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Platelet-derived growth factor-C functions as a growth factor in mouse embryonic stem cells and human fibrosarcoma cells

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Abstract

Background: Platelet-derived growth factor-C (PDGF-C) has been shown to be involved in several biological processes, such as embryonic development, wound healing and angiogenesis, as well as in diseases including tumor formation and fibrotic diseases. However, its role in fibrosarcoma and embryonic stem (ES) cells has not been elucidated.

Methods: The expression level of PDGF-C was measured using RT-PCR. The activity of PDGF-C was suppressed using RNA interference or a neutralizing antibody and the effect on cell growth was examined using the WST and soft agar assays.

Results: In the tumor cell lines studied, the highest level of PDGF-C expression was in human HT1080 fibrosarcoma cells. In ES cells, it was highly expressed in the self-renewal state but not in the differentiated state. PDGF-C knockdown suppressed anchorage-dependent and -independent growth of HT1080 and ES cells. In addition, the suppression of PDGF-C activity by a neutralizing antibody retarded ES cell growth.

Conclusion: Our results suggest that PDGF-C plays an important role in the proliferation of fibrosarcoma and ES cells.

Keywords: Embryonic stem cells, Cancer, PDGF-C, Fibrosarcoma, Anchorage-independent growth

Background

Embryonic stem (ES) cells, which are derived from pluripotent cells of the early mammalian embryo, can grow without limit in the undifferentiated state. In mouse ES cells, the addition of leukemia inhibitory factor (LIF) to the culture medium enables them to maintain their self-renewal and pluripotency. Several transcription factors, including Oct3/4 and STAT3, play important roles in the self-renewal of ES cells by forming transcription factor networks to stimulate the expression of a set of genes that promote self-renewal in ES cells [1].

Interestingly, ES cells share many biological properties with cancer cells [2]. Both ES and cancer cells can survive and grow in the absence of anchorage, and thus produce tumors when injected into nude mice. Several oncogenic pathways, including the STAT3 and Wnt/β-catenin pathways, play important roles in ES cell self-renewal [3–8]. Moreover, there is an overlap in the expression of certain genes in ES and cancer cells [9].

These similarities indicate that some genes expressed in self-renewing ES cells might possibly play important roles in cancer cell growth. To further evaluate this, we have been searching for genes that are expressed in self-renewing ES cells, but not in differentiated ES cells. We have identified several self-renewal-specific genes, including Zfp57, Gabpα, Eed, Dax1, Sddr/Ooep, Zfp296 and E2f3a [10–16]. Of these, we have demonstrated that Zfp57 can function as a novel oncogene [17].

The results of a preliminary microarray analysis conducted during this search raised the possibility that platelet-derived growth factor-C (PDGF-C) is expressed in self-renewing ES cells. PDGF-C is a member of the PDGF family, which regulates various cellular processes, such as cell proliferation and motility, both under normal physiological conditions and during the pathogenesis of a number of human diseases, including tumorigenesis [18–21].

While PDGF-A and -B are secreted as active hetero- or homo-dimers, PDGF-C and -D are secreted as latent homodimers consisting of an N-terminal CUB domain and a C-terminal growth factor domain (GFD) [18, 21]. Proteases, such as plasmin and tissue plasminogen activator, proteolytically cleave the latent PDGF-C, and thus release the GFD from the CUB domain. The GFD binds to and stimulates its cognate receptor, PDGF receptor alpha (PDGF-Rα) [19, 22]. The GFD of PDGF-C can also activate PDGF-Rβ through the formation of the PDGF-Rαβ heterodimer.

Several studies have suggested that PDGF-C plays an important role in tumorigenesis. For example, oncogenic chimeric protein EWS/FLI stimulated PDGF-C expression in Ewing family tumors [23]. In addition, the expression of a dominant-negative mutant of PDGF-C suppressed anchorage-independent growth in Ewing sarcoma cells [24]. These observations suggest that PDGF-C regulates cell growth in Ewing sarcoma. However, little is known about the role of PDGF-C in the growth of fibrosarcoma, another type of sarcoma. Therefore, we first examined whether PDGF-C promotes fibrosarcoma cell growth. Considering the similarity between ES and cancer cells, we then examined a possible involvement of this growth factor in ES cell growth. Our results suggest that PDGF-C functions as a growth factor of both fibrosarcoma cells and ES cells.

Methods

Cell lines and reagents

The fibrosarcoma cell line HT1080 was obtained from the Health Science Research Resources Bank (HSRRB). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere of 5% CO2. The ES cell lines were E14TG2a (E14), obtained from the American Type Culture Collection (ATCC) and ZHBTc4 [25], donated by Dr. H. Niwa. They were cultured in ES culture medium, as described previously [17]. Goat anti-mouse PDGF-C-neutralizing antibody and control goat IgG were purchased from R&D Systems. Synthesized RNAs were purchased from Operon Biotechnologies: 5'-GCCACAACGUCUAUAUCAUGG-3′ and 5'-AUGAUAUAGACGUUGUGGCUG-3′ were used to knockdown green fluorescent protein (GFP) [26], and 5'-UUGUACUCCG UUCUGUUCCUU-3′ and 5'-GGAACAGAACGGAGUACAAG-3′ were used to knockdown human PDGF-C.

Plasmid construction and establishment of stable cell lines

For knockdown experiments, oligonucleotides containing target sequences for PDGF-C – 5'-GCCAGGTTGTCTCCTGGTTAA-3′ for hPDGFC(1304) and 5'-GTGGAG GAAATTGTGCCTGTT-3′ for mPDGFC(1860) – were introduced into the artificial miRNA expression vector pcDNA6.2-GW/EmGFP miR (Invitrogen) to obtain pGW/EmGFP-hPDGFC(1304) and pGW/EmGFP-mPDGFC(1860), respectively. Similarly, an oligonucleotide containing a target sequence for mPDGFC (5'-TGCATCCACTACA GTATTA-3′) was inserted into pSilencer H1 puro (Thermo Fisher Scientific) to produce pSi-H1p-mPDGFC(469).

An inducible expression vector, pTRE-tTA2p-EmGFP-hPDGFC(1304), was constructed by transferring an EmGFP-hPDGFC miRNA(1304) cassette from pGW/EmGFP-hPDGFC(1304) to pTRE-tTA2p [12]. To establish HT1080/hPDGFC(1304) cells, HT1080 cells were transfected with pTRE-tTA2p-EmGFP-hPDGFC(1304) using Lipofectamine 2000 (Invitrogen) and selected with 0.4 μg/ml puromycin in the presence of doxycycline.

Two plasmids, pCAGIP-EmGFP-control miRNA and pCAGIP-EmGFP-mPDGFC(1860), were constructed by transferring EmGFP-negative control miRNA and EmGFP-mPDGFC(1860) cassettes from pcDNA6.2-GW/EmGFP-miR-neg control (Invitrogen) and pGW/EmGFP-mPDGFC(1860) into pCAG-IP [27], respectively. The construction of pCAG-dnSTAT3-IP, an expression vector for a dominant-negative STAT3, STAT3[Y705F], was described previously [10].

RT-PCR

Total RNA was extracted from individual cultured cells using Sepasol RNA I super G (Nacalai Tesque) or the RNeasy Plus Mini Kit (Qiagen), and cDNA synthesis was performed with ReverTra Ace (Toyobo) or using a high-capacity cDNA reverse transcription kit (Applied Biosystems). After PCR with the primers listed in Additional file 1: Table S1, amplified products were subjected to agarose gel electrophoresis.

WST-1 assay and soft agar assay

The proliferation of HT1080 cells was analyzed using WST-1 Cell Proliferation Reagent (Roche) according to the manufacturer's instructions. In brief, cells were seeded in 96-well microplates and cultured for 3 days. WST-1 reagent was then added to each well, and the number of viable cells was estimated by measuring absorbance at 450 nm.

For the soft agar assay, the appropriate number of cells was seeded in 2.4 ml of culture medium containing 0.4 or 0.5% Sea Plaque agarose (Lonza) and overlaid on 3 ml of culture medium containing 0.53% Sea Plaque agarose in 6-cm petri dishes. Cultures were maintained for approximately 2 weeks. Viable colonies were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Nacalai Tesque), and stained colonies were counted by Image-J (NIH).

Results

PDGF-C is highly expressed in HT1080 cells

We examined the expression level of PDGF-C in the HT1080 fibrosarcoma cell line. When we compared mRNA expression levels of PDGF-C among 6 different tumor cell lines, PDGF-C was the most highly expressed in fibrosarcoma HT1080 cells (Fig. 1a). In addition,

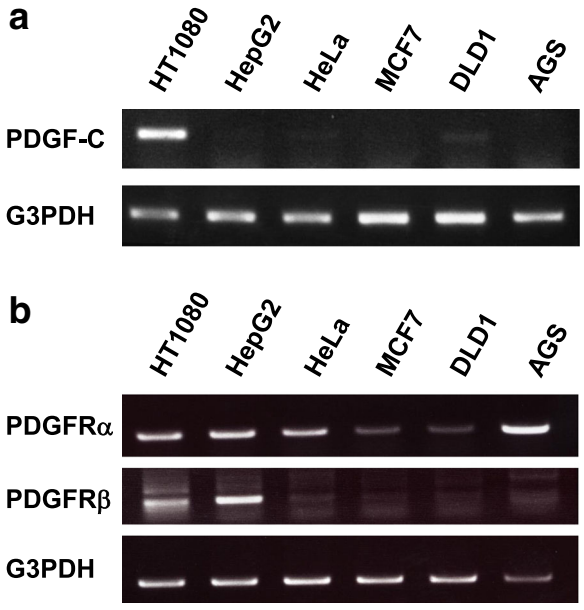

Fig. 1 The expression levels of PDGF-C (**a**) and PDGF receptor (**b**) for six different tumor cell lines: HT1080 (fibrosarcoma), HepG2 (hepatocellular carcinoma), HeLa (cervical carcinoma), MCF7 (breast carcinoma), DLD1 (colon carcinoma), and AGS (stomach carcinoma). In each case, total RNA was prepared and subjected to RT-PCR. Glycerol-3-phosphate dehydrogenase (G3PDH) was used as a loading control, and the results are representative of three independent experiments

as reported previously [28], we confirmed that HT1080 cells express both PDGF-Rα and β (Fig. 1b). Furthermore, the growth of HT1080 cells was significantly suppressed when exposed to 20 μM of the chemical inhibitor AG1296, which inhibits the kinase activity of PDGF-Rα and β (Fig. 2a, Additional file 1: Figure S2a). These observations suggest that PDGF-C plays an important role in HT1080 cells.

PDGF-C is required for anchorage-dependent and -independent HT1080 cell growth

We examined the requirement of PDGF-C for cell growth in HT1080 cells through knockdown experiments with artificial micro RNA (miRNA) against PDGF-C. We introduced an inducible expression plasmid carrying PDGF-C miRNA into HT1080 cells and established a transfectant, which we designated HT1080/hPDGFC(1304) (Additional file 1: Figure S1). In this transfectant cell line, the expression of PDGF-C can be maintained by the addition of doxycycline (Dox) to the culture medium. When we cultured HT1080/hPDGFC(1304) cells in the absence of Dox, PDGF-C expression was knocked down (Additional file 1: Figure S1c) and the growth of this transfectant was suppressed, although not significantly (Fig. 2b, Additional file 1: Figure S2b). When we performed another knockdown experiment using small interfering RNA (siRNA) against PDGF-C, PDGF-C knockdown significantly reduced the growth (Fig. 2c, Additional file 1: Figure S2c, S3). These results suggest that PDGF-C is required for the growth of HT1080 cells.

Unlike normal cells, transformed cells can grow in the absence of anchorage. We therefore examined whether PDGF-C is also required for anchorage-independent growth of HT1080 cells. PDGF-C knockdown induced by Dox removal suppressed the

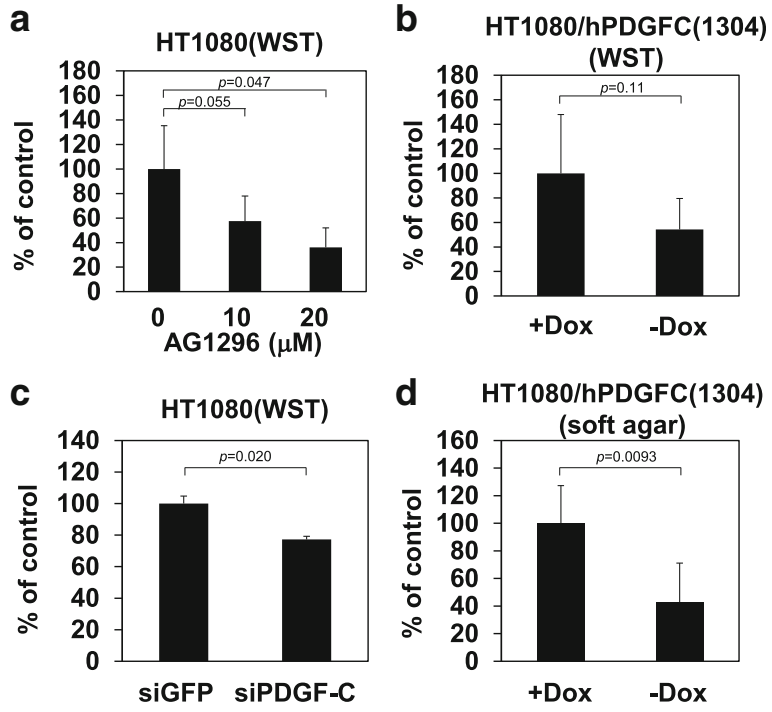

Fig. 2 The inhibition of PDGF-C results in HT1080 cell growth retardation. **a** The PDGF-R inhibitor suppresses the growth of HT1080 cells. HT1080 cells were cultured for 3 days with the indicated concentrations of AG1296 and subjected to the WST assay. **b and c** PDGF-C knockdown reduces the growth of HT1080 cells. **b** HT1080/hPDGFC(1304) cells were treated with (+) or without (–) doxycycline (Dox) for 3 days and subjected to the WST assay. Notably, the expression of PDGF-C was repressed in the absence of doxycycline in HT1080/hPDGFC(1304) cells. **c** HT1080 cells were transfected with 500 nM of PDGF-C-specific siRNA (siPDGF-C) or negative control siRNA (siGFP), and subjected to the WST assay after a 3-day culture. **d** PDGF-C knockdown inhibits anchorage-independent growth of HT1080 cells. HT1080/hPDGFC(1304) cells were subjected to the soft agar assay with or without Dox for 2 weeks. In all the experiments, the average result for the control samples was set to 100%, and the bars and error bars respectively represent the means and standard deviations ($n = 3$). Statistical analysis was performed using the two-tailed paired t test. Statistical significance is defined as $p < 0.05$. The raw data are shown in Additional file 1: Figure S2

anchorage-independent growth of HT1080/hPDGFC(1304) cells in soft agar (Fig. 2d, Additional file 1: Figure S2d). These results suggest that PDGF-C is involved in both anchorage-dependent and -independent growth of HT1080 cells.

PDGF-C is expressed in self-renewing ES cells and downregulated during ES cell differentiation

Our preliminary analysis suggested that PDGF-C is also expressed in self-renewing ES cells. We therefore examined the possible involvement of PDGF-C in ES cell growth. First, we confirmed that PDGF-C is expressed in self-renewing ES cells. E14 ES cells were cultured with or without LIF, and the expression levels of PDGF-C and the self-renewal markers Oct3/4 and Rex1 were examined. RT-PCR analyses revealed that PDGF-C is highly expressed in LIF-treated self-renewing ES cells and that its expression level decreases upon ES cell differentiation induced by LIF depletion (Fig. 3a).

Similar results were obtained when ES cell differentiation was induced through Oct3/4 depletion and STAT3 inhibition. In the Oct3/4 conditional knockout ES cell line ZHBTc4

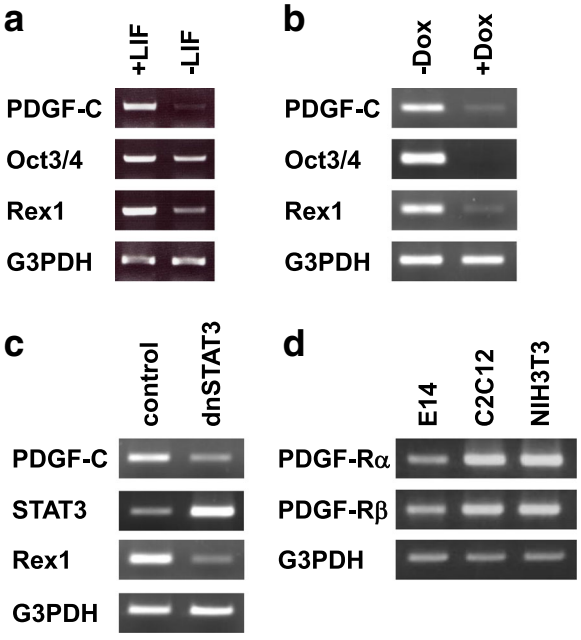

Fig. 3 PDGF-C is expressed in self-renewing ES cells and downregulated during ES cell differentiation. **a** Reduction of PDGF-C mRNA expression by LIF depletion. After culture with LIF for 3 days (+LIF) or without LIF for 6 days (–LIF), the expression levels of PDGF-C, Oct3/4 and Rex1 in the E14 ES cells were examined using RT-PCR. **b** The reduction in PDGF-C mRNA expression by Oct3/4 depletion. ZHBTc4 ES cells were cultured with (+) or without (–) doxycycline (Dox) for 2 days. Expression of the indicated genes was examined by RT-PCR. **c** Reduction of PDGF-C mRNA expression by the expression of a dominant-negative mutant of STAT3. After transfection with pCAG-IP (control) or pCAG-dnSTAT3-IP (dnSTAT3), E14 ES cells were cultured with puromycin for 2 days. Expression of the indicated genes was examined using RT-PCR. **d** Expression of PDGF receptors in ES cells. Total RNA was prepared from E14 ES cells, C2C12 myoblasts and NIH3T3 fibroblasts and subjected to RT-PCR. In all experiments, the results shown are representative of three independent experiments

[25], Dox-stimulated Oct3/4 repression led to a dramatic decrease in PDGF-C expression (Fig. 3b). Overexpression of a dominant-negative mutant of STAT3 in E14 ES cells also resulted in reduced expression levels of PDGF-C (Fig. 3c).

We also observed that both PDGF-Rα and β are expressed in E14 ES cells, although at lower levels than those in myoblasts and fibroblasts (Fig. 3d). Furthermore, the cell growth of ES cells showed a tendency to decrease when these receptors were inhibited by AG1296 (Fig. 4a, Additional file 1: Figure S4a). These results indicate that the expression of PDGF-C is restricted to undifferentiated ES cells and raise the possibility that PDGF-C functions as a growth factor in ES cells.

PDGF-C is required for anchorage-dependent and -independent growth of ES cells

We also examined the effect of PDGF-C knockdown on anchorage-dependent ES cell growth. When artificial miRNA and short-hairpin RNA (shRNA) against PDGF-C were expressed in E14 ES cells, their growth was reduced (Fig. 4b, Additional file 1: Figure S4b). Using RT-PCR, we confirmed that both miRNA and shRNA suppress the expression of endogenous PDGF-C mRNA (Additional file 1: Figure S5). Next, we inhibited PDGF-C activity using an anti-PDGF-C neutralizing antibody. Treatment with the neutralizing antibody led to the suppression of E14 ES cell growth (Fig. 4c, Additional file 1: Figure S4c). These results suggest that PDGF-C is involved in anchorage-dependent growth of ES cells.

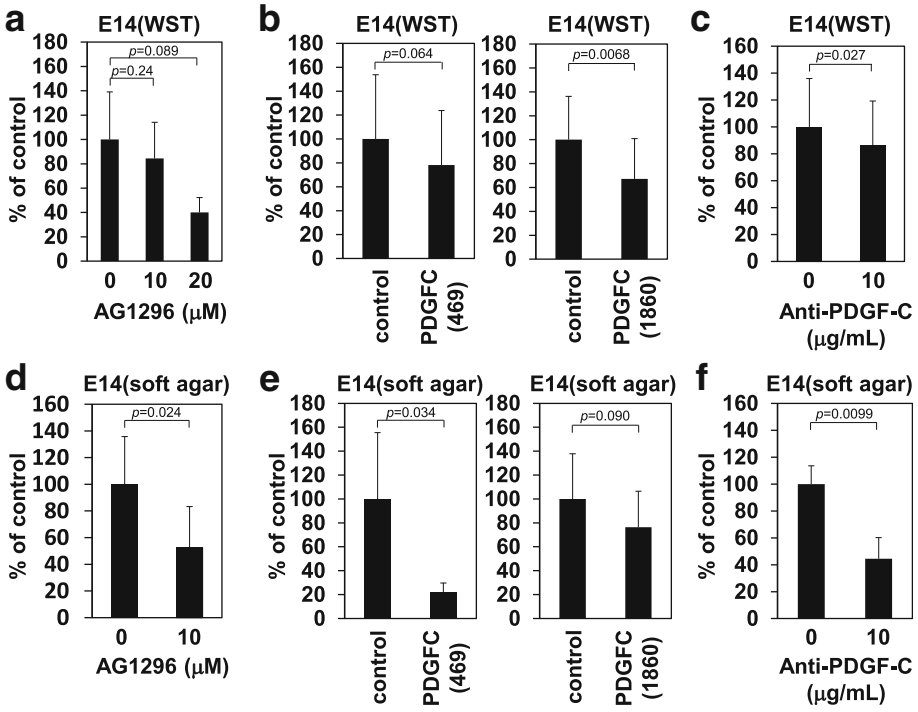

Fig. 4 PDGF-C is involved in ES cell growth. **a** PDGF-R inhibitor suppresses the growth of ES cells. E14 ES cells were cultured for 3 days with the indicated concentrations of AG1296 and subjected to the WST assay. **b** Knockdown of PDGF-C inhibits growth in ES cells. E14 ES cells were transfected with pSi-H1p-control shRNA or pSi-H1p-mPDGFC(469) (left panel) or with pCAGIP-EmGFP-control miRNA or pCAGIP-EmGFP-mPDGFC(1860) (right panel), and their growth was examined using the WST assay after 3-day culture. **c** Anti-PDGF-C neutralizing antibody reduces ES cell growth. E14 ES cells were cultured for 3 days in the presence or absence of 10 μg/ml neutralizing antibody against PDGF-C and subjected to the WST assay. **d** Treatment with PDGF-R inhibitor decreases soft agar growth of ES cells. E14 ES cells were subjected to the soft agar assay with or without 10 μM AG1296. **e** Knockdown of PDGF-C inhibits the growth of ES cells in soft agar. E14 ES cells were transfected with pSi-H1p-mPDGFC(469) (left panel) or pCAGIP-EmGFP-mPDGFC(1860) (right panel) and subjected to the soft agar assay for 1 week. **f** Neutralization of PDGF-C suppresses anchorage-independent growth of ES cells. E14 ES cells were subjected to the soft agar assay in the presence or absence of 10 μg/ml PDGF-C-neutralizing antibody. In all experiments, the average result for the control samples was set to 100%. The bars and error bars represent the means and standard deviations, respectively ($n = 3$, except the left panel of Fig. 4e, where $n = 4$). Statistical analysis was performed using two-tailed paired t test. Statistical significance is defined as $p < 0.05$. The raw data are shown in Additional file 1: Figure S4

ES cells can grow under anchorage-independent conditions [17, 29], as expected since they can give rise to benign tumors called teratomas when injected into immunodeficient mice [30]. The PDGF-R inhibitor AG1296 suppressed anchorage-independent growth of E14 ES cells in soft agar (Fig. 4d, Additional file 1: Figure S4d). Thus, we explored the possibility that PDGF-C is also involved in anchorage-independent ES cell growth. PDGF-C knockdown suppressed the growth of E14 ES cells in soft agar (Fig. 4e, Additional file 1: Figure S4e). Similarly, the soft agar growth of E14 ES cells decreased when PDGF-C activity was inhibited by the presence of the PDGF-C neutralizing antibody (Fig. 4f, Additional file 1: Figure S4f). These results suggest that PDGF-C plays an essential role in anchorage-dependent and -independent ES cell growth.

Discussion

PDGF-C autocrine signaling has been suggested for the growth of several tumor cells. Overexpression of this growth factor has been observed in brain tumors, colorectal tumors and rhabdomyosarcoma [31–33]. In colorectal tumors, PDGF-C overexpression is significantly associated with worse overall and relapse-free survival [31]. The enforced expression of PDGF-C has also been shown to promote the growth of breast cancer cells [34]. In this study, we demonstrated that PDGF-C promotes the growth of fibrosarcoma cells and ES cells.

In contrast to PDGF-A and -B, which are processed intracellularly, PDGF-C is secreted as a latent growth factor, and it requires proteolytic removal of its N-terminal CUB domain for its activation [18–21]. Two serine proteases, plasmin and tissue plasminogen activator (tPA), can remove the CUB domain of PDGF-C [19, 22].

In primary mouse fibroblasts, tPA is endogenously expressed and activates PDGF-C expressed by the same cells, forming a tPA-mediated growth stimulatory loop [22]. Similarly, fibrosarcoma HT1080 cells have been shown to express both plasmin and tPA [35], suggesting that PDGF-C is activated by these two proteases in HT1080 cells. On the other hand, the expression and activity of tPA and urokinase-type plasminogen activator (uPA) are almost undetectable in ES cells [36]. Since plasmin is converted from plasminogen by either tPA or uPA, these observations suggest that ES cells lack both plasmin and tPA. Therefore, other proteases may be involved in PDGF-C activation in ES cells.

It is well-established that PDGF-C transmits its signal by binding to and activating PDGF-R. Our results using a PDGF-R inhibitor support this concept. Well-known downstream signaling pathways of this receptor are the PI3 kinase(PI3K)/Akt and the Ras/Erk pathways. Both play a critical role in cell growth and survival. In HT1080 fibrosarcoma cells, a decrease in Ras signaling due to the loss of an activated mutation in the N-*ras* gene reduced their anchorage-independent growth, and this reduction was restored through the ectopic expression of activated MEK [37]. By contrast, the overexpression of PTEN, an inhibitor of the PI3K/Akt pathway, has shown no effect on the cells' ability to form colonies in soft agar [28]. These results suggest that PDGF-C activates the Ras/Erk pathway, but not the PI3K/Akt pathway, to promote HT1080 cell growth.

In ES cells, the activation of the Ras/Erk pathway induces ES cell differentiation [27, 38], which leads to the suppression of cell growth, while the activation of Akt promotes ES cell self-renewal [39]. Therefore, in contrast to HT1080, the PI3K/Akt pathway may be a major downstream pathway of PDGF-C for the promotion of ES cell growth.

In this study, we used AG1296 as an inhibitor of PDGF receptors. This compound has been shown to inhibit c-kit, a close relative of the PDGF receptor [40]. HT1080 cells have been shown to highly express c-kit, probably through downregulation of miR-152 [41]. Knockout of c-kit in ES cells resulted in reduced growth rates, probably through the loss of spontaneously differentiating cells [42]. It is possible that c-kit or its ligand, stem cell factor, may also play a role in the growth of fibrosarcoma and ES cells.

PDGF-C promoted both anchorage-dependent and anchorage-independent growth of ES cells. This suggests that PDGF-C promotes the formation of teratoma when ES cells are transplanted. Because ES cells and induced pluripotent stem (iPS) cells have similar gene expression profiles, it is highly likely that PDGF-C is expressed in iPS cells. Considering the transforming potential of this growth factor, it is possible that high

expression of PDGF-C in iPS cells increases the risk of tumor formation during cell therapy using iPS-derived cells.

LIF is a well-known factor that maintains self-renewal and promotes cell growth in mouse ES cells. Several other factors have been implicated in mouse ES cell growth and self-renewal, such as Wnt3a, which promotes the latter [5–7]. The hedgehog family seems to promote the former: Gli, a downstream molecule of hedgehog signaling, has been shown to be involved in ES cell growth [43, 44]. In addition, adrenocorticotropic hormone (ACTH) has been shown to promote the propagation of ES cells [45].

This study adds PDGF-C to the list of these growth factors for mouse ES cells. Considering that human ES cells are thought to be powerful materials for regenerative medicine, it would be interesting to examine whether PDGF-C can also promote the growth of human ES cells.

Conclusions

Our results show that PDGF-C is highly expressed in self-renewing mouse ES cells and in human fibrosarcoma cells. Suppression of PDGF-C results in growth retardation of both cell types. These findings indicate that PDGF-C plays an important role in the proliferation of fibrosarcoma and ES cells. Although further studies are needed, our results shed light on the potential application of PDGF-C in regenerative medicine that uses ES cells, as well as on the potential of this growth factor as a therapeutic target for fibrosarcoma.

Additional file

Additional file 1: Table S1. List of primers used for RT-PCR. **Figure S1.** Establishment of the HT1080 transfectant that expresses PDGF-C miRNA in a doxycycline-dependent manner. **Figure S2.** Inhibition of PDGF-C results in HT1080 cell growth retardation. **Figure S3.** Confirmation of PDGF-C knockdown by siRNA in HT1080 cells. **Figure S4.** PDGF-C is involved in ES cell growth. **Figure S5.** Confirmation of PDGF-C knockdown by shRNA (left panel) and miRNA (right panel) in E14 ES cells. (PDF 5629 kb)

Abbreviations

Dox: Doxycycline; ES: Embryonic stem; iPS: induced pluripotent stem; LIF: Leukemia inhibitory factor; miRNA: micro RNA; PDGF: Platelet-derived growth factor; PDGF-R: PDGF receptor; PI3K: PI3 kinase; shRNA: short-hairpin RNA; siRNA: small interfering RNA; tPA: tissue plasminogen activator

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Availability of data and materials

The data sets generated and/or analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

TK, CS, TI, TI and HK performed the experiments and analyzed the data. TK, YT, TA, TY and HK developed the concept of the project. TK and HK were involved in writing the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

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