Integrative genomic analyses of *CXCR4*: Transcriptional regulation of *CXCR4* based on TGFß, Nodal, Activin signaling and POU5F1, FOXA2, FOXC2, FOXH1, SOX17, and GFI1 transcription factors

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Abstract. CXCR4, CD133, CD44 and ABCG2 are representative transmembrane proteins expressed on the surfaces of normal and/or cancer stem cells. CXCR4 is co-expressed with POU5F1 in endodermal precursors and adult-tissue stem cells. *CXCR4* is expressed in a variety of human tumors, such as breast cancer, prostate cancer, pancreatic cancer, and gastric cancer. CXCR4 is a G protein-coupled receptor (GPCR) for CXCL12 (SDF1) chemokine, and the CXCL12-CXCR4 signaling axis is involved in proliferation, survival, migration, and homing of cancer cells. Integrative genomic analyses of *CXCR4* gene were carried out to elucidate the mechanisms of *CXCR4* expression in stem cells, because CXCR4 is a key molecule occupying the crossroads of oncology, immunology, gerontology and regenerative medicine. Human *CXCR4* promoter region with binding sites for HIF1α, ETS1, NF-κB and GLI was not conserved in mouse and rat *Cxcr4* orthologs. Proximal enhancer region with palindromic Smad-binding sites, FOX-binding site, POU-binding site, triple SOX17-binding sites, bHLH-binding site, TCF/LEF-binding site, and double GFI1-binding sites was almost completely conserved among human, chimpanzee, mouse, and rat *CXCR4* orthologs. TGFß, Nodal, and Activin signals induce *CXCR4* upregulation based on Smad2/3 and FOX family members, such as FOXA2, FOXC2, and FOXH1. *CXCR4* is expressed in endodermal precursors due to the existence of triple SOX17-binding sites around the POU-binding site instead of the POU5F1-SOX2 joint motif. Because CXCR4 is downregulated by p53-GFI1 signaling axis, p53 mutation in cancer stem cells leads to CXCR4 upregulation. CXCR4 is also upregulated by TGFß and Hedgehog signals in tumor cells at the invasion front. Small molecule compound or human antibody targeted to CXCR4 will be applied for cancer therapeutics focusing on cancer stem cells at the primary lesion as well as metastasis or recurrence niches, such as bone marrow and peritoneal cavity.

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Introduction

Normal stem cells, characterized by self-renewal and multiple lineage-generating potentials, are involved in fetal-tissue morphogenesis and adult-tissue homeostasis (1-3). Epigenetic changes and genetic alterations in normal stem cells or progenitor cells give rise to cancer stem cells, which are involved in a various steps during carcinogenesis (4-6).

CD133 (7,8), CD44 (9,10), ABCG2 (11,12), and CXCR4 (13,14) are representative transmembrane proteins expressed on the surface of normal and/or cancer stem cells, although some are also expressed on differentiated cells. Because stem cell markers are expressed in similar but distinct domains, a combinatory approach using multiple stem-cell markers is useful to enrich stem cells from normal or cancerous tissues.

CXCR4, functioning as a receptor of CXCL12 (SDF1) chemokine and a co-receptor of human immunodeficiency virus (HIV), is a G protein-coupled receptor (GPCR) belonging to the γ-group of the Rhodopsin family (13,15-17). CXCL12 signaling through CXCR4 inhibits the cAMP-PKA signaling cascade via Gi family of proteins, and activates the SRC-RAS-ERK, PI3K-AKT, NF-κB, IP3-Ca^{2+}, and RHO-ROCK signaling cascades (18-20). CXCL12-CXCR4 signaling axis is involved in the regulation of cellular proliferation, survival, migration, and homing of cancer cells to bone, lung, and peritoneal cavity.

CXCR4 is expressed in a variety of human tumors, such as breast cancer (21), colorectal cancer (22), lung cancer (23), prostate cancer (24), bladder cancer (25), pancreatic cancer (26), gastric cancer (22,27-29), esophageal cancer (22,30), glioblastoma (31), basal cell carcinoma (32), acute myelogenous leukemia (33), and B-cell leukemia/lymphoma (34). Because CXCR4 is a key molecule occupying the crossroads

of oncology, immunology, gerontology, and regenerative medicine, integrative genomic analyses of *CXCR4* gene were carried out in this study to elucidate the mechanisms of CXCR4 expression on stem cells.

Materials and methods

Comparative genomic analyses. Human genome sequences corresponding to human CXCR4 RefSeq (NM_003467.2) were searched for by using BLAST programs, as previously described (35,36). *CXCR4* expressed sequence tags (ESTs) were searched for to identify *CXCR4* splicing variants, and also to determine the putative transcription start site (TSS) (37,38). Conserved transcription factor-binding sites within the regulatory regions of the *CXCR4* gene were then searched for based on manual inspection, as previously described (39,40).

Regulatory network analyses. Literature on TGFß/Nodal/Activin, WNT, Hedgehog, and receptor tyrosine kinase (RTK) signaling molecules, POU family transcription factors, FOX family transcription factors, and SOX family transcription factors in PubMed databases were critically evaluated to extract knowledge on the Smad, TCF/LEF, GLI, POU, FOX, and SOX target genes. The mechanisms of *CXCR4* transcription were then investigated based on our data of conserved transcription factor-binding sites within the regulatory regions of the *CXCR4* gene and in-house knowledgebase of transcription factors regulated by the stem-cell signaling network (41,42).

Results

CXCR4 splicing variants. BLAST programs using NM_003467.2 RefSeq as a query sequence revealed that human *CXCR4* gene is located within human genome sequence AC068492.2 (Fig. 1). Human *CXCR4* gene was found consisting of two exons as previously reported by Caruz *et al* (43). BLAST programs using the *CXCR4* genome sequence as a query sequence next revealed multiple human ESTs derived from the *CXCR4* gene (data not shown). Most *CXCR4* ESTs corresponded to the NM_003467.2 RefSeq with splicing out of intron 1, while a few *CXCR4* ESTs corresponded to NM_001008540.1 RefSeq transcribed by using alternative promoter located at the 3'-portion of intron 1. In addition, several ESTs corresponded to another type of *CXCR4* transcript with retained intron 1. Among three types of splicing variants mentioned above, the *CXCR4* splicing variant corresponding to the NM_003467.2 RefSeq was the major *CXCR4* transcript.

Comparative genomics on mammalian CXCR4 orthologs. Chimpanzee *CXCR4* gene, mouse *Cxcr4* gene, and rat *Cxcr4* gene are located within NW_001232106.1, AC161170.7, and AC122097.4 genome sequences, respectively. Comparative genomic analyses of mammalian *CXCR4* orthologs revealed that the proximal promoter region 5'-adjacent to the exon 1 of human CXCR4 gene was not well conserved, and that the 5'-proximal enhancer region (nucleotide position -2317 to -1993 of TSS) and the intronic enhancer region (nucleotide position +987 to +1551 of TSS) were well conserved (Fig. 1).

Conserved transcription factor-binding sites within CXCR4 regulatory regions. Although it has been reported that binding sites for HIF1α, ETS1, NF-κB and GLI were located within the proximal promoter region, 5'-adjacent to the first exon of human *CXCR4* gene (44-47), the proximal promoter region was not well conserved among mammals (Fig. 1). To elucidate the general mechanism for *CXCR4* transcription, we then searched for evolutionarily conserved transcription factor-binding sites within the proximal and intronic enhancer regions.

Based on manual inspection, we identified palindromic Smad-binding sites, basic helix-loop-helix (bHLH)-binding site, TCF/LEF-binding site, FOX-binding site, POU-binding site, triple SOX17-binding sites, and double GFI1-binding sites within the proximal enhancer region of human *CXCR4* gene, and also double Smad-binding sites, double bHLH-binding sites, double FOX-binding sites, and TCF/LEF-binding site within the intronic enhancer region of human *CXCR4* gene (Fig. 1). These transcription-binding sites were almost completely conserved among human, chimpanzee, mouse, and rat *CXCR4* orthologs (Fig. 1).

TGFß/Nodal/Activin signals involved in CXCR4 upregulation. TGFß, Nodal, and Activin signals are transduced through receptor-type serine/threonine kinases to phosphorylate R-Smads (Smad2 or Smad3) (48,49), and then phosphorylated R-Smads are associated with Smad4 to regulate TGFß/Nodal/Activin target genes in corporation with other DNA-binding proteins, such as FOX and TCF/LEF transcription factors (50,51). Jiang *et al* reported CXCR4 upregulation by using Activin and sodium butylate in human embryonic stem (ES) cells (52). Takenaga *et al* reported Nodal-induced Cxcr4 upregulation in mouse ES cells (53). Javelaud *et al* reported that *CXCR4, PTHLH (PTHrH), IL11*, and *Osteopontin (OPN)* are TGFß-target genes (54). These reports indicate that the TGFß/Nodal/Activin signals are involved in *CXCR4* upregulation; however, precise mechanisms remained to be elucidated.

We identified that Smad-, TCF/LEF- and FOX-binding sites within the regulatory regions of human *CXCR4* gene were conserved among mammalian *CXCR4* orthologs as mentioned above (Fig. 1). In addition, the FOX-binding site in the proximal enhancer region was consistent with more stringent criteria for FOXH1-binding site. FOXH1 is involved in the transcriptional co-regulation of Nodal-target genes during embryogenesis (50). Co-existence of conserved Smad-binding sites with FOX- and TCF/LEF-binding sites within the regulatory regions of *CXCR4* orthologs (Fig. 1) strongly supports the *CXCR4* transcription based on cross-talk of TGFß/Nodal/Activin signals with FOX or TCF/LEF transcription factors.

Indirect CXCR4 downregulation by p53. Mehta *et al* reported that wild-type but not mutant-type p53 represses CXCR4 expression (55). Because precise mechanisms remained unclear, we next investigated the mechanism of p53-mediated *CXCR4* downregulation. p53-binding site was not identified around or within the human *CXCR4* gene (data not shown), which suggest that p53 might indirectly downregulate *CXCR4* transcription.

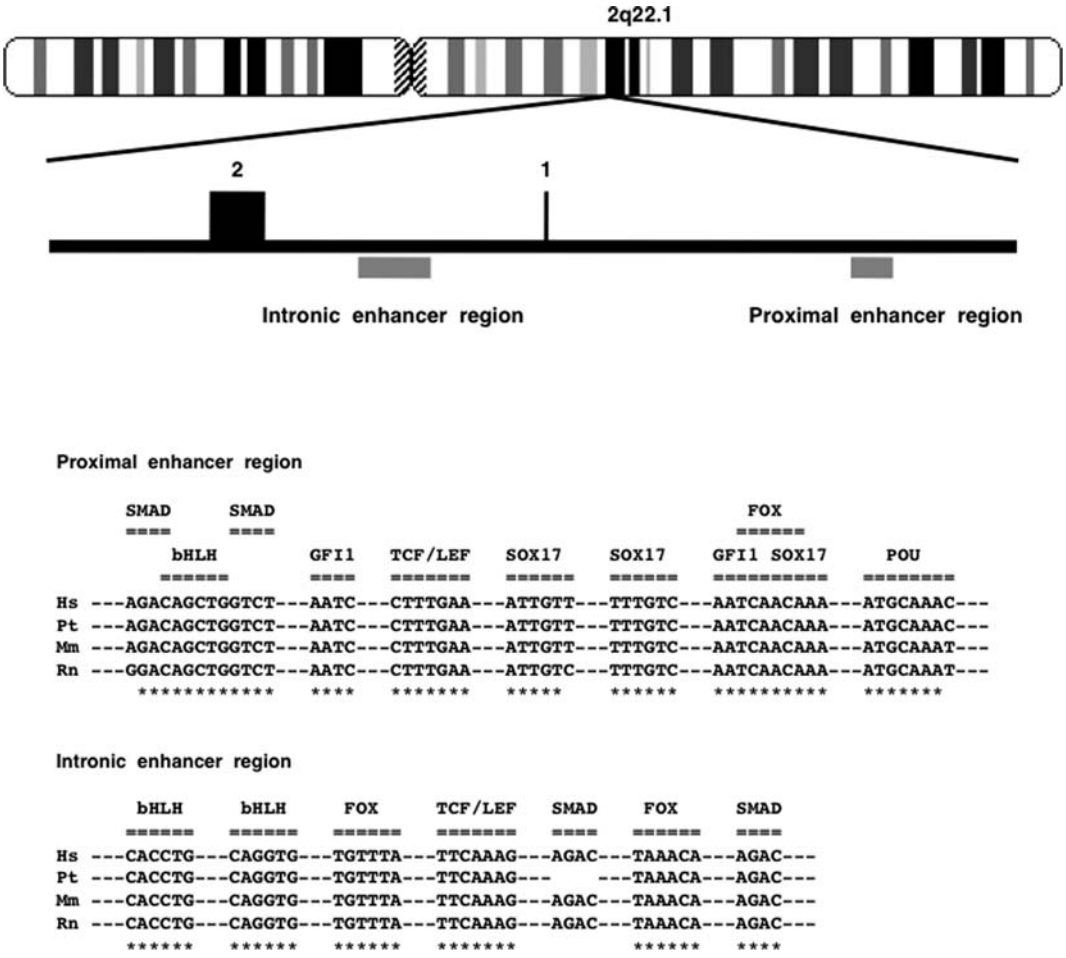

Figure 1. Integrative genomic analyses of *CXCR4*. Schematic representation of *CXCR4* gene at human chromosome 2q22.1 is shown in the upper part. *CXCR4* gene encodes three splicing variants. The major *CXCR4* isoform consists of two exons. Conserved transcription factor-binding sites within *CXCR4* regulatory regions are shown in the lower part. Hs, human; Pt, chimpanzee; Mm, mouse; Rn, rat.

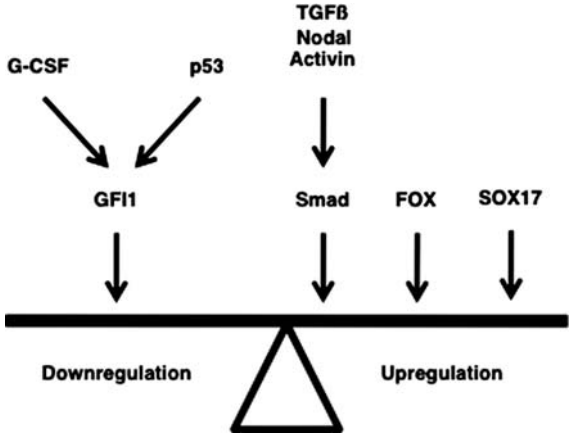

Figure 2. Transcriptional regulation of *CXCR4*. TGFß, Nodal, and Activin signaling, FOX, and SOX17 transcription factors are involved in transcriptional upregulation of *CXCR4*. G-CSF signaling and p53 are involved in transcriptional downregulation of *CXCR4* via GFI1 transcriptional repressor.

p53 is known to upregulate target genes, such as GFI1 (56) and Notch1 (57,58). GFI1 is a SNAG-domain transcriptional repressor (59,60), while Notch1 is a transmembrane receptor inducing HES/HEY family transcriptional repressors (61,62). GFI1-binding sites rather than HES/HEY-binding N-box were identified within the proximal enhancer region of human *CXCR4* gene (Fig. 1). Based on the conserved GFI1-binding sites within the proximal enhancer region, it was predicted that that p53 induces *CXCR4* downregulation indirectly via GFI1 (Fig. 2).

Discussion

Integrative genomic analyses of *CXCR4* were carried out to elucidate the mechanisms of *CXCR4* expression. We searched for evolutionarily conserved enhancer regions, and identified proximal and intronic enhancer regions of *CXCR4* orthologs (Fig. 1). Palindromic Smad-binding sites, FOX-binding site, POU-binding site, triple SOX17-binding sites, bHLH-binding site, TCF/LEF-binding site, and double GFI1-binding sites within the proximal enhancer region and double Smad-binding sites, double bHLH-binding sites, double FOX-binding sites, and TCF/LEF-binding site within the intronic enhancer region were almost completely conserved among human, chimpanzee, mouse, and rat *CXCR4* orthologs (Fig. 1).

Conservation of Smad- and FOX-binding sites within the proximal enhancer region of mammalian *CXCR4* orthologs

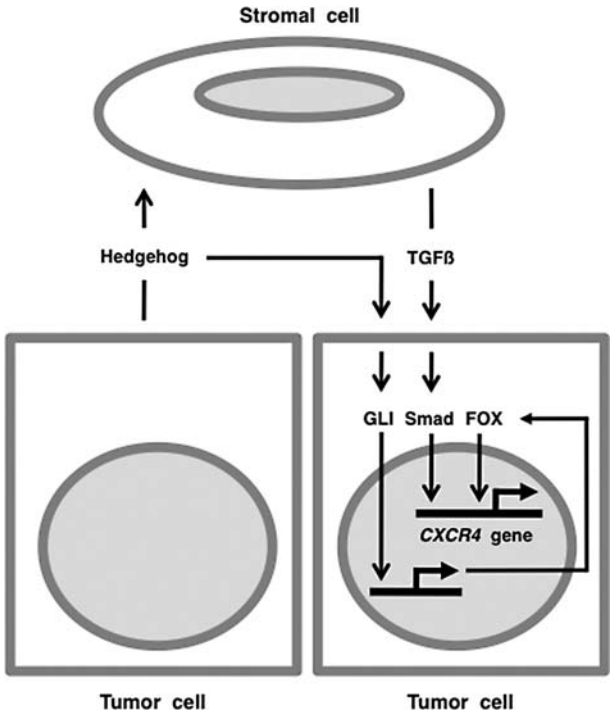

Figure 3. Tumor-stromal interaction involved in CXCR4 upregulation. Tumor cells secrete Hedgehog family ligands, while stromal cells secrete TGFß and SDF1. Paracrine TGFß signal and autocrine Hedgehog signal induce *CXCR4* upregulation in tumor cells at the invasion front.

(Fig. 1) was the most exiting finding in this study. TGFß signaling synergizes with FOXC2 to induce epithelial-to-mesenchymal transition (EMT) during carcinogenesis (63,64). Nodal signaling synergizes with FOXH1 during early embryogenesis (65). Activin signaling synergizes with FOXA2 during endodermal induction (66). Together these facts indicate that TGFß/Nodal/Activin signaling to Smad2 or Smad3 induces *CXCR4* upregulation in cooperation with FOX family members, such as FOXA2, FOXC2, and FOXH1 (Fig. 2).

Conserved POU- and SOX17-binding sites within the proximal enhancer region of mammalian *CXCR4* orthologs (Fig. 1) was another exiting finding in this study. CXCR4 and POU5F1 are co-expressed in ES cells with endodermal differentiation, and also in adult-tissue stem cells (66,67). *SOX17* and *CXCR4* are preferentially expressed in ES cells differentiated to endodermal precursors (66,68-70), and POU-binding site within the proximal enhancer region of mammalian *CXCR4* orthologs was surrounded by triple SOX17-binding sites (Fig. 1). On the other hand, POU-binding site within the regulatory region of *POU5F1, SOX2, NANOG* and *FGF4* genes, preferentially expressed in undifferentiated ES cells, is linked to SOX2-binding site (71,72). *CXCR4* is expressed in endodermal precursors rather than undifferentiated ES cells due to the lack of POU5F1-SOX2 joint motif and the existence of the triple SOX17-binding sites around the POU-binding site.

CXCR4 transcription is repressed by wild-type p53 (55), whereas p53-binding site was not identified around or within the human *CXCR4* gene (data not shown). Because p53 upregulates GFI1 in hematopoietic stem cells (56), double GFI1-binding sites within the proximal enhancer region of mammalian *CXCR4* orthologs (Fig. 1) indicate that p53 downregulates *CXCR4* indirectly via GFI1 induction. CXCR4 is also downregulated by G-CSF signaling to promote the release of hematopoietic stem cells and granulocytic cells in the bone marrow via GFI1 induction (73). p53 is a tumor suppressor gene, which is frequently inactivated in a variety of human cancer (74,75). CXCR4 is downregulated by p53-GFI1 signaling axis in physiological conditions (Fig. 2), whereas CXCR4 is upregulated in cancer stem cells with p53 mutation.

Hedgehog and TGFß signals induce EMT to promote gastrulation and neurulation during embryogenesis as well as invasion and metastasis during multi-stage carcinogenesis (76-80). Hedgehog signaling through Patched receptor and Smoothened signal transducer induces transcriptional activation of Hedgehog target genes via GLI activators (80-83). Yoon *et al* reported *CXCR4* upregulation in medulloblastoma with Hedgehog signaling activation (47). Although two putative GLI-binding sites with one base substitution were identified within the proximal promoter region of human *CXCR4* (-212 and -179 positions from TSS), these sites were not conserved in mouse *Cxcr4* proximal promoter (data not shown). Because Hedgehog signals induce upregulation of FOX family members and TGFß signaling activation (80), Hedgehog signals indirectly upregulate *CXCR4* via FOX and Smad transcription factors. Based on these facts, the following model was proposed: Cancer cells secrete Hedgehog family ligands, and stromal cells at the invasion front secrete TGFß and SDF1. Paracrine TGFß signal and autocrine Hedgehog signal induce *CXCR4* upregulation in tumor cells at the invasion front, and then paracrine SFD1 signal induces CXCR4 signaling activation in tumor cells to promote metastasis (Fig. 3).

CXCR4 is a functional stem-cell marker to be applied for the basic research of endodermal progenitors and adult-tissue stem cells, and for the clinical research of therapeutics targeted to cancer stem cells. Small molecule compound or human antibody targeted to CXCR4 will be applied for cancer therapeutics focusing on cancer stem cells at the primary lesion as well as metastasis or recurrence niches, such as bone marrow and peritoneal cavity.

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