and that inhibition of such endogenous signaling by SB-431542 decreased ES cell propagation in serum-free conditions. These findings suggest that endogenously activated autocrine loops of activin-Nodal signaling promote ES cell self-renewal.

Supplementary material available online at
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Key words: Embryonic stem cell, Self-renewal, Propagation, TGFβ superfamily signaling, Activin-Nodal, Serum-free

Introduction

One of the most important characteristics of stem cells is their ability to self-renew. Self-renewal is achieved by suppression of differentiation and stimulation of proliferation. Embryonic stem (ES) cells are self-renewing cells derived from the inner cell mass (ICM) of blastocysts (Niwa, 2001). They have the ability to maintain pluripotency to differentiate into all types of cells of the three germ layers, and are expected to be of great use in regenerative medicine. The signaling instructions that govern these characteristics are provided by growth factors in the stem cell niche microenvironment (Schofield, 1978). Identification of these growth factors and extending such knowledge to control ES cell propagation would improve understanding of the basic biology of ES cells and may yield therapeutic benefits in regenerative medicine. However, the signaling pathways that govern the proliferation of ES cells remain largely unknown.

At present, mouse ES (mES) cells can be propagated in medium containing fetal calf serum (FCS) and cytokine leukemia inhibitory factor (LIF) without the support of feeder cells (Smith et al., 1988; Niwa, 2001). The effect of LIF is mediated through a cell-surface complex composed of LIFRβ and gp130. Upon ligand binding, gp130 activates Janus-associated tyrosine kinases (JAK) and their downstream component signal transducer and activator of transcription (STAT)-3. Although activation of STAT3 is necessary and sufficient for suppression of differentiation of mES cells (Niwa et al., 1998; Matsuda et al., 1999), LIF does not appear to regulate the proliferation of mES cells directly (Raz et al., 1999; Viswanathan et al., 2002). These findings suggest that unidentified growth factors provided by serum or feeder cells and/or in an autocrine fashion by ES cells could contribute to self-renewal of ES cells.

Several lines of evidence suggest that the signaling pathways mediated by the members of the transforming growth factor β (TGFβ) superfamily play important roles in the biology of epiblasts and ES cells. The TGFβ superfamily includes nearly 30 proteins in mammals, e.g. TGFβ, activin, Nodal and bone morphogenetic proteins (BMPs), and its members have a broad array of biological activities. Members of the TGFβ superfamily signal via heteromeric complexes of type I and type II receptors (Heldin et al., 1997). Upon ligand binding,

the constitutively active type II receptor kinase phosphorylates the type I receptor which, in turn, activates intracellular signaling cascades including Smad pathways. Activins, TGFβs and Nodal bind to type I receptors known as activin receptor-like kinase (ALK)-4, ALK-5 and ALK-7, respectively. In addition, Cripto serves as a co-receptor for Nodal in conjunction with ALK-4. The activated type I receptors phosphorylate receptor-regulated Smad proteins (R-Smads). Smad2 and Smad3 transduce signals for TGFβ, activin and Nodal, whereas Smad1, Smad5 and Smad8 are activated by BMP type I receptors (Massague, 1998). Activated R-Smads form complexes with common-partner Smad (Co-Smad, i.e. Smad4), translocate into the nucleus, and regulate the expression of target genes in cooperation with various transcription factors such as those of the FAST-FoxH family. Smad6 and Smad7 are inhibitory Smads (I-Smads) (Imamura et al., 1997; Nakao et al., 1997), and have been reported to exhibit significant differences in the manner of inhibition of TGFβ superfamily signaling. BMP signaling is inhibited by both Smad6 and Smad7, whereas activin-Nodal-TGFβ signaling is more potently inhibited by Smad7 (Hata et al., 1998; Itoh et al., 1998; Hanyu et al., 2001).

Genetic studies have shown that embryos deficient in *Smad4* display defective epiblast proliferation and retarded ICM outgrowth (Sirard et al., 1998), and that *Nodal* null mice display very little *Oct3/4* expression and substantial reduction in size of the epiblast cell population (Conlon et al., 1994; Robertson et al., 2003). Furthermore, large-scale gene profiling of embryonic and adult stem cells has revealed that TGFβ signaling networks are likely to play important roles in maintenance of the unique properties of ES cells (Ramalho-Santos et al., 2002; Ivanova et al., 2002; Brandenberger et al., 2004). Nodal signaling, in particular, has been speculated to be active in undifferentiated human ES (hES) cells, since components of Nodal signals (human orthologs of *Cripto* and *FAST1*) and a target gene (a human homolog of *Lefty2*) are transcriptionally enriched (Brandenberger et al., 2004). Moreover, phosphorylation and the nuclear localization of Smad2/3 were detected in undifferentiated hES cells and shown to play important roles in maintenance of their pluripotency (James et al., 2005). However, the precise roles of Smad2/3 signaling mediated by activin-Nodal-TGFβ in self-renewal of mouse and human ES cells have yet to be elucidated.

In the present study, we investigated the effects of TGFβ superfamily members on mES cell self-renewal. When activin-Nodal-TGFβ signaling was inhibited by Smad7 expression or the specific inhibitor SB-431542, mES cell propagation was dramatically decreased, whereas inhibition of BMP signaling by Smad6 expression did not. In clonal cultures with serum-free medium, supplementation of recombinant Nodal and activin increased the ES cell proliferation ratio with maintenance of the pluripotent state, but supplementation with BMP-4 did not. These findings indicate that Nodal and activin signaling promotes mES cell propagation, and that mES cells themselves produce this activity.

Results

Inhibition of activin-Nodal-TGFβ signaling decreases mES cell proliferation

To study the roles of TGFβ superfamily signaling in the proliferation of ES cells cultured in FCS-containing medium, we first used various natural inhibitors such as Smad6 and 7. We confirmed the specificity of TGFβ superfamily signaling, which is inhibited by Smad6 and Smad7 in MGZ5 ES cells, with luciferase reporter assays using BMP-specific Id-1-luc and activin-Nodal-specific activin responsive elements (ARE)-luc (Chen et al., 1996; Korchynskyi and ten Dijke, 2002). Transient expression of Smad6 inhibited BMP-dependent reporter activity, whereas that of Smad7 inhibited both BMP-dependent and activin-Nodal-TGFβ-dependent reporter activities (Fig. 1A). These results suggested that the pathway specificity of inhibitory Smads is conserved in mES cells, and that BMP and activin-Nodal-TGFβ signaling is autonomously activated in mES cells in FCS-containing medium.

To examine the effects of inhibition of BMP and activin-Nodal-TGFβ signaling on self-renewal of mES cells, we overexpressed mouse *Smad6* or *Smad7* using an episomal vector system, which allows efficient transfection and strong expression of transgenes. *Smad6* and *Smad7* cDNAs were introduced into pCAG-IP supertransfection vector and then transfected into MGZ5 ES cells. Quantitative RT-PCR analysis showed that expression of Smad7 was nine times higher in the Smad7-transfected cells than in the control (supplementary material Fig. S1A). Forced expression of *Smad7* significantly reduced cell number and colony size after culture in FCS-containing medium, whereas that of *Smad6* yielded a smaller reduction of cell number and colony size compared with empty transfectants (Fig. 1B-D). We also calculated the cell number per colony to determine the relative proliferation. Forced expression of *Smad7* decreased the ES cell proliferation ratio by about 75%, whereas that of *Smad6* decreased ES cell proliferation ratio by only 10%, which was not significant (Fig. 1D).

To examine whether the growth-inhibitory effect of Smad7 is due to inhibition of activin-Nodal-TGFβ signaling, we blocked the same signal pathway with SB-431542, a synthetic molecule that inhibits the kinases of receptors for activin-Nodal-TGFβ but not those of BMPs (Laping et al., 2002; Inman et al., 2002). The size of colonies without inhibition of TGFβ signaling was larger than that of colonies of mock transfectants in episomal transfection, possibly because of the absence of drug (puromycin) selection (Fig. 1B and Fig. 2A). Addition of 10 μM SB-431542 to FCS-containing medium significantly inhibited mES cell proliferation (Fig. 2). However, SB-431542 did not reduce the cell number as strongly as forced expression of Smad7, which may inhibit the plating efficiency of mES cells. These results strongly suggest that autonomously activated activin-Nodal-TGFβ signaling, not BMP signaling, contributes to proliferation of mES cells in FCS-containing medium.

The growth-inhibitory effect of Smad7 is reversible

We further examined whether growth inhibition by *Smad7* expression affects the characteristics of mES cells, using a reversible *Smad7* expression system. As shown in Fig. 3A, fSmad7-10$^+$ ES cells were generated by stable integration of the floxed-*Smad7* cDNA transgene into EB3 ES cells. fSmad7-10$^+$ ES cells expressing five times as many *Smad7* transcripts as the control cells (supplementary material Fig. S1B), but not the *DsRed* transgene, were cultured in FCS-containing medium with 1 μg/ml puromycin for 1 month, and then transfected with

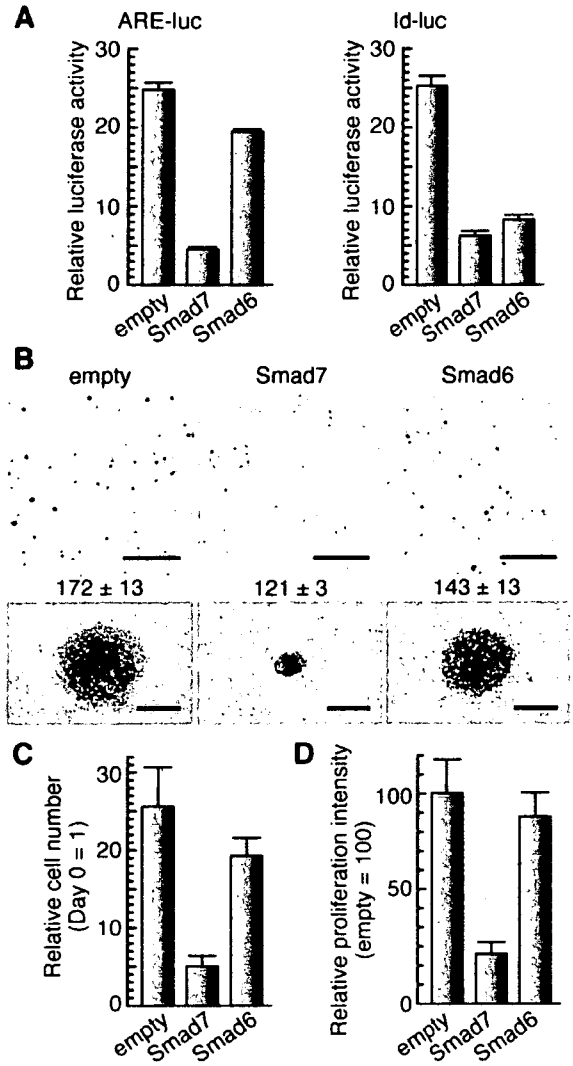

Fig. 1. Forced Smad7 expression inhibited ES cell propagation. (A) Luciferase reporter assay in MGZ5 cells transfected with reporter constructs (ARE-luc reporter or Id-1-luc reporter) and each expression plasmid. Each bar represents the mean ± s.e.m. (n=3). (B) Colony morphologies of supertransfectants. MGZ5 cells were transfected with Smad7-expressing plasmid (middle), Smad6 plasmid (right), or empty vector (left), seeded at 2000 cells/well in six-well plates, and cultured for 1 week in medium supplemented with puromycin. Numbers indicate numbers of colonies appearing (n=3). The lower panel shows AP staining. Bars, 5 mm (upper panels); 200 μm (lower panels). (C) Relative numbers of each transfectant present on Day 7 of culture compared with that at Day 0. Each bar represents the mean ± s.e.m. (n=3). (D) Relative proliferation intensity is shown as total cell number/number of colonies appearing, compared with that of empty vector transfectants (100%). Each bar represents the mean ± s.e.m. (n=3).

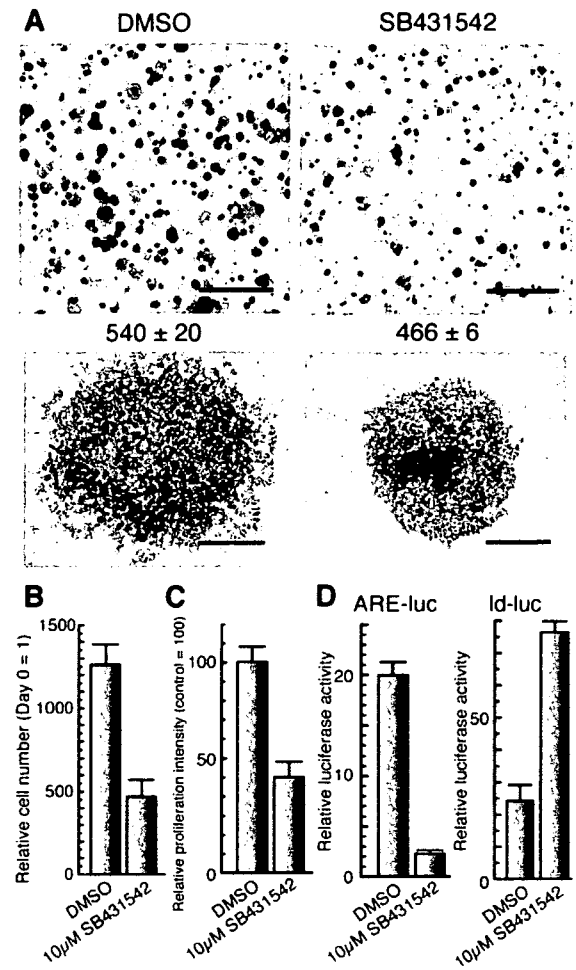

Fig. 2. SB-431542 inhibited ES cell propagation. (A) Colony morphologies of MGZ5 cells treated with SB-431542. MGZ5 cells were seeded at 2000 cells/well in six-well plates and cultured in FCS-containing medium supplemented with 10 μM SB-431542 (right) or DMSO (as control, left) for 1 week. Numbers indicate numbers of colonies appearing (n=3). Each lower panel shows AP staining. Bars, 5 mm (upper panels); 200 μm (lower panels). (B) Relative numbers of cells treated with SB-431542 or DMSO present on Day 7 of culture compared with that at Day 0. Each bar represents the mean ± s.e.m. (n=3). (C) Relative proliferation intensity is shown as total cell number/number of colonies appearing, compared with that of cells treated with DMSO (100%). Each bar represents the mean ± s.e.m. (n=3). (D) Luciferase reporter assay of MGZ5 cells transfected with reporter constructs (ARE-luc reporter or Id-1-luc reporter) and treated with SB-431542 or DMSO (as a control). Each bar represents mean ± s.e.m. (n=3).

pCAGGS-*Cre*. Cre-mediated recombination resulted in the generation of fSmad7-10$^-$ ES cells, in which the *Smad7* transgene had been excised and the *DsRed* transgene activated (Fig. 3A,B). Excision of the *Smad7* transgene was confirmed by RT-PCR analysis (supplementary material Fig. S1B), which showed that the elevated expression of *Smad7* reverted to normal in Smad7-10$^-$ ES cells. We measured the growth ratios of fSmad7-10$^+$, fSmad7-10$^-$ and EB3 ES cells by determining cell numbers at 1, 3 and 5 days after seeding. Proliferation and plating efficiency of fSmad7-10$^+$ ES cells was significantly decreased by expression of *Smad7* transgene, as found with the episomal expression system (Fig. 3C-E). However, upon removal of the *Smad7* transgene, proliferation of fSmad7-10$^-$