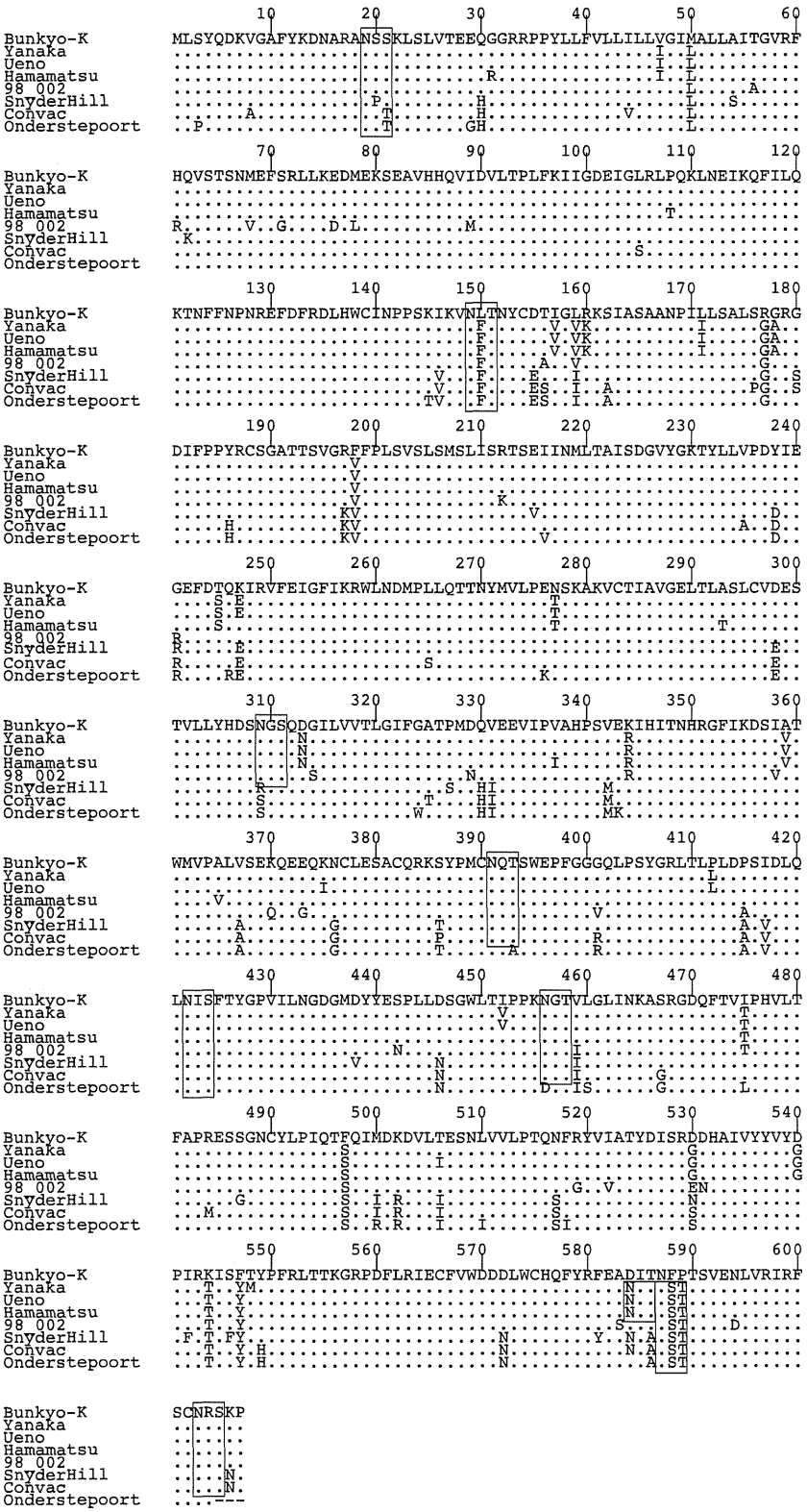


Fig. 3. Alignment of deduced amino acid sequences of the H protein from the Bunkyo-K strain compared with Japanese field isolates (Yanaka, Ueno, Hamamatsu, and 98-002 strains), a well-characterized virulent strain (Snyder Hill), and current vaccine strains (Convac and Onderstepoort). Dots represent amino acid identities. Potential N-linked glycosylation sites are boxed.

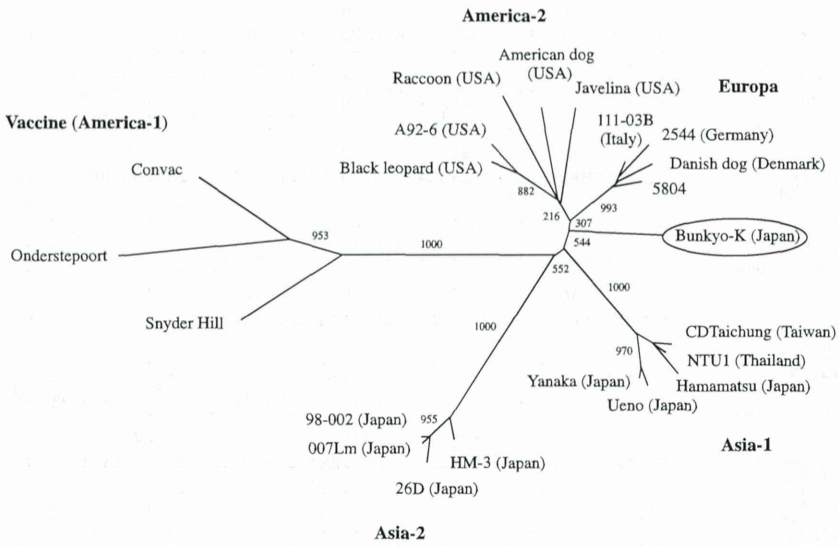


Fig. 4. Phylogenetic analysis of the amino acid sequences of the CDV H proteins. The Bunkyo-K strain is circled.

cluster (which includes the Onderstepoort, Snyder Hill, and Convac strains). Bunkyo-K was 94.9–96.4% identical to four Japanese field isolates (Yanaka, Ueno, Hamamatsu, and 98-002 strains) and higher than the old strain, America-1.

When aligned with other strains (Fig. 3), the predicted amino acid sequence of the Bunkyo-K strain H gene contained seven potential asparagine (N)-linked glycosylation sites at the same positions as the recent Japanese field isolates (Iwatsuki et al., 1997), but lacked tandem two potential glycosylation sites at the C-terminal region.

Interestingly, the Bunkyo-K strain is not grouped in the Asia-1 cluster, which includes the Yanaka strain, or the Asia-2 cluster, which includes several recent Japanese isolates, although it is closely related to these clusters and falls within a major group of recent worldwide prevalent strains (Fig. 4).

3.4. Vaccine efficacy against virulent strain challenge

Based on virulence testing and phylogenetic tree analysis, we speculated that the Yanaka strain could induce neutralizing antibodies and confer protective immunity against former prevalent and recent prevalent CDV strains. To evaluate this hypothesis, we compared the protection efficacy of the Yanaka strain with that of a current vaccine strain, the Onderstepoort, against virulent strains.

First, dogs were subcutaneously inoculated with two doses of 10^4 $TCID_{50}$ of the Yanaka or Onderstepoort strains. Neutralizing antibody titers in sera were analyzed using the respective inoculating viruses. Both strains induced neutralizing antibodies, but titers induced by the Yanaka strain were higher than the Onderstepoort strain (Table 1). Subcutaneously inoculated dogs and two unimmunized dogs were then challenged with the Snyder Hill strain, classified as an old strain similar to the Onderstepoort strain (Fig. 4) and used as a virulent challenge virus for quality control of current CDV vaccines. As summarized in Table 1, unimmunized control animals were leukopenic and developed overt clinical signs, diminished activity and diarrhea at 4–5 dpi, and depression, diarrhea, convulsion and lethargy at 6 dpi in dog 3-1, and depression, convulsion, sialorrhea at 7–8 dpi, and further diarrhea, hematochezia and lethargy at 8 dpi in dog 4-2. Both dogs developed viremia at 7 dpi. However, two dogs immunized with the Onderstepoort strain did not show marked clinical symptoms except a transient and slight fever in one dog. Similarly, two dogs inoculated with the Yanaka strain did not develop clinical signs of canine distemper. Virus in the peripheral blood could not be detected in these

Table 1
Summary of clinical outcomes after challenge with the Snyder Hill strain.

Dog no.	Immunized with	Neutralizing antibodies	Challenged with	Febrile	Leukopenic	Clinical score	Virus in PBMC
3-1	None	<10	SnyderHill	+	+	2,2,4 (4,5,6 dpi)	+ (7 dpi)
3-2		<10		+	–	3,6 (7,8 dpi)	+ (7 dpi)
3-3	Onderstepoort (10^4)[a]	190	SnyderHill	–	–	–	–
3-4		113		±	–	–	–
3-5	Yanaka (10^4)[a]	2076	SnyderHill	–	–	–	–
3-6		3044		–	–	–	–

[a] Three weeks old at first vaccination. Dogs received two vaccine doses.
PBMC, peripheral blood mononuclear cells; dpi, days post-infection.

dogs (Table 1). Thus, the Yanaka strain showed protective effects against the old CDV virulent strain that was equal to the Onderstepoort strain.

In the second experiment, dogs were inoculated with a single dose of the Ondersepoort or Yanaka strain at 10^3 $TCID_{50}$, or with two doses at 10^4 $TCID_{50}$. It was confirmed that the Yanaka strain induced higher levels of neutralizing antibodies than the Onderstepoort strain even with a single low dose (Table 2).

Subcutaneously inoculated dogs and two unimmunized dogs were challenged with the Bunkyo-K strain. The unimmunized dogs developed marked leukopenia and clinical signs, anorexia in two dogs at 11–12 dpi, and further diarrhea and hematochezia in one dog at 12 dpi (Table 2). Virus was detected by RT-PCR from peripheral blood throughout the experimental period (Table 2). The PCR product sequence was identical to that of the Bunkyo-K strain (data not shown). Dogs immunized with a single low dose of the Onderstepoort strain developed no clinical signs or viremia, but developed consecutive leukopenia. Notably, two dogs immunized twice with a high dose of the Onderstepoort strain developed significant neutralizing antibody titers, and developed a mild but transient leukopenia.

In contrast, none of the dogs immunized with the Yanaka strain developed clinical signs including leukopenia. In particular, single immunization with a low dose of the Yanaka strain efficiently induced protective immunity against the Bunkyo-K strain (Table 2). This result indicated the Yanaka strain has stronger protective effect against current prevalent virulent CDVs than the Onderstepoort strain.

3.5. Virus detection and histopathology

None of the dogs immunized with the Onderstepoort or Yanaka strains had histopathological lesions in any of the examined tissues (data not shown). Lesions consistent with CDV infection were observed in all tissues collected from challenged control dogs (Fig. 5A). To confirm virus replication in tissues of the control dogs, tissues were homogenized and serially diluted 10-fold, followed by RT-PCR for the CDV-H gene (Fig. 5B). In a dog inoculated with the Snyder Hill strain, CDV was detected at a 10^3 dilution of the cerebrum and spleen, and above a 10^5 dilution of the mesenteric lymph nodes and popliteal lymph nodes. In a dog inoculated with the Bunkyo-K strain, CDV was detected in a 10^4 dilution of the cerebrum and mesenteric lymph nodes and a 10^3 dilution of popliteal lymph nodes, but was not detected in the spleen. Virus RNA was not detected in urinary bladders from either dog. There is therefore no obvious difference in pathogenicity and tissue tropism between the Snyder Hill strain and the Bunkyo-K strains.

4. Discussion

In the present study, we characterized two recent Japanese isolate strains, the Yanaka strain and Bunkyo-K strain. In the virulence test, the Yanaka strain was avirulent in dogs. In contrast, the Bunkyo-K strain represented a typical disease of canine distemper (Figs. 1 and 2), thus the Bunkyo-K strain should be utilized as a novel virulent strain.

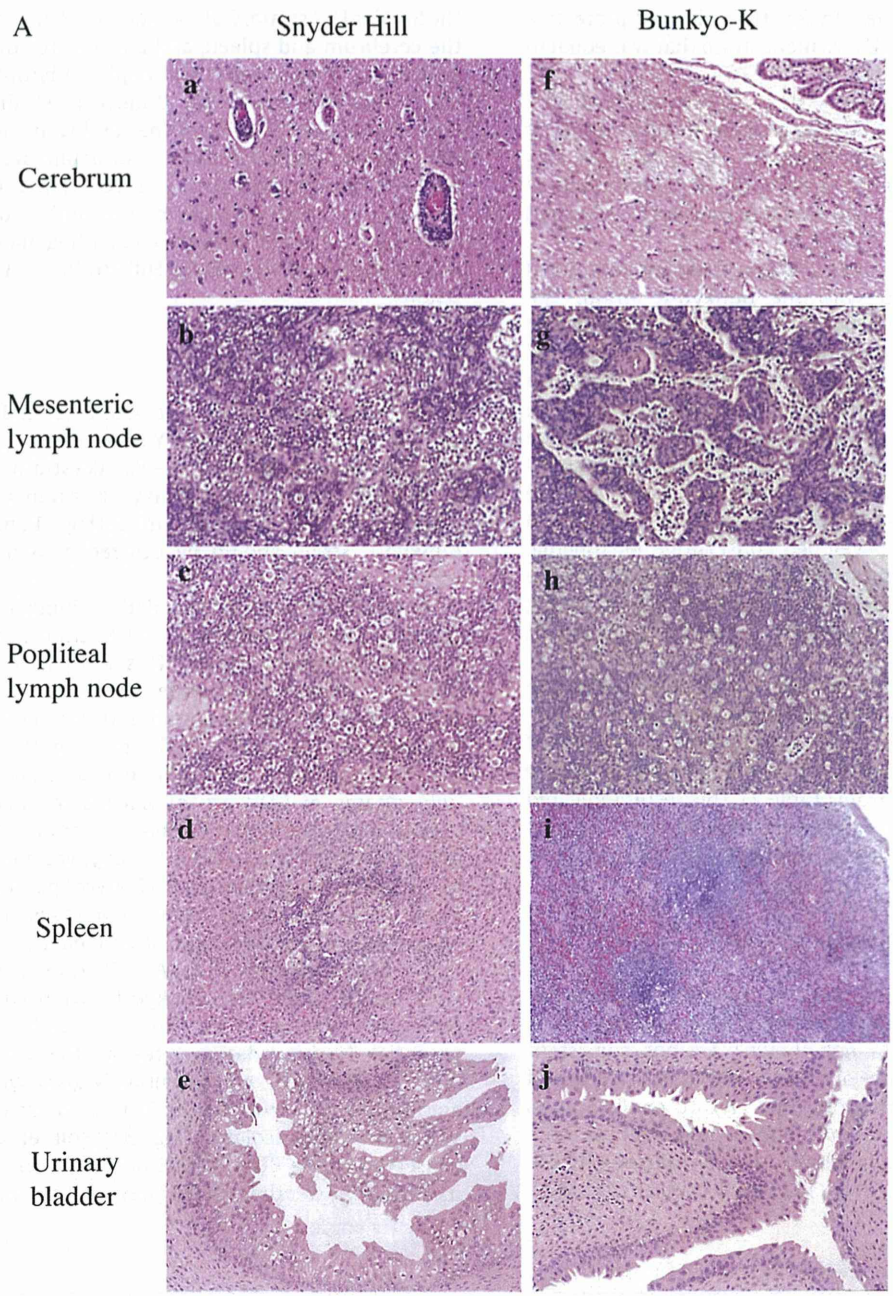
Phylogenetic analysis revealed the Bunkyo-K strain was distinct from the current vaccine strains, grouped with recently prevalent strains. This is consistent with our previous study that revealed a neutralizing antigenic region in the H protein was altered in recent field isolates (Iwatsuki et al., 2000). Differences in the potential N glycosylation site in the H protein between the vaccine and wild strains of CDV are of particular interest. Studies suggest H protein glycosylation site variants play a crucial role in antigenic differences. Usually, vaccine strains have four (Onderstepoort) or seven (Convac) potential glycosylation sites. The Yanaka strain possesses nine N-linked glycosylation sites, similar to other Japanese isolates in the Asia-1 group and close to the Asia-2 group, which has eight sites. Interestingly, the Bunkyo-K strain contains seven potential N-glycosylation sites and lacks two tandem potential N-glycosylation sites at the C-terminal end (Fig. 3). However, one potential N-glycosylation site at amino acid 309, which is unique to most of the recent worldwide field isolates (Fig. 3) (Bolt et al., 1997), is conserved in the Bunkyo-K H protein, further supporting its similarity to other recent prevalent strains.

Table 2
Summary of clinical outcome after challenge with the Bunkyo-K strain.

Dog No.	Immunized with	Neutralizing Ab	Challenged with	Febrile	Leukopenia	Clinical score	Virus in PBMC
3-7	None	ND	Bunkyo-K	–	+	1,1 (11,12 dpi)	+ (4,8,12 dpi)
3-8		ND		–	+	1,3 (11,12 dpi)	+ (4,8,12 dpi)
3-9	Onderstepoort (10^3)[a]	8	Bunkyo-K	–	+	–	–
(3-10)		(Accidental loss)					
3-11	Onderstepoort (10^4)[b]	381	Bunkyo-K	–	+	–	–
3-12		226		–	+	–	–
3-13	Yanaka (10^3)[a]	1076	Bunkyo-K	–	–	–	–
3-14		381		–	–	–	–
3-15	Yanaka (10^4)[b]	2560	Bunkyo-K	–	–	–	–
3-16		4305		–	–	–	–

[a] Four weeks old at vaccination. Single vaccine dose.
[b] Five weeks old at first vaccination. Two vaccine doses.
ND: No data.

A



B

	Cerebrum	Popliteal lymph nodes	Mesenteric lymph nodes	Spleen	Urinary bladder
Snyder Hill	10^3	$10^5<$	$10^5<$	10^3	ND
Bunkyo-K	10^4	10^3	10^4	ND	ND

Fig. 5. Histopathology and virus detection. (A) Representative lesions are shown for each virus (left panels: Snyder Hill, right panels: Bunkyo strain). (a and f) Spongiform demyelination-like lesions, and (a) perivascular infiltration of mononuclear cells in cerebral cortex and white matter. (b, c, g and h) Starry-sky effect, which indicates activation of macrophages and propagation of lymphocytes, and sinus catarrh in lymphoid organs. (d) Mitosis in red pulp and starry-sky lesions, and (i) follicular atrophy and proliferation of megakaryocytes in spleen. (e and j) Hyperplasia of transitional epithelium in urinary bladders. Magnification: i × 100, a–h and j× 200. (B) Tissue RT-PCR for virus detection. ND: not detected.

Differences were also evident when comparing vaccination efficacy. In the vaccine examination, the Yanaka strain produced higher levels of neutralizing antibodies at all doses and vaccination schedules than the Onderstepoort strain, and was completely protective against both the Snyder Hill and Bunkyo-K strains. In nature, the outcome of CDV infection and the severity of clinical signs vary markedly based on strain virulence, the age of the animal, and its immune status. If dogs develop a weak immune response, insufficient vaccination efficacy will increase the probability of infection.

Incomplete protection from CDV by vaccine may also represent a threat to wildlife. For example, CDV epizootics caused mass mortality of Baikal seals in 1988 and Caspian seals in early 2000 (Anonymous, 1989; Kennedy et al., 2000). Inter-species CDV transmission between terrestrial and aquatic mammals is thought to be due to canine populations residing in lake regions. In addition, current prevalent CDV strains in dogs transmitted the infection to wild masked palm civets or raccoon dogs in Japan (Hirama et al., 2004; Machida et al., 1992, 1993; Ohashi et al., 2001; Takayama et al., 2009). Therefore, control of recent prevalent strains of CDV is imperative for control of distemper in wildlife, requiring effective vaccination protocols.

While results of the Yanaka strain as a novel vaccine strain are promising, ideally, vaccine development should be always performed carefully. Indeed, in the 1970s and 1990s, vaccine-related diseases such as post-vaccination encephalitis were reported on several occasions (Appel, 1978; Cornwell et al., 1988; Hartley, 1974; Krakowka et al., 1982; McCandlish et al., 1992). These cases were considered to be caused by virulence reversion and occurred in both egg- and cell-adapted live vaccines. In particular, recovery of the Rockborn vaccine strains from dogs and other carnivores with distemper has been reported (Appel, 1978; Bestetti et al., 1978; Cornwell et al., 1988; Hartley, 1974), and analogous viruses have been found circulating in field cases (Martella et al., 2011). For the development of safer vaccines in the future, reverse genetics to generate recombinant CDV might be useful. Recent reports of CDV and MeV demonstrated that SLAM-blind viruses, with mutations in the H protein amino acid residues corresponding to SLAM-binding, could establish a non-pathogenic infection (Leonard et al., 2010; von Messling et al., 2006). We established a reverse genetics system for the Yanaka strain (Fujita et al., 2007). Furthermore, reverse genetics enables us to generate recombinant CDV expressing foreign genes, which may be useful to generate immunity against CDV and other antigens simultaneously, creating a divalent vaccine.

5. Conclusion

Through animal testing, we demonstrated significant differences in the virulence of recent prevalent strains of CDV. Immunization with the Yanaka strain completely protected dogs from subsequent challenge with either the Snyder Hill or Bunkyo-K strains. As a result, the Yanaka strain is a potential candidate for a novel live attenuated CDV vaccine against old or more recent prevalent strains of CDV.

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ORIGINAL ARTICLE

The pleiotrophin-ALK axis is required for tumorigenicity of glioblastoma stem cells

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Increasing evidence suggests that brain tumors arise from the transformation of neural stem/precursor/progenitor cells. Much current research on human brain tumors is focused on the stem-like properties of glioblastoma. Here we show that anaplastic lymphoma kinase (ALK) and its ligand pleiotrophin are required for the self-renewal and tumorigenicity of glioblastoma stem cells (GSCs). Furthermore, we demonstrate that pleiotrophin is transactivated directly by SOX2, a transcription factor essential for the maintenance of both neural stem cells and GSCs. We speculate that the pleiotrophin-ALK axis may be a promising target for the therapy of glioblastoma.

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Keywords: ALK; cancer stem cell; glioblastoma; kinase; pleiotrophin; SOX2

INTRODUCTION

Glioblastoma is one of the most aggressive human cancers with a median survival of around 1 year.[1] Increasing evidence suggests that glioblastoma may arise from the transformation of neural stem/precursor/progenitor cells.[2] Consistent with this idea, glioblastoma cells cultured in serum-free media, which favor the growth of neural stem cells (NSCs), maintain stem-like properties and tumorigenicity.[3] However, when grown in the presence of serum, they undergo irreversible differentiation and lose their tumorigenicity.[3] This finding raises the possibility that differentiation therapy might be effective for glioblastoma.[4]

Almost all cell proliferative signaling involves phosphotransfer cascades, and accordingly protein kinases have been intensely pursued as drug targets. Indeed, a number of small-molecule inhibitors and antibodies targeting kinases are currently being used for cancer treatment.[5] Therefore, one approach to developing differentiation-inducing therapies for glioblastoma would be to identify kinases that regulate their stem-like properties.

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that is bound by the growth factor pleiotrophin or the closely related midkine.[6,7] ALK was initially discovered as a protein fused to nucleophosmin (NPM) in an anaplastic large cell lymphoma (ALCL). This fusion was shown to cause the ligand-independent autophosphorylation and activation of ALK. Studies using mouse models further showed that this NPM-ALK fusion is a primary driver of oncogenesis in ALCL.[8] Moreover, oncogenic fusions or mutations of ALK have also been described in various other cancers, including inflammatory myofibroblastic tumors, non-small cell lung cancer (NSCLC), diffuse large B-cell lymphoma, squamous cell carcinoma of the esophagus and neuroblastoma.[7] Importantly, the ALK inhibitor crizotinib has recently been approved for the treatment of ALK-positive NSCLC.[9,10] In addition, an anti-ALK antibody has been shown to repress the invasive capacity of the glioblastoma cell line U87 (Stylianou *et al.*[11]).

The Sry-related transcription factor SOX2 was identified as a partner of Oct3/4 in embryonic stem cells (ESCs) and is known to be essential for pluripotent cell development.[12–14] SOX2 is also expressed in NSCs and has an important role in neural development and homeostasis of the adult central nervous system.[14,15] On the other hand, it has also been shown that SOX2 is overexpressed in glioblastoma.[16] Furthermore, it has been reported that knockdown of SOX2 by RNA interference (RNAi) suppresses the tumorigenicity of glioblastoma stem cells (GSCs) xenografted into immunodeficient mice.[17]

In the present study, we show that the pleiotrophin-ALK axis is activated by SOX2 and is required for the self-renewal and tumorigenicity of GSCs.

RESULTS

ALK and its ligand pleiotrophin are required for the self-renewal and stem-like properties of GSCs

We established four GSC lines under serum-free conditions, GB2–5, and found that they are of the proneural type.[18,19] These cell lines exhibited enrichment for GSCs as they maintained sphere-forming ability and expressed high levels of the NSC markers, SOX2 and Nestin, as reported previously[3] (Figure 1). The GB2 cell line possesses the highest tumorigenic activity among our GSC lines, and we used these cells to perform an unbiased kinome-wide RNAi screen. We transfected GB2 cells that had been maintained in serum-free medium with a library of small interference RNAs (siRNAs) that target each of 704 kinases and kinase-related genes and then measured the expression levels of the stem cell markers *CD133*[20] and *Lgr5*.[21] We found 15 kinase genes whose suppression affected *CD133* or *Lgr5* expression, including 4 kinases known to be involved in the proliferation of GSCs[22] (Table 1 and Supplementary Table S1). One of these top 15 genes, ALK was also previously reported to be involved in

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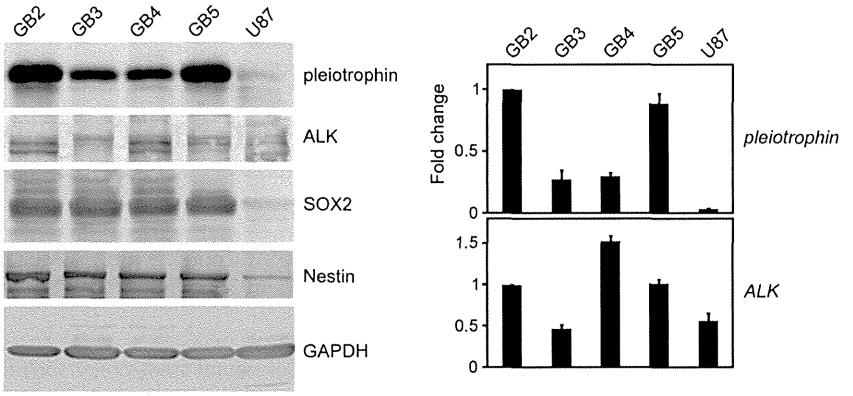

Figure 1. Expression levels of ALK and pleiotrophin in GSCs. GB2–5 cells were maintained in serum-free medium. U87 cells were maintained in serum-containing medium. Cell lysates were subjected to immunoblotting with antibodies to the indicated proteins (left). The mRNA levels of *ALK* or *pleiotrophin* were evaluated by quantitative RT–PCR and shown as the fold change over mRNA levels in GB2 cells (right). Error bars represent the s.d. ($n = 3$).

Table 1. Kinome-wide RNAi screen in GSCs

	Gene symbol	*CD133*	*Lgr5*	*Average*
1	TSSK6	0.55	0.50	0.52
2	GRK4	0.49	0.81	0.65
3	FRK	0.58	0.76	0.67
4	CDC42BPA*	0.45	0.89	0.67
5	PICK1	0.71	0.70	0.71
6	TAL1	0.78	0.64	0.71
7	DVL3	0.80	0.62	0.71
8	PCTK2	0.78	0.65	0.71
9	MARK1	0.70	0.73	0.72
10	OXSR1*	0.75	0.70	0.72
11	FYN*	0.53	0.91	0.72
12	DYRK2	0.76	0.70	0.73
13	MAPKAPK3*	0.68	0.80	0.74
14	**ALK**	**0.69**	**0.80**	**0.75**
15	ETNK2	0.89	0.60	0.75

Abbreviations: GSC, glioblastoma stem cell; RNAi, RNA interference. Note: Average fold changes in the top 15 genes whose knockdown resulted in the greatest change in CD133 and/or Lgr5 expression. ALK data are indicated in bold and the asterisks indicate genes identified in a similar RNAi screen.[22] The entire results are shown in Supplementary Table S1.

glioblastomagenesis.[11,23] However, these studies mainly analyzed the commonly used glioblastoma cell line U87, which does not exhibit any stem-like properties.[3,24] Furthermore, although U87 cells are highly tumorigenic, U87-derived tumors do not show any glioblastoma-specific features.[3,24] We therefore set out to study the role of ALK in the tumorigenicity of GSCs. Lentiviral introduction of a short hairpin RNA (shRNA) targeting ALK resulted in a decrease in both sphere formation and stem cell marker expression (Figures 2a and b). Although the levels of *nestin* mRNA were downregulated, the levels of Nestin protein did not change drastically, probably due to the stability of the Nestin protein in GB2 cells.

We next examined whether the ALK ligand pleiotrophin is required for the stem-like properties of GSCs. We found that pleiotrophin was expressed at high levels in GB2–5 cells compared with U87 cells and mainly as an 18-kDa protein (Figure 1). When pleiotrophin expression was knocked down by shRNA, both sphere formation and expression levels of the stem cell markers examined were suppressed (Figures 2a and b). We found that GSCs infected with a lentivirus expressing an shRNA targeting ALK or pleiotrophin had increased levels of the neural marker mitogen-activated protein kinase 2 (*MAP2*; Supplementary Figure S1A). By contrast, knockdown of either ALK or pleiotrophin resulted in decreased expression of the astrocyte marker glial fibrillary acidic protein (*GFAP*) and the oligodendrocyte marker *Olig2* (Supplementary Figure S1A). We observed that knockdown of either ALK or pleiotrophin did not cause apoptosis (Supplementary Figure S1B) or any drastic morphological change (Supplementary Figure S1C). In addition, knockdown of pleiotrophin resulted in a decrease in ALK protein and mRNA levels, suggesting that pleiotrophin stimulates not only ALK kinase activity but also *ALK* gene expression. These results suggest that ALK and pleiotrophin are important for the self-renewal and stem-like properties of GSCs.

ALK and pleiotrophin activate the Myc and ESC-like transcriptional programs in GSCs

To study the role of the pleiotrophin-ALK axis in GSCs, we investigated the gene expression profiles of GB2 cells in which either ALK or pleiotrophin expression had been suppressed by siRNA. DNA microarray analyses revealed that the MAP kinase, phosphoinositide 3-kinase (PI3-kinase) and Janus kinase/signal transducer and activator of transcription factor (JAK/STAT) pathways are activated in GSCs (Figure 2c and Supplementary Tables S2–S4). Pleiotrophin, but not ALK, was also found to activate the Wnt signaling pathway. We found about a 30% overlap in the genes whose expression was reduced by suppression of ALK and pleiotrophin (Figure 2d and Supplementary Tables S2–S4). Furthermore, we found that downstream target genes of ALK and pleiotrophin overlap with those enriched in ESCs, which are known to be overexpressed in poorly differentiated tumors, including glioblastoma[25] (Figure 2d, upper panel and Supplementary Tables S2–S4). It had been previously reported that the NPM-ALK fusion protein induces Myc expression.[26] We also found that ALK and pleiotrophin target genes overlap those targeted by Myc and its related proteins, the Myc module, which has been reported to account for most of the similarity between ESCs and cancer cells[27] (Figure 2d, lower panel and Supplementary Tables S2–S4). These results suggest that ALK and pleiotrophin may confer a more aggressive oncogenic phenotype to glioblastomas by activating the Myc and ESC-like transcriptional programs.

ALK and pleiotrophin are critical for the tumorigenicity of GSCs

It has been suggested that the stem-like properties of GSCs are indispensable for their tumorigenicity.[3,28] We therefore attempted to clarify the involvement of the pleiotrophin-ALK axis in the tumorigenicity of GSCs. We took GB2 cells containing a

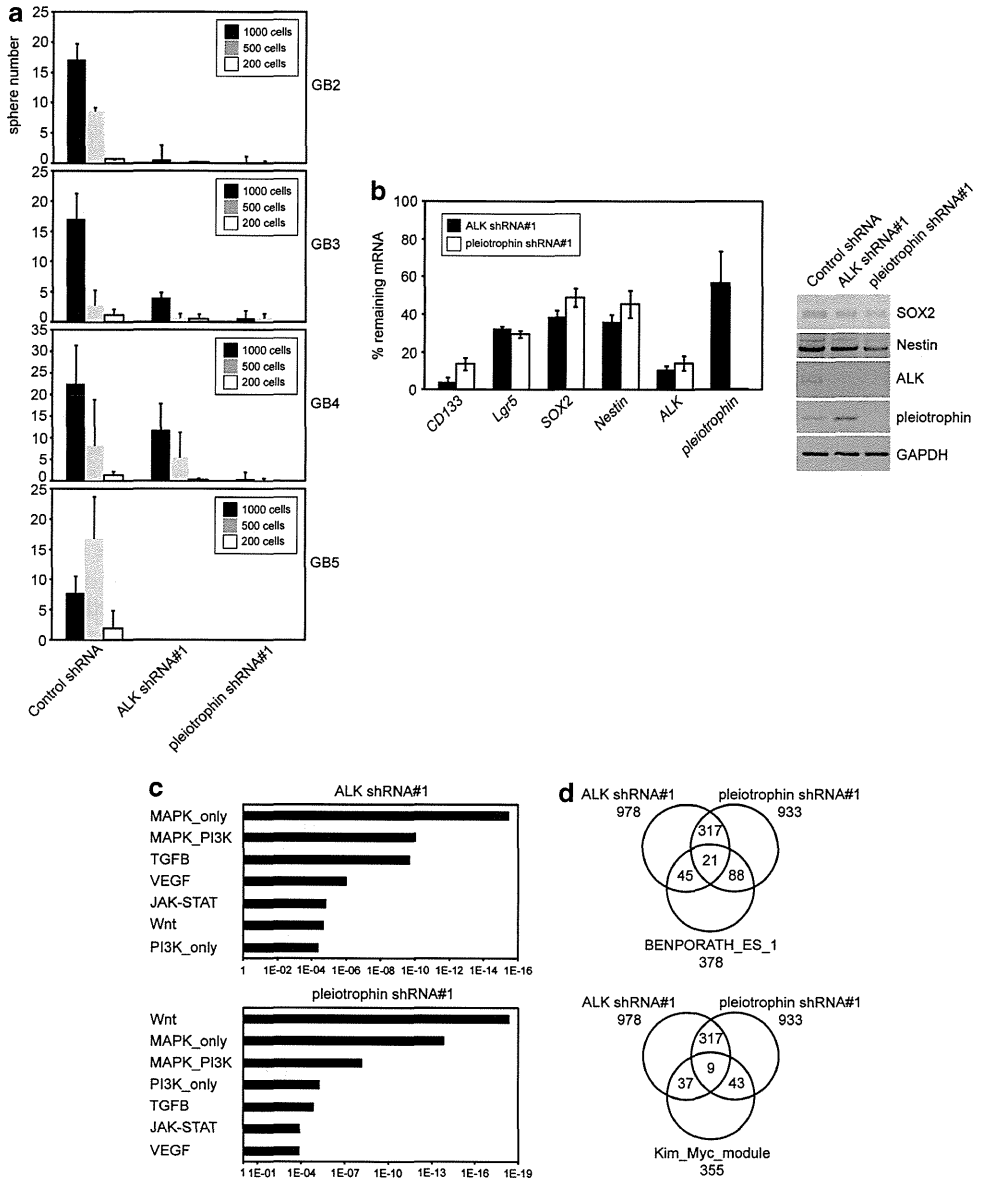

Figure 2. ALK and pleiotrophin are required for the self-renewal and stem-like properties of GSCs. (**a**) GB2–5 cells maintained in serum-free medium were infected with a lentivirus expressing an shRNA-targeting ALK or pleiotrophin. Three days after lentivirus infection, cells were trypsinized and plated at the indicated cell numbers into 96-well tissue culture plates. Two weeks after plating, the number of spheres was counted. Error bars represent the s.d. ($n = 7$–8). (**b**) GB2 cells maintained in serum-free medium were infected with a lentivirus expressing an shRNA-targeting ALK or pleiotrophin. One week after infection, the mRNA levels of the indicated genes were evaluated by quantitative RT–PCR and shown as the percentage of the remaining mRNA compared with cells expressing control shRNA (left). Error bars represent the s.d. ($n = 3$). Cell lysates were subjected to immunoblotting with antibodies to the indicated proteins (right). CD133 and Lgr5 could not be detected by immunoblotting because of their low expression levels. (**c**) GB2 cells maintained in serum-free medium were treated as described in panel (**b**). One week after infection, expression data were generated using HG-U133 plus 2.0 GeneChips. Bar graph represents signaling pathways downregulated by knockdown of ALK (upper) and pleiotrophin (lower). (**d**) Venn diagram showing the overlap among genes downregulated by knockdown of ALK (ALK_signature: ALK shRNA#1), those downregulated by knockdown of pleiotrophin (PTN_signature: pleiotrophin shRNA#1) and those enriched in ESCs (ES signature: BENPORATH_ES_1) (upper) or those targeted by Myc and its related proteins (Myc human: Kim_Myc_module) (lower). The significance of the overlap between each pair of signature is shown in Supplementary Table S4 (hypergeometric *P*-value).

lentivirus-delivered shRNA that stably suppresses ALK or pleiotrophin expression and intracranially transplanted these into immunodeficient mice. Mice receiving the ALK- or pleiotrophin-suppressed cells were found to survive longer than those transplanted with GB2 cells infected with a control lentivirus (Figure 3). Histopathological analysis of the tumor xenografts demonstrated that silencing of either ALK or pleiotrophin inhibited glioblastoma progression, whereas control GB2 cells formed invasive glioblastoma (Supplementary Figure S2). Taken together, these results suggest that the pleiotrophin-ALK axis maintains the tumorigenicity of GSCs.

High expression of pleiotrophin in GSCs

Aberrant activation of ALK by oncogenic fusion or mutation can drive tumorigenesis.[7] However, we could not identify any

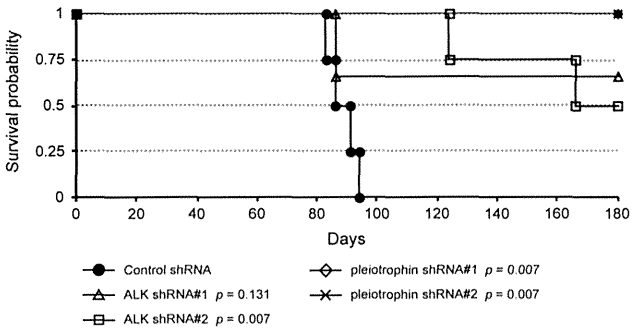

Figure 3. ALK and pleiotrophin are critical for the tumorigenicity of GSCs. GB2 cells maintained in serum-free medium were infected with a lentivirus expressing an shRNA-targeting ALK or pleiotrophin. One week after infection, cells were transplanted into the frontal lobe of immunodeficient mice. *P*-values with comparison to control shRNA by log rank test are shown.

mutation in the *ALK* gene in GB2 cells, consistent with the results of a comprehensive genome analysis reported previously.[29,30] It is known that pleiotrophin is highly expressed in GSCs (Figure 1). We therefore examined the transcriptional control of *pleiotrophin* in GSCs. We found that pleiotrophin protein and mRNA expression levels decreased during serum-induced differentiation, similar to what was observed for the stem cell markers (Figure 4a). Moreover, retinoic acid-induced differentiation also resulted in a decrease in the levels of pleiotrophin[31] (Figure 4b). We also investigated the expression profiles of *pleiotrophin* obtained from a public microarray database.[3] Almost all patient glioblastomas and two GSC lines as well as NSCs expressed substantial levels of *pleiotrophin* (Figure 4c). By contrast, glioma cell lines and two GSC lines cultured in serum-containing medium expressed relatively low levels of *pleiotrophin*, presumably because they had undergone 'differentiation'.[3] These results suggest that high expression of pleiotrophin is a common feature of GSCs.

SOX2 directly transactivates the expression of pleiotrophin in GSCs

To further investigate the mechanisms underlying the expression of *pleiotrophin*, we performed reporter assays using a *luciferase* reporter under the control of the full-length *pleiotrophin* 5′ region (−1401 to +309) and several deletion variants. When transfected into GB2 cells, a reporter containing the region between −251 and +309 showed a substantial level of activity (Figure 5a, left panel). Furthermore, this activity was repressed upon serum-induced differentiation. We therefore attempted to identify the transcription factor(s) involved in modulating this promoter activity. We first searched the region −251 and +309 for transcription factor consensus binding sites and then compared the expression patterns of the selected transcription factors and pleiotrophin using a microarray database.[3] We found that the expression pattern of *pleiotrophin* is similar to that of *SOX2* (Figure 4c). Furthermore, a mutant reporter lacking the SOX2-binding site (−40 to −21) showed reduced promoter activity (Figure 5a, right panel). Chromatin immunoprecipitation analysis demonstrated that endogenous SOX2 was present at the *pleiotrophin* promoter in GSCs (Figure 5b and Supplementary Figure S3A). Consistent with these results, silencing of SOX2 by shRNA resulted in a decrease in the levels of pleiotrophin protein and mRNA (Figure 5c and Supplementary Figure S3B). Similar results were obtained by transfecting two different siRNAs targeting SOX2 (Supplementary Figure S3C). These results suggest that SOX2 stimulates transcription of the *pleiotrophin* gene, thereby maintaining high expression levels of pleiotrophin in GSCs.

DISCUSSION

It has been reported that ALK acquires oncogenic potential when truncated and fused to a partner protein, such as NPM, as can occur via chromosomal rearrangement.[7,8] It has also been reported that ALK is activated by point mutations in its kinase domain in some neuroblastoma.[7,32] Although we could not identify any chromosomal rearrangement or point mutation in ALK in GB2 cells, we found that ALK is highly expressed in GSC lines cultured in serum-free medium, consistent with previous reports.[33] Furthermore, we showed that knockdown of ALK results in a decrease in the self-renewing capacity and tumorigenicity of GB2 cells. We also found that pleiotrophin is overexpressed both in patient glioblastomas and in GSC lines cultured in serum-free medium and that knockdown of pleiotrophin leads to a reduction in the self-renewing capacity and tumorigenicity of GB2 cells. Thus, our results suggest that the pleiotrophin-ALK axis is required for the self-renewal and tumorigenicity of GSCs.

NPM-ALK, the most thoroughly studied ALK-fusion protein, has been reported to signal via the MAP kinase, phospholipase Cγ, PI3-kinase and JAK/STAT pathways.[7] Consistent with these reports, our DNA array analysis revealed that the MAP kinase, PI3-kinase and JAK/STAT pathways are activated in GSCs. Furthermore, we found that ALK and pleiotrophin activate the Myc and ESC-like transcriptional programs, which are known to be associated with more aggressive phenotypes in human cancers.[25,27] Activation of these pathways by ALK has not been previously reported, presumably because earlier studies utilized cultured cell lines grown in serum-containing media that do not exhibit any stem cell-like properties. We found that there was only about a 30% overlap between the genes dependent on ALK and pleiotrophin expression. Furthermore, only pleiotrophin, but not ALK, activated the Wnt signaling pathway. These results appear to be in line with the fact that pleiotrophin signals not only via ALK but also via other receptors, such as RPTPβ/ζ (receptor protein tyrosine phosphatase β/ζ) and N-syndecan.[34,35] Interestingly, pleiotrophin is known to stimulate tyrosine phosphorylation of β-catenin through inactivation of RPTPβ/ζ.[34] Furthermore, pleiotrophin stimulates tumor angiogenesis and remodeling of the microenvironment.[36] Consistent with these findings, we found that repression of pleiotrophin has a more dramatic effect on the tumorigenicity of GSCs than repression of ALK.

It has been reported that SOX2 is involved in the tumorigenesis of several cancers, including lung cancer and breast cancer.[37–39] We found that SOX2 directly transactivates the expression of pleiotrophin in GSCs. Our results appear to be consistent with a previous report showing that knockdown of SOX2 suppresses the tumorigenicity of GSCs in immunodeficient mice.[17] Thus, SOX2-mediated transactivation of pleiotrophin may be important for the tumorigenicity of GSCs. However, knockdown of SOX2 only partially reduced the expression levels of pleiotrophin (Figure 5c and Supplementary Figures 3B and C), and mutation of the SOX2-binding site in the pleiotrophin promoter only partially reduced activity of a luciferase reporter (Figure 5a, right panel). In addition, we found that the levels of pleiotrophin decreased faster than those of SOX2 during serum-induced differentiation (Figure 4a) and that retinoic acid-induced differentiation resulted in a drastic decrease in the expression levels of pleiotrophin but not of SOX2 (Figure 4b). Thus, the pleiotrophin-ALK axis may be regulated by other transcription factors in addition to SOX2.

SOX2 is well known to have critical roles in the maintenance of neural stem and progenitor cells.[14,15] For example, it has been reported that multipotent neural stem-like cells transfected with an siRNA targeting SOX2 express increased neurofilaments but decreased GFAP and Nestin levels.[40] These results suggest that SOX2 inhibits the differentiation of neural stem-like cells into neurons and maintains their stem-like properties. Intriguingly, our results appear to be consistent with these results. We found that