Nanog Increases Focal Adhesion Kinase (FAK) Promoter Activity and Expression and Directly Binds to FAK Protein to Be Phosphorylated*

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Background: Nanog and focal adhesion kinase are overexpressed in cancer cells.

Results: Nanog binds the FAK promoter, up-regulates FAK, and directly binds and is phosphorylated by FAK that regulates cell morphology, growth, and invasion.

Conclusion: Nanog binds the FAK promoter and up-regulates FAK, binds to FAK to be tyrosine-phosphorylated, and regulates cancer cell functions.

Significance: The Nanog and FAK interaction is critical for cancer biology.

Nanog and FAK were shown to be overexpressed in cancer cells. In this report, the Nanog overexpression increased FAK expression in 293, SW480, and SW620 cancer cells. Nanog binds the FAK promoter and up-regulates its activity, whereas Nanog siRNA decreases FAK promoter activity and FAK mRNA. The FAK promoter contains four Nanog-binding sites. The site-directed mutagenesis of these sites significantly decreased up-regulation of FAK promoter activity by Nanog. EMSA showed the specific binding of Nanog to each of the four sites, and binding was confirmed by ChIP assay. Nanog directly binds the FAK protein by pulldown and immunoprecipitation assays, and proteins co-localize by confocal microscopy. Nanog binds the N-terminal domain of FAK. In addition, FAK directly phosphorylates Nanog in a dose-dependent manner by in vitro kinase assay and in cancer cells in vivo. The site-directed mutagenesis of Nanog tyrosines, Y35F and Y174F, blocked phosphorylation and binding by FAK. Moreover, overexpression of wild type Nanog increased filopodia/lamellipodia formation, whereas mutant Y35F and Y174F Nanog did not. The wild type Nanog increased cell invasion that was inhibited by the FAK inhibitor and increased by FAK more significantly than with the mutants Y35F and Y174F Nanog. Down-regulation of Nanog with siRNA decreased cell growth reversed by FAK overexpression. Thus, these data demonstrate the regulation of the FAK promoter by Nanog, the direct binding of the proteins, the phosphorylation of Nanog by FAK, and the effect of FAK and Nanog cross-regulation on cancer cell morphology, invasion, and growth that plays a significant role in carcinogenesis.

One of the main properties of cancer cells is extensive proliferation that is common in embryonic stem cells and is important for embryogenesis and tumorigenesis. The molecular mechanisms that regulate embryonic stem cell self-renewal can be similar to the mechanism of cancer cell renewal. The Nanog is one of the main transcription factors, together with Oct3/4 and Sox 2 factors, that plays a major role in the regulation of pluripotency in mammalian embryonic and induced stem cells. Nanog is a homeobox transcription factor with a homeobox N-terminal DNA-binding domain and plays a critical role in the embryonic cell fate specification following formation of the blastocyst. The Nanog transcription factor binds the following consensus sequences: 5' - TAA(TG)(TG)-3' or 5' - (CG)- (GA)(CG)(GC)ATTAN(GC)-3' (3). Loss of Nanog is embryonically lethal (E5.5) (3, 4), whereas constitutive overexpression of Nanog allows self-renewal of the embryonic stem cells. Nanog has been shown to be expressed not only in germ tumor carcinoma but also in different types of cancer, such as oral squamous cell carcinoma (5), gastric adenocarcinoma (6), endometrial carcinoma (7), prostate (8), brain (9), colon (10), and other tumors. In cancer cells, Nanog induction has been shown to lead to drug resistance in breast and prostate cancer cells (11). The detailed molecular mechanisms that contribute to the role of Nanog in human tumorigenesis are still unknown.

Recent studies demonstrate that a small subpopulation of cells called cancer stem cells, due to their ability to self-renew and regenerate, are responsible for tumor growth in cancer, and these cells are resistant to chemotherapies (12, 13). Focal adhesion kinase is a protein that is located in the cytoplasm, in the focal adhesions, and recently was found to be in the nucleus and is overexpressed in many types of tumors (14). FAK plays an important role in cell adhesion, motility, survival, proliferation, invasion, and angiogenesis (15). The autophosphorylation of the Tyr-397 site of FAK in response to changes in the extracellular matrix, integrin clustering, or growth factor receptor signaling turns on downstream signaling leading to cytoskeletal

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changes. Recently, novel functions of FAK have been identified such as binding to p53 (16) and Mdm-2 proteins that demonstrate the novel nuclear function of FAK (17). This is consistent with the role of FAK protein in gene expression (18) and up-regulation of cyclin-D1 (19) and WNT-3a (20). Several recent reports indicate the important role of FAK in the proliferation of micrometastatic cancer cells (21) and during cancer stem cell function (22, 23).

Recently, exogenous expression of Nanog transcription factor in 293 cells has been shown to increase cell proliferation, growth in soft agar, and tumorigenesis in a xenograft model and caused increased FAK expression by proteomics analysis (24). Recently, increased expression of Oct3/4 was found in glioblastoma that up-regulated FAK expression and resulted in increased cell motility (25), suggesting the link between stem cell markers and FAK. In this report, we show that Nanog over-expression increases FAK protein expression, and induction of endogenous Nanog expression by hyaluronan (HA) increased FAK expression. In addition, in this report we demonstrate the direct role of Nanog transcription factor in the regulation of FAK promoter activity and FAK expression in different cancer cells. We analyzed the FAK promoter sequence and found that the FAK promoter has four Nanog-binding sites: site 1, TAATGG (−650 to −645 bp); site 2, TAATGC (−626 to −621 bp); site 3, TAATCC (−373 to −368 bp); and site 4, GACAAATTACG (−218 to −208 bp). The overexpression of Nanog in protein 293 cells significantly increases the FAK promoter activity by a Dual-Luciferase assay, and two Nanog siRNAs and site-directed mutagenesis of Nanog-binding sites significantly inhibited Nanog-induced FAK promoter activity. In addition, EMSA showed direct binding and specific binding of Nanog to each of the four Nanog-binding sites in the FAK promoter in cancer cells that was inhibited by cold FAK promoter oligonucleotide probe and Nanog antibody. We also demonstrate that Nanog binds the FAK promoter by chromatin immunoprecipitation (ChiP) assay, which confirms EMSA and Dual-Luciferase data. Moreover, two different Nanog siRNAs decreased binding of Nanog to the FAK promoter and decreased FAK promoter activity and FAK mRNA expression. In addition, we show by pulldown assay and by immunoprecipitation that FAK and Nanog proteins directly interact in different cancer cells, and we also show by confocal laser microscopy that these proteins are co-localized in the nucleus and perinuclear areas in the cancer cells. We detect that Nanog binds the N-terminal domain of FAK but not the kinase or C-terminal domains of FAK. Confocal laser microscopy demonstrated co-localization of Nanog with GFP-FAK-NT and GFP-FAK but not with the control GFP and GFP-FAK-CD proteins. Moreover, we show that FAK directly phosphorylates Nanog protein in a dose-dependent manner by in vitro kinase assay, and the phosphorylation inhibitor of FAK decreases direct phosphorylation of Nanog by FAK. The tyrosine phosphorylation of endogenous Nanog was detected in different cancer cells, NCCIT and SW620 cells. The site-directed mutagenesis of tyrosines Y35F and Y174F blocked phosphorylation of Nanog by FAK and the binding of FAK and Nanog proteins. Overexpression of FAK in 293 cells increased tyrosine phosphorylation and binding of wild type Nanog that was significantly less in the case of Y35F and Y174F Nanog mutants. Overexpression of wild type Nanog in 293 cells increased lamellipodia and filopodia formation and increased cell polarization and cell invasion that was decreased by the FAK phosphorylation inhibitor. The mutants Nanog Y35F and Y174F did not increase filopodia and lamellipodia formation and did not affect cell polarization compared with the wild type Nanog. Moreover, overexpression of FAK induced cell invasion that was stimulated by wild type Nanog, but it less significantly increased in the case of mutants Y35F and Y174F Nanog. Moreover, Nanog siRNA decreased tumor cell growth similarly to FAK siRNA and the FAK phosphorylation inhibitor that was reversed by the overexpression of FAK. Thus, this report for the first time identifies four Nanog-binding sites in the FAK promoter, demonstrates that Nanog binds the FAK promoter by a Dual-Luciferase assay, site-directed mutagenesis, and EMSA and ChiP methods, and shows that Nanog up-regulates FAK promoter activity and expression. This report demonstrates the direct interaction of FAK and the N-terminal domain of FAK and Nanog proteins by pulldown assays in vitro and by immunoprecipitation and confocal microscopy in vivo. It demonstrates direct phosphorylation of Nanog by FAK using in vitro kinase assays and in cancer cells using immunoprecipitation of Nanog with phospho-specific antibody, and it shows the functional significance of wild type Nanog and mutants Y35F and Y174F Nanog for phosphorylation and binding with FAK and for cancer cellular morphology and invasion. Thus, this study provides the mechanism of Nanog and FAK cross-regulation and interaction in different cancer cells, which is important for the fields of cancer cell biology, stem cell biology, and cancer research.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Human epithelial kidney 293T cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS) and 1 μg/ml penicillin/streptomycin. The human pluripotent embryonal carcinoma, teratocarcinoma NCCIT (ATTC, CRL-2073), and embryonal pluripotent teratocarcinoma NTERA-2clD1 (ATTC, CRL-1973) cell lines were obtained from the ATCC and cultivated in RPMI 1640 medium with 10% FBS. The human colon cancer cell lines SW480 and SW620 were obtained from Dr. Bullard Dunn and grown in DMEM with 10% FBS and 1 μg/ml penicillin/streptomycin.

**Tumor Samples**—The protein lysates were prepared by a standard protocol from four colon cancer tumor samples, and matched control tissues were described previously (26). All samples were obtained according to approved IRB protocol.

**Plasmids**—The pcDNA3-Hyg-Nanog and pMX-hNanog plasmids were obtained from Addgene. The FAK promoter p723-pGL3 construct was described previously (27). The Nanog-GST plasmid was generated by re-cloning the Nanog cDNA insert from pMXs-hNanog into the EcoRI and NotI site of pGEX-4T1 GST vector. The Y174F and Y35F GST- and pcDNA3 Nanog mutants were generated by site-directed mutagenesis using Nanog wild type plasmids. The GST-FAK-NT, FAK kinase, and C-terminal (FAK-CD)-pGEX4T1 plasmids were generated by PCR and were described previously (16). The GFP, GFP-FAK-NT, GFP-FAK-CD and GFP-FAK

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plasmids were described previously (16) and used for expression of different domains of FAK for confocal laser microscopy.

Nanog and FAK siRNA—Control siGenome nontargeting siRNA and Nanog siRNA ON Target plus number 1 (GGAAGG-CUUAAUGUAUAUA), number 2 (GGACAGUCCCUUCUAAUA), number 3 (GCAUCCACUGUAAGAAAU), and number 4 (GGACAGUCCCUUCAAUAA) were obtained from Dharmacon Inc. The FAK siRNA and control luciferase siRNA vectors have been described previously (18). The Nanog siRNA was transfected into the cells with Lipofectamine, according to the manufacturer’s protocol.

Antibodies, Reagents, and Recombinant Proteins—For the immunological analyses, antibodies used were as follows: anti-FAK 4.47 from Upstate Biologials; anti-Nanog from Abcam and Santa Cruz Biotechnology; anti-β-actin from Sigma; and anti-phosphotyrosine RC-20-HRP-conjugated antibody was from Transduction Laboratories. Baculoviral FAK protein and Pyk-2 proteins, described previously (28), were used for pull-down assay. FAK protein was obtained from Invitrogen. For the kinase assay, human c-Src and FAK proteins were obtained from Transduction Laboratories. Baculoviral FAK protein and Pyk-2 proteins, described previously (28), were used for pull-down assay. FAK protein was obtained from Invitrogen. For the kinase assay, human c-Src and FAK proteins were obtained from BIOSOURCE. For pulldown with Nanog protein, the different fragments of FAK in the PGEX-4T1 vectors, encoding GST-FAK-NT (1–423 amino acids), GST-FAK kinase (416–676 amino acids), and GST-FAK-CD (677–1052 amino acids) proteins, were generated by PCR, as described previously (16), and used for pulldown assays. All GST proteins were confirmed with fragment-specific antibodies. The C-terminal FAK, FAK-CD, was confirmed with FAK-C20 antibody from Santa Cruz Biotechnology; the N-terminal domain of FAK was confirmed with fragment-specific antibodies. The C-terminal FAK, FAK-CD, was confirmed with FAK-C20 antibody from Santa Cruz Biotechnology; the N-terminal domain of FAK was confirmed with FAK 4.47 antibody (Upstate Biologials), and the FAK kinase domain inside was confirmed with polyclonal BC-2 antibody (a kind gift of Dr. Michael Schaller, West Virginia University, Morgantown, WV). The GST-Nanog protein was isolated using the Nanog-pGEX-4T1 plasmid. For the kinase assay, we either used a human recombinant Nanog protein (ab50053) or Nanog protein isolated from GST-Nanog-conjugated protein by cleavage with thrombin. The thrombin was used either from Sigma or from Novagen thrombin cleavage capture kit according to the manufacturer’s protocol. The thrombin was removed with the p-a-minobenzamidine-agarose from Sigma according to the manufacturer’s protocol. The HA (41,000–65,000) was obtained from Life Core Biomedical and added to a serum-free medium in accordance with the manufacturer’s protocol. The HA (41,000–65,000) was obtained from Life Core Biomedical and added to a serum-free medium in accordance with the manufacturer’s protocol. The HA (41,000–65,000) was obtained from Life Core Biomedical and added to a serum-free medium in accordance with the manufacturer’s protocol.

RNA Isolation—Total cellular RNA was isolated from cultured cells with a NucleoSpin RNA II purification kit (Clontech) according to the manufacturer’s protocol.

Real Time PCR—For FAK cDNA amplification by RT-PCR, TaqMan one-step RT-PCR master mix was used (Applied Biosystems). RT-PCR primers and TaqMan probes and PCR conditions were described previously (32). The forward FAK primers were as follows: forward primer 5'-GTGCTCTTGTTACAAGCTGGAAT-3' and reverse primer 5'-ACTTGAGTGAAGGCAGAAGATGTG-3' and the FAK probe 5'-6-carboxy-fluorescein-TCACCTAAGGACAAGGCTGCAATCC-6-carboxymethylrhodamine-3'. For normalization purposes, we used RT-PCR with primers for GAPDH, forward 5'-GGAAGG-TGAAGGTCCGAGTC-3' and reverse 5'-GAAGATTGGTAGTG-3' and the FAK probe 5'-6-carboxy-fluorescein-CAAGCTTCCCGCTTACGCT-6-carboxymethylrhodamine-3'. The ABI PRISM 7700 cycler software calculated a threshold cycle number (Ct) at which each PCR amplification reached a significant threshold level.

Dual-Luciferase Assay—The Dual-Luciferase assay was performed with the Dual-Luciferase Reporter Assay System kit (Promega). For normalization of luciferase activity, the pRL-TK control vector, encoding Renilla luciferase was used for co-transfection with FAK promoter-PGL3 luciferase plasmids, as described previously (27). Luminescence was measured on a luminometer in three or five independent experiments.

Site-directed Mutagenesis—Site-directed mutation of the Nanog-binding sites in the FAK promoter was generated with a QuickChange II XL site-directed mutagenesis kit (Stratagene) using p700-pGL3 FAK promoter plasmid as a template, according to the manufacturer’s protocol. To generate Nanog site 1 mutation, we used the following oligonucleotides (mutation in Nanog site is underlined): site 1, forward 5'-CATCTTTCCAAAATCAA-ACAATGCGCCACCGCAACTAGC-3' and reverse 5'-GTAGTGGTGTGGGTTCGATT-3'; site 2, forward 5'-CCAGCGCAACTTAGCTAAGATGTC-3' and reverse 5'-GGCTCAAGATTCTTTTCATGGCATTCTTTGCTGGG-3'; site 3, forward 5'-GGAACCTTCCCAGAAGTCTGCCAATCTCCGCTGGGAGGAGG-3' and reverse 5'-GCGCTTCCGCAACGCGGATTGAGGAAGTGCTGGGAGGAGG-3'; and site 4, forward 5'-GCACCGCGGTCACTACAAGGAGAATTCAGC-3' and reverse 5'-GTGCTCTTGTCACCTAAGGCTATGAGCTTCCGCAAGGCGGAG-3'. All mutant plasmids were sequenced in both directions at the Automated DNA Sequencing Facility at the Roswell Park Cancer Institute (Buffalo, NY).

Electrophoretic Mobility Shift Assay (EMSA)—For EMSA, the Nanog protein was used with oligonucleotides with Nanog-binding sites and binding buffer from a gel-shift kit (Pierce). The sequence of oligonucleotide containing the consensus Nanog-binding site and one shown in bold font, was 5'-CAATGCTGTCGAC-3'; and the oligonucleotide with binding site 2 (shown in bold font) was 5'-CACCTTAGCTATAA-
TGGCATGAAAG-3′; site 3 (shown in bold font) was 5′-GAA-GTCCGTCTAATCCGCTGGCGG-3′; and site 4 (shown in bold font) was 5′-CTACAGGACAAATTACCGGACAGA-G-3′. The oligonucleotides with corresponding complementary oligonucleotides were labeled using the biotin 3′ end DNA labeling kit from Pierce, according to the manufacturer’s instructions. The labeled oligonucleotides were annealed, phenol/chloroform-purified, and used for the binding reaction in a double-stranded form. The DNA binding reaction of biotin-labeled double-stranded oligonucleotides with 2–10 μg of cell extract was performed at room temperature for 20 min, according to the manufacturer’s protocol. The DNA–protein complexes were analyzed with Novex 6% DNA retardation gels. Electrophoretic mobility shift assays were performed with the Light Shift chemiluminescent kit from Pierce, according to the manufacturer’s recommendations. Biotin-labeled DNA was then detected by chemiluminescence. For control of binding specificity, the binding reactions were performed in the presence of excess of cold probe or with 0.1–1 μg of Nanog antibody.

Western Blotting and Immunoprecipitation—Western blot analysis and immunoprecipitation were performed as described previously (32). In brief, cells were washed twice with cold 1× PBS and lysed on ice for 30 min in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 50 mM NaF, 1 mM NaVO₄, 10% glycerol and for protease inhibitors containing 10 μg/ml leupeptin, 10 μg/ml PMSF, and 1 μg/ml aprotinin. The lysates were cleared by centrifugation at 10,000 rpm for 30 min at 4°C. Protein concentrations were determined using a Bio-Rad kit. The boiled samples were loaded on Ready SDS-10% polyacrylamide gels (Bio-Rad) and used for Western blot analysis with the protein-specific antibody. The blots were stripped in a stripping solution (Bio-Rad) at 37 °C for 15 min and then re-probed with the primary antibody to check equal loading of proteins. Immunoblot membranes were developed with chemiluminescence Renaissance reagent (PerkinElmer Life Sciences).

Expression of Recombinant GST Fusion Proteins—GST fusion proteins were engineered by PCR. The fusion proteins were expressed in Escherichia coli bacteria by incubation with 0.2 mM isopropyl β-D-galactopyranoside for 6 h at 37°C. The bacteria were lysed by sonication, and the fusion proteins were purified with glutathione-agarose beads, as described previously. GST-paxillin was used as a positive control and GST protein as a negative control in reactions with [γ-32P]ATP in a buffer: 20 mM Hepes (pH 7.4), 5 mM MgCl₂, 5 mM MnCl₂, 1 mM DTT, 0.1 mM Na₃VO₄ at 30°C for 30–40 min, as described previously (29). 0.025 μg of kinase and 0.3 μg of substrate were used in most reactions.

In Vitro Kinase Assay—The in vitro kinase assay was performed with purified FAK, Pyk-2, c-Src, and Nanog proteins. GST-paxillin was used as a positive control and GST protein as a negative control in reactions with [γ-32P]ATP in a buffer: 20 mM Hepes (pH 7.4), 5 mM MgCl₂, 5 mM MnCl₂, 1 mM DTT, 0.1 mM Na₃VO₄ at 30°C for 30–40 min, as described previously (29). 0.025 μg of kinase and 0.3 μg of substrate were used in most reactions.

Chromatin Immunoprecipitation (ChIP) Assay—The ChIP assay was performed with the Upstate ChIP kit, according to the manufacturer’s protocol, as described previously (26). The lysates were immunoprecipitated with Nanog antibody, and PCR was performed with the FAK promoter and GAPDH primers. The primers for PCR for FAK promoter were forward 5′-CCAAAATCAAATTGCCCCAGCCG-3′ and reverse 5′-CTCTGGAGAACCTCCCTC-3′. The control GAPDH primers were described previously (26).

In Vivo Kinase Assay—The in vitro kinase assay was performed with purified FAK, Pyk-2, c-Src, and Nanog proteins. GST-paxillin was used as a positive control and GST protein as a negative control in reactions with [γ-32P]ATP in a buffer: 20 mM Hepes (pH 7.4), 5 mM MgCl₂, 5 mM MnCl₂, 1 mM DTT, 0.1 mM Na₃VO₄ at 30°C for 30–40 min, as described previously (29). 0.025 μg of kinase and 0.3 μg of substrate were used in most reactions.

Immunostaining and Confocal Microscopy—Immunostaining was performed with Nanog and FAK primary antibodies and secondary Alexa 488- and Alexa 546-conjugated antibodies, as described previously (16). The Zeiss fluorescent microscope was used for detection of FAK and Nanog immunostaining. Co-localization analysis of FAK and Nanog proteins was performed by confocal microscopy at the Flow Cytometry Facility (Roswell Park Cancer Institute) using a Leica confocal laser microscope. Leica confocal software was used for analysis of z-stack slice images.

Lamellipodia and Filopodia Quantification—The cells were stained with phalloidin-FITC detecting actin and filopodia and lamellipodia formations. The 50–80 cells for each sample in different fields were analyzed under a Zeiss microscope, and the average percent of lamellipodia/filopodia per cell was calculated.

Cell Invasion—Cells were plated in the serum-free medium and analyzed after 24 h for invasion on Boyden chambers using cell invasion kit (Chemicon International) according to manufacturer’s protocol. The experiments were performed in triplicate.

Cell Growth—Cells were plated on 6-well plates in complete medium and analyzed for cell growth by counting cells on a hemocytometer, as described previously (33).

Statistical Analyses—The Student’s t test was performed to determine significance. The difference between data with p < 0.05 was considered significant.

RESULTS

Nanog Overexpression Induces FAK Expression and Silencing of Nanog with siRNA Decreases FAK Expression in Cancer Cell Lines—To test the effect of Nanog on FAK expression in different cell lines, we overexpressed the Nanog protein in 293 cells, transfected with Nanog-pcDNA3 plasmid (Fig. 1A). Overexpression of Nanog in 293 cells induced FAK expression compared with control pcDNA3-transfected 293 cells (Fig. 1A). To test the effect of induced expression of endogenous Nanog, we treated 293 cells with HA, which is a major component of extracellular matrix that has been shown to induce Nanog expression (34). In 293 cells treated with HA (100 μg/ml) in serum-free medium for 24 h, Nanog and FAK expressions were
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FIGURE 1. Nanog up-regulates FAK expression and Nanog siRNAs decrease FAK expression in cancer cells. A, 293 cells were transfected with Nanog-pcDNA3 or pcDNA3 (Vector) control plasmid for 24 h, and Western blotting (WB) was performed with Nanog and FAK antibodies. FAK is up-regulated in 293 cells transfected with Nanog plasmid compared with vector control cells. Western blotting with β-actin was performed to show equal protein loading. B, left panel, HA induces Nanog and FAK expression in 293 cells. 293 cells were treated with 100 μg/ml hyaluronan for 24 h after overnight cell starvation in a serum-free medium. Western blotting with FAK and Nanog antibodies shows increased FAK and Nanog protein expression in the HA-treated 293 cells. Right panel, colon cancer cells SW620 and SW480 were treated with 100 μg/ml HA in serum-free medium for 4 h, and Western blotting with Nanog and FAK antibodies was performed. C–E, SW480 cells (C), NCCIT (ATCC, CRL-2073) (D), and 293 cells (E) were transfected with two different Nanog siRNAs sites 1 and 2 and control siRNA for 24 h, and Western blotting was performed as in A. Cells with down-regulation of Nanog with both siRNAs express less FAK protein compared with control siRNA in three cancer cell lines. F, Nanog and FAK proteins are overexpressed in breast and colon cancer tumors. The lysates from one breast and three colon cancer tumors and matched normal tissues were analyzed by Western blotting with Nanog, FAK, and β-actin antibody. Nanog and FAK were overexpressed in tumor (T) samples compared with matched normal (N) tissues. The image is a composite from the same film.

Nanog Overexpression Induces FAK Promoter Activity, whereas Nanog siRNA Decreases Its Activity and Decreases FAK mRNA Levels in Cancer Cells—To test if induction of Nanog expression with HA induces FAK promoter activity, we transfected the FAK core promoter p723, described previously (27), into the 293 cells without addition of HA or with HA (Fig. 2A, left panel). The HA treatment increased FAK promoter activity (Fig. 2A, left panel) that is consistent with induced Nanog and FAK expression (Fig. 1B). In addition, two different Nanog siRNAs blocked HA-induced FAK promoter activity compared with control siRNA (Fig. 2A) that shows Nanog-dependent HA-induced FAK promoter activity. To test if Nanog directly induces FAK promoter activity, we co-transfected FAK core promoter plasmid (p723) (27) with Nanog-pcDNA3 plasmid or with control pcDNA3 vector plasmid, and we performed a Dual-Luciferase assay (Fig. 2B). Nanog overexpression significantly increased activity of the FAK-p723 promoter construct (Fig. 2B). The same increased activity of FAK promoter by Nanog was observed in SW480 colon cancer cells transfected with Nanog plasmid (Fig. 2C). Thus, Nanog overexpression up-regulates FAK promoter activity.

To test if down-regulation of endogenous Nanog expression in 293 cells decreases FAK promoter activity, we transfected the cells with either control siRNA or with two different Nanog siRNAs, numbers 1 and 2 (Fig. 2D). Down-regulation of Nanog with two Nanog siRNAs significantly decreased FAK promoter activity in 293 cells, whereas control siRNA did not (Fig. 2D, left panel). The same effect of FAK luciferase activity was observed in an NCCIT teratocarcinoma cell line, where two Nanog siRNAs decreased luciferase activity of the FAK promoter, whereas control siRNA did not (Fig. 2D, right panel). The down-regulation of FAK promoter activity by Nanog siRNA
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FIGURE 2. Nanog induces FAK promoter activity in 293 cells. A, HA induces FAK promoter activity. 293 cells were co-transfected with the FAK promoter construct p723 and with Renilla plasmid and also control siRNA or Nanog siRNAs sites 1 and 2 either without HA (−HA) or with the addition of HA 100 μg/ml (+HA). The Dual-Luciferase assay shows induction of FAK promoter by HA, although both Nanog siRNAs decreased HA-induced FAK promoter activity. The bars are averages of three independent experiments ± S.E. *, p < 0.05, p723 (+HA) versus p723 (−HA); **, Nanog siRNA + (HA) versus p723 + HA and Control siRNA + HA, Student's t test. B, overexpression of Nanog increases FAK promoter activity in 293 and SW480 cells. 293 (B) and SW480 (C) cells were co-transfected with Nanog-pcDNA3 or control (Vector) pcDNA3 plasmids with FAK promoter luciferase p723-pGL3 construct together with Renilla construct for normalization, and a Dual-Luciferase assay was performed. The luciferase activity of FAK promoter was significantly increased by Nanog plasmid compared with control vector plasmid in both cell lines. The bars show averages of three independent experiments ± S.E. *, p < 0.05, Student's t test. C, down-regulation of Nanog decreases FAK promoter activity by a Dual-Luciferase assay in 293 and NCCIT cells. The 293 (left panel) and NCCIT (right panel) cells were transfected with p723-pGL3 plasmid in the presence of control siRNA or two Nanog siRNAs sites 1 and 2, and Dual-Luciferase assay was performed as in A. Both Nanog siRNAs significantly decreased FAK promoter activity in contrast to control siRNA. Bars are an average of three independent experiments ± S.E. *, p < 0.05, Student's t test. D, Nanog siRNA decreases FAK mRNA level. 293 cells were transfected with control siRNA or Nanog siRNA sites 1 and 2, and RT-PCR was performed on isolated RNA. FAK mRNA is significantly decreased by two Nanog siRNAs. *, p < 0.05, Nanog siRNA versus control siRNA, Student's t test.

was also observed in SW480 colon cancer cells and transfected with Nanog siRNA (data not shown). Thus, down-regulation of Nanog expression with two different Nanog siRNAs decreases FAK promoter activity.

To test the effect of down-regulation of Nanog on FAK mRNA level, we isolated RNA from 293 cells transfected with two Nanog siRNAs and analyzed the FAK mRNA level (Fig. 2E). Both Nanog siRNAs significantly decreased FAK mRNA level versus control siRNA (Fig. 2E). Thus, Nanog overexpression significantly increases FAK promoter activity, whereas down-regulation of Nanog with two different Nanog siRNAs decreases FAK promoter activity and decreases FAK mRNA level.

FAK Promoter Contains Four Nanog-binding Sites—To identify the potential binding sites of Nanog in the FAK promoter, we analyzed the FAK core promoter p723-pGL-3 sequence, described previously (27), for the presence of Nanog-binding sites. The FAK promoter contains four potential binding sites with conserved TAA(T/A)TTA sequence (underlined): site 1 (−650–645 bp) TAAATGG; site 2, −626–621, TAATGC; site 3 −373–368, TAATCC; and site 4 −218–208, GACAAAT-TACG (Fig. 3A). To test the effect of mutation in each site, we performed the site-directed mutagenesis in the conserved TAAT or ATTA sequence of the consensus Nanog site of the p723 FAK promoter; as shown in Fig. 3, and generated mutations in sites 1–4 and then performed a Dual-Luciferase assay with either wild type or mutant constructs co-transfected with either control pcDNA3 or with Nanog-pcDNA3 plasmid into 293 cells (Fig. 3B). Mutation of all Nanog sites significantly decreased up-regulation of FAK promoter activity by Nanog (Fig. 3B). Thus, the data demonstrate that mutation of the Nanog-binding sites in the FAK promoter inhibits up-regulation of FAK promoter activity, confirming that Nanog binds the four sites in the FAK promoter sequence.

EMSA Assay Demonstrates That Nanog Binds Four Sites in the FAK Promoter—To detect binding of Nanog protein to the Nanog-binding sites in the FAK promoter, we synthesized and biotin-labeled oligonucleotides, as described under “Experimental Procedures,” with Nanog-binding sites 1–4 from the FAK promoter, and we performed EMSA with nuclear extracts.
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A

FAK promoter

-723
-650 -626
-373 -213

Nanog consensus binding sites:

5’-TAAT[TG][TG]-3’ or 5’-[CG][GA][CG]C[GC]ATTAN[GC]-3’

Nanog binding sites

1. TAATGG  -650-645
2. TAATGC  -626-621
3. TAATCC  -373-368
4. GACAAATTACG  -218-208

Nanog binding site mutations

nan 1  CAATGG
nan 2  GAATGC
nan 3  CAATCC
nan 4  GACAACTTACG

B

![Graph showing relative luciferase activity](image)

- Relative luciferase activity (fold)
- WT, Mut 1, Mut 2, Mut 3, Mut 4

293 cells
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FIGURE 4. A and B, EMSA detects binding of Nanog protein to the four binding sites in the FAK promoter. The four oligonucleotides containing Nanog-binding sites 1–4 were synthesized, labeled with biotin, and used for EMSA with NCCIT (A) or HeLa cells (B). Nanog binds all four sites in the FAK promoter in both cell lines. C, competitor Nanog cold oligonucleotide and Nanog antibody inhibit binding of the Nanog proteins to the binding sites in the FAK promoter. The EMSA was performed either without addition of cold oligonucleotide or with increased concentration of cold oligonucleotides (0.01, 1, and 1 μM). EMSA was also performed in the presence of 0.2 μg of Nanog antibody. Both cold oligonucleotide and Nanog antibody inhibited binding of Nanog protein to the four Nanog-binding sites 1–4 in the FAK promoter. The images are composites from the same film. D, EMSA was performed on extracts of NCCIT cells transfected with either control siRNA or two Nanog siRNAs with the site 1 oligonucleotide. The binding of Nanog to FAK promoter probe was significantly decreased by Nanog siRNA versus control siRNA. The same result was observed with the other site oligonucleotides. E, ChIP demonstrates the binding of Nanog to the FAK promoter chromatin. We performed ChIP assay on NCCIT cells as described under “Experimental Procedures.” ChIP assay was performed either without antibody (No Ab) and with Nanog antibody (Nanog Ab). Then PCR was performed with FAK promoter or with the negative control GAPDH promoter primers. Nanog binding was detected with Nanog antibody but was not detected without Nanog antibody. The binding was detected with FAK promoter primers but was not detected with GAPDH promoter primers. Nanog binds FAK promoter chromatin by ChIP assay.

of NCCIT cells that expressed endogenous Nanog (Fig. 4A) or with HeLa nuclear extract that was also shown to express the Nanog protein (Fig. 4B) (35). EMSAs show that Nanog protein binds four sites in the FAK promoter in NCCIT cells (Fig. 4A). The same result was obtained with HeLa cells, where Nanog was binding all four sites in the FAK promoter (Fig. 4B). To test the specificity of the Nanog binding, we performed EMSA in the presence of the cold oligonucleotides or with Nanog antibodies (Fig. 4C). The addition of increased concentration of cold oligonucleotide with Nanog-binding site inhibited in a dose-dependent manner binding of Nanog to all four sites (Fig. 4C). The binding was also decreased in the presence of Nanog antibody (Fig. 4C). In addition, two Nanog siRNAs decreased binding of Nanog to the FAK promoter compared with control siRNA (Fig. 4D). To detect binding of Nanog to the FAK promoter chromatin in vivo, we performed ChIP assay in NCCIT cells, expressing high levels of endogenous Nanog (Fig. 4E). We immunoprecipitated Nanog and performed PCR with FAK promoter primers and with negative control GAPDH promoter primers. We detected binding with Nanog immunoprecipitation using FAK promoter primers, although it was not detected with the negative control GAPDH primers (Fig. 4E). No binding

FIGURE 3. A, scheme of the four Nanog-binding sites in the FAK promoter. The Nanog-binding sites 1–4 in the FAK promoter are shown on the left. The conservative TAA(T/A)TTA motif is shown in bold (left panels). The mutation inside the conservative consensus TAA(T/A)TTA motif in the Nanog (non)-binding sites are shown in bold and underlined font on the right panel by site-directed mutagenesis. Luc, luciferase. B, Nanog up-regulates the FAK promoter, and mutation of the four binding sites abolishes this up-regulation. The Dual-Luciferase assay was performed in 293 cells co-transfected with Nanog-pcDNA3 (Nanog) or with the control pcDNA3 plasmid (Vector) together with the FAK promoter, p723 wild type (WT), or with mutant constructs with mutations 1–4 (Mut1, Mut2, Mut 3, and Mut 4), and the Dual-Luciferase assay was performed as described under “Experimental Procedures.” The mutation of the Nanog-binding sites 1–4 did not cause significant up-regulation of FAK promoter activity by Nanog in contrast to wild type Nanog. Three independent experiments were performed. Bars represent the average luciferase activity ± S.E. *, p < 0.05, Student’s t test; luciferase activity in the 293 cells was transfected with Nanog-pcDNA3 plasmid compared with control vector (pcDNA3 plasmid)-transfected cells.
was detected in control immunoprecipitation without the Nanog antibody. The ChIP assay shows that Nanog binds FAK promoter chromatin (Fig. 4E), which confirms EMSA data and demonstrates that Nanog binds FAK promoter on a chromatin level. Thus, Nanog binds all four sites in the FAK promoter by EMSA, which is consistent with a Dual-Luciferase assay with the FAK promoter containing mutations in the Nanog-binding site.

FAK and Nanog Proteins Directly Interact in Vitro and in Vivo—To test if FAK and Nanog proteins directly interact in vitro, we performed a pulldown assay with GST, GST-Nanog, and GST-paxillin control proteins and with the recombinant FAK protein (Fig. 5A), as described previously (16). The pulldown assay shows that FAK binds to GST-Nanog and control GST-paxillin, although it does not bind to control GST protein. Western blotting with GST confirmed GST proteins, Western blotting with paxillin antibody confirmed paxillin protein, and Western blotting with Nanog antibody confirmed Nanog protein (Fig. 5A). Thus, Nanog and FAK proteins directly interact in vitro.

To test if FAK and Nanog proteins interact in vivo, we performed immunoprecipitation of Nanog and FAK proteins in NCCIT teratocarcinoma cells that express high levels of endogenous proteins in both directions. Immunoprecipitation (IP) of FAK detected a complex of FAK with Nanog protein in NCCIT cells, whereas IP with control His antibody did not detect binding of the proteins (Fig. 5B, left upper panel). In the reverse direction, immunoprecipitation of Nanog protein detected a complex of Nanog with the FAK protein, whereas the control His tag antibody did not detect binding of the proteins (Fig. 5B, right upper panel). To show specificity of FAK and Nanog binding, we performed Western blotting with Nanog antibody, and no complex was detected with control HA-tag antibody (Fig. 5B). We also detected complex of FAK and Nanog in the reverse direction, when we immunoprecipitated Nanog and performed Western blotting with FAK antibody, and no complex was detected with control HA-tag antibody (Fig. 5B, upper right panels). To show specificity of FAK and Nanog binding, we performed immunoprecipitation of Nanog in FAK+/+ and FAK–/– MEF cells transfected with Nanog plasmid, and Western blotting with FAK antibody was performed (Fig. 5B, lower panels). The complex of FAK and Nanog was detected in FAK+/+ cells but was not detected in FAK–/– cells. We also detected binding of FAK and Nanog proteins in SW620 colon cancer and NCCIT teratocarcinoma cells. Thus, FAK and Nanog proteins directly interact in vitro and in vivo.

Nanog Binds to the N-terminal Domain of FAK—To map the domain of FAK that is involved in binding with Nanog, we isolated the GST-FAK-NT (N-terminal domain) (1–423 amino acids), GST kinase domain (416–676 amino acids), and GST-FAK-CD (677–1052 amino acids) domain of FAK and performed pulldown with purified Nanog protein, which is shown in Fig. 5C. The pulldown of Nanog with GST-conjugated fragments of FAK (Fig. 5D, upper panel) demonstrates that Nanog binds the N-terminal domain of FAK but does not bind the C-terminal and kinase domain (Fig. 5D, middle panel). Coomassie Blue staining shows GST-FAK proteins (Fig. 5D, lower panel), and the GST-FAK domain proteins were confirmed with FAK antibodies specific to each domain (data not shown) (16). Thus, Nanog directly binds the N-terminal domain of FAK.

Nanog and FAK Proteins Co-localize in Cancer Cells—To detect FAK and Nanog protein co-localization, we performed immunostaining of Nanog and FAK and confocal laser-scanning microscopy in cancer cells with high endogenous Nanog and FAK. We performed immunostaining of Nanog with Nanog antibody followed by staining with the secondary Alexa 546 (Red) antibody. We performed immunostaining of FAK with FAK 4.47 antibody and Alexa 488 (Green) antibody in NCCIT teratocarcinoma cells, expressing endogenous Nanog and FAK proteins. The confocal laser-scanning microscopy detected co-localization of Nanog and FAK proteins mainly in the nucleus (white arrows) and perinuclear regions in NCCIT cells (white arrowheads) (Fig. 5E). The co-localization of FAK and Nanog proteins was also detected in 293 cells with overexpressed Nanog protein. The co-localization was observed in the nucleus (white arrows) and perinuclear cytoplasmic speckles (Fig. 5E, middle panel, white arrowheads). We also detected co-localization of FAK and Nanog proteins in the nucleus (white arrows) and perinuclear areas (white arrowheads) in 480 colon cancer cells, transfected with Nanog plasmid (Fig. 5E, lower panel). The data are consistent with reported nuclear and cytoplasmic localization of both proteins and clearly show co-localization of both proteins in several cancer cell lines.

To demonstrate co-localization of Nanog with the N-terminal domain of FAK, we overexpressed GFP, GFP-FAK-NT (N-terminal), GFP-FAK-CD (C-terminal), and GFP-FAK proteins in 480 cells and performed confocal laser-scanning microscopy to detect protein co-localization (Fig. 5F). The GFP-FAK-NT is localized in the nucleus and co-localizes with the Nanog protein in the nucleus, cell junctions, and lamellepodia (Fig. 5F, white arrows), although it did not co-localize with control GFP and GFP-FAK-CD proteins. GFP-FAK co-localizes with Nanog in the nucleus, perinuclear areas, and focal adhesions (Fig. 5F, white arrows). Thus, Nanog co-localizes with GFP-FAK-NT and GFP-FAK, which is consistent with the pulldown experiment showing binding of Nanog with the N-terminal domain of FAK. The same result was obtained in 293 cells. Thus, FAK, N-terminal domain of FAK, and Nanog proteins co-localize in the nucleus and perinuclear cytoplasmic regions in different cancer cells in vivo.

FAK Directly Phosphorylates Nanog Protein—To test if FAK directly phosphorylates the Nanog protein and to test the functional significance of FAK and Nanog protein interaction, we performed an in vitro kinase assay with purified recombinant FAK and Nanog proteins. We performed the in vitro kinase assay with the Nanog protein and used GST-paxillin as a positive control and GST protein as a negative control protein (Fig. 6A). FAK directly phosphorylates Nanog and control paxillin, although it does not phosphorylate the GST protein (Fig. 6A). To test the dose-dependent effect of Nanog phosphorylation,
we performed an *in vitro* kinase assay with increasing amounts of Nanog protein. FAK phosphorylates Nanog in a dose-dependent manner (Fig. 6B, left panels). To test if c-Src is able to phosphorylate Nanog, we performed an *in vitro* kinase assay with Nanog and c-Src protein (Fig. 6B, right panels). The c-Src protein is also able to phosphorylate Nanog (Fig. 6B). To test if FAK homologous protein Pyk-2 phosphorylates Nanog, we performed an *in vitro* kinase assay with Pyk-2 kinase (Fig. 6C).
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The FAK homologous Pyk-2 kinase was able to phosphorylate Nanog protein (Fig. 6C).

To test the effect of FAK inhibition on Nanog phosphorylation, we inhibited FAK activity with the FAK-specific inhibitor PF-228 (Pfizer) (Fig. 6D). The FAK inhibitor PF-228 decreases FAK autophosphorylation activity and significantly decreases Nanog phosphorylation, in a dose-dependent manner (Fig. 6D).

To test if FAK protein expressed in 293 cells is able to phosphorylate Nanog protein, we overexpressed HA-tagged FAK, immunoprecipitated FAK with HA-antibody, and performed an in vitro kinase assay (Fig. 6E). FAK expressed in 293 cells directly phosphorylates Nanog and control paxillin proteins (Fig. 6E). We also performed immunoprecipitation of Nanog and performed Western blotting with tyrosine antibody, and we detected tyrosine-phosphorylated Nanog in different cancer cells in vitro and in vivo (Fig. 7, D and E). Thus, in vitro kinase and immunoprecipitation assays show that FAK directly phosphorylates the Nanog protein.

Site-directed Mutagenesis of Nanog Y35F and Y174F Blocks Binding and Phosphorylation by FAK Compared with Wild Type Nanog—Because FAK is a tyrosine kinase, we analyzed Nanog protein for the presence of potential tyrosines to be phosphorylated with NetPhos 2.0 software. The software analyzed all tyrosines in the sequence of Nanog protein (Fig. 7A) and predicted four tyrosine sites, Tyr-35, Tyr-136, Tyr-174, and Tyr-283, out of nine tyrosines that can be phosphorylated with the highest scores (Fig. 7A). The two tyrosines Tyr-35 and Tyr-174 had the highest scores to be phosphorylated (0.95 and 0.913, respectively). To test the effect of these tyrosines 35 and 174 on Nanog phosphorylation by FAK, we performed site-directed mutagenesis of tyrosines Tyr-35 and Tyr-174 that were changed to phenylalanine, Y35F and Y174F, sites inside the GST-Nanog protein. We isolated Nanog wild type and mutant Nanog proteins and performed an in vitro kinase assay with FAK. The Y35F and Y174F Nanog had significantly decreased phosphorylation by FAK compared with the wild type Nanog (Fig. 7B). Western blotting with Nanog antibody detected expression of all Nanog proteins (Fig. 7B). Thus, Nanog mutations of tyrosines Y35F and Y174F blocked direct Nanog phosphorylation by FAK.

To test if mutations of Y35F and Y174F have an effect on the binding of FAK and Nanog, we performed pulldown of GST-Nanog wild type protein and GST-Nanog Y35F and Y174F mutant proteins with FAK protein (Fig. 7C, upper panel). The wild type Nanog protein binds FAK, whereas Nanog mutants do not bind FAK (Fig. 7C). Coomassie Blue staining demonstrates an equal Nanog protein amount used for pulldown (Fig. 7C, lower panel). Thus, Tyr-35 and Tyr-174 are critical for direct binding of Nanog and FAK and for tyrosine phosphorylation by FAK.

To show phosphorylation of Nanog by FAK in vivo and to test the effect of tyrosine mutations on phosphorylation, we overexpressed HA-tagged FAK in 293 cells transfected with wild type Nanog and performed immunoprecipitation of Nanog and
Western blotting with phosphotyrosine antibody (Fig. 7D). The overexpressed FAK significantly increased tyrosine phosphorylation of wild type Nanog compared with 293 cells without FAK overexpression (Fig. 7D, right panel). When we overexpressed mutant Nanog Y35F and 174F with HA-FAK, the phosphorylation level of Nanog was significantly decreased compared with the wild type Nanog (Fig. 7D). To detect the effect of Nanog mutations on the binding with FAK, Western blotting with FAK antibody was performed and demonstrated increased binding of Nanog and FAK in the presence of overexpressed FAK.
FAK and wild type Nanog, but it was less in the case of with mutant Y35F and Y174F Nanog. Thus, the in vitro data confirm the in vivo kinase and pulldown assays and show decreased tyrosine phosphorylation and binding of Y35F and Y175F Nanog mutants by FAK compared with wild type Nanog.

Nanog Is Tyrosine-phosphorylated in Cancer Cells—To demonstrate tyrosine phosphorylation of endogenous Nanog in different cancer cells and confirm data of in vitro kinase assay, we immunoprecipitated Nanog in two colon cancer cell lines SW620 and SW480 and colon cancer and two teratocarcinoma cell lines NCCIT and NTERA-2, and we performed Western blotting with phosphotyrosine antibody (Fig. 7E, upper panel). We performed control immunoprecipitation without antibody and with His tag or HA tag antibodies (Fig. 7E). The Nanog was tyrosine-phosphorylated in all cancer cell lines in vivo. No tyrosine-phosphorylated Nanog was detected by immunoprecipitation without antibody or with His tag and HA tag antibodies (Fig. 7E). As additional control, we performed immunoprecipitation of Nanog and Western blotting with phosphotyrosine antibody in NCCIT cells, transfected with two Nanog siRNAs (Fig. 7E, left lower panel). We detected decreased tyrosine phosphorylation of Nanog in NCCIT cells with decreased Nanog expression by two Nanog siRNAs compared with control siRNA (Fig. 7E, lower left panel). In addition, we performed immunoprecipitation of Nanog in colon cancer SW480, teratocarcinoma NCCIT and NTERA-2 cells, treated with two FAK phosphorylation inhibitors inhibitor 14 (Y15) (29, 36) or TAE-226 inhibitor (31), respectively. Both FAK inhibitors inhibited FAK Tyr-397 phosphorylation and FAK in NCCIT and NTERA-2 cancer cells (Fig. 7E, lower middle and right panels) and in SW480 cells (data not shown). The Nanog tyrosine phosphorylation was decreased in the presence of FAK inhibitor in three different cancer cells: SW480, NCCIT, and NTERA-2 cells (Fig. 7E, upper panels). Thus, inhibition of FAK with two...
different FAK inhibitors decreased tyrosine phosphorylation of endogenous Nanog in different cancer cells. The data show that endogenous Nanog is tyrosine-phosphorylated in different cancer cells and that its inhibition of FAK decreased Nanog tyrosine phosphorylation.

Overexpression of Wild Type Nanog Increases Lamellipodia and Filopodia Formation but Overexpression of Nanog Y35F and Y174F Mutants Does Not—To test the effect of Nanog wild type and mutant Nanog Y35F and Y174F on cell morphology, FAK, and focal adhesion, we performed immunostaining of FAK and Nanog in 293 control cells and cells transfected with Nanog wild type and Nanog Y35F and Nanog Y174F pcDNA3 plasmids (Fig. 8A). Nanog is localized in the nucleus and perinuclear cytoplasmic regions in 293 cells, and overexpressed wild type Nanog had nuclear (white arrows), perinuclear, and cytoplasmic localization (Fig. 8A, white arrows). The cells with overexpressed wild type Nanog change cell morphology (Fig. 8, A and B). The cells transfected with wild type Nanog increased lamellipodia and filopodia formation. Both FAK and Nanog were detected in the nucleus (white arrows) and perinuclear regions (white arrowheads) (Fig. 8A). The same effect was observed in 480 cells. The 293 cells transfected with mutants Nanog Y35F and Y174F expressed less nuclear localization of Nanog, less co-localization with FAK, and less filopodia and lamellipodia formation than wild type Nanog (Fig. 8, A and B).

To quantify the effect of wild type Nanog and Y35F and Y174F Nanog on lamellipodia and filopodia formation, we stained actin with phalloidin-FITC in these cells and counted percent of lamellipodia and filopodia formation (Fig. 8B, upper panel). The Nanog wild type overexpression significantly increased lamellipodia and filopodia formation, whereas overexpression of mutant Nanog Y35F and Y174F did not cause a significant increase of lamellipodia and filopodia (Fig. 8B, upper panel). In addition, 293 cells transfected with Nanog wild type have polarized morphology, whereas mutant Nanog Y35F and Y174F do not (Fig. 8B, lower panel). Thus, overexpression of Nanog wild type but not the mutant Nanog Y35F and 174F increased lamellipodia and filopodia formation.

Overexpression of Wild Type Nanog Increases Cell Invasion That Is Decreased by FAK Phosphorylation Inhibitor and Induced by FAK More Significantly Than with Mutant Tyr-35 and Tyr-174 Nanog—To test the effect of Nanog overexpression on cell invasion, we performed an invasion assay with 293 cells transfected with pcDNA3 plasmid and with Nanog wild type pcDNA3 plasmid. Overexpression of wild type Nanog significantly increases cell invasion (Fig. 8C, left panel), which is consistent with increased lamellipodia and filopodia formations and cell polarization in these cells. To test the dependence of Nanog-induced invasion from FAK phosphorylation, we performed analysis of invasion in the presence of FAK phosphorylation inhibitor 14 or Y15 (29, 30). The FAK inhibitor Y15 significantly decreased Nanog-induced cell invasion (Fig. 8C, left panel). To test the effect of FAK overexpression on Nanog wild type and Nanog mutant-stimulated invasion, we analyzed cell invasion without FAK overexpression and with FAK overexpression in 293 cells transfected with Nanog wild type, and with mutant Y35F and Y174F Nanog plasmids. FAK overexpression increased Nanog wild type-stimulated cell invasion but FAK significantly less stimulated or did not stimulate Nanog-induced invasion in the case of mutants Y35F and Y174F Nanog, respectively (Fig. 8C, right panel), which is consistent with less tyrosine phosphorylation and binding of Y35F and Y174F Nanog.
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by FAK (Fig. 7D). The cells transfected with mutant Y35F and Y174F Nanog had significantly less FAK-induced invasion than wild type Nanog (Fig. 8C, right panel). Thus, overexpression of wild type Nanog increases cell invasion that is inhibited by FAK phosphotyrosine inhibitor or is increased by FAK more significantly than in case of the mutant Y35F and Y174F Nanog. Nanog siRNA Decreases Cancer Cell Growth Reversed by FAK Overexpression—Because Nanog was shown to increase cancer cell growth (6, 37), we tested the effect of Nanog down-regulation on cell growth. We transfected NCCIT teratocarcinoma cells with high expression of Nanog with two Nanog siRNAs and with control siRNA, and we show that both Nanog siRNAs that decreased Nanog expression (Fig. 1D) significantly decreased cell growth in NCCIT cells in contrast to control siRNA (Fig. 8D). Thus, cancer cell growth is dependent on Nanog expression and is significantly decreased by the silencing of Nanog expression. To test the inhibition of cell growth by Nanog down-regulation and its dependence on FAK signaling, we co-transfected 293 cells with control siRNA and two Nanog siRNAs that decreased Nanog expression (Fig. 1E) and measured cell growth (Fig. 8E). Both Nanog siRNAs decreased 293 cell growth, and FAK overexpression reversed its inhibition (Fig. 8E). When we down-regulated FAK expression with FAK siRNA or decreased FAK phosphorylation with FAK inhibitor Y15 at 1 and 10 μM, the 293 cell growth was inhibited by FAK siRNA compared with control siRNA transfected or untreated cells (Fig. 8F). The cancer cell growth was inhibited by FAK phosphorylation inhibitor in a dose-dependent manner (Fig. 8F). Thus, regulation of FAK expression by Nanog and FAK regulation of Nanog and its tyrosine phosphorylation affects cancer cell growth and plays a significant role in carcinogenesis.

DISCUSSION

In this report, we show that Nanog overexpression or induction of endogenous Nanog expression by HA induces FAK expression, although down-regulation of Nanog with siRNA decreases FAK expression in cancer cells. In addition, one breast cancer and three colon cancer tumors overexpressed Nanog, which correlated with FAK overexpression compared with normal colon tissues. Recent study with immunohistochemical analysis of 75 colon cancer tumors showed overexpression of Nanog in tumors compared with normal tissues, which correlated with poor prognosis, lymph node status, and tumor stage (10). Thus, overexpression of Nanog and FAK proteins in breast and colon cancer tumors can be associated with more aggressive tumor phenotype.

In this report, we, for the first time, identify four potential binding sites in the FAK core promoter sequence and demonstrate that Nanog up-regulates FAK promoter activity. The site-directed mutagenesis and Dual-Luciferase assay demonstrates that mutation of the four Nanog sites blocks induction of FAK promoter activity by Nanog. EMSA confirmed and demonstrated direct binding of Nanog to the four sites in the FAK promoter. This is consistent with data on the crystal structure of Nanog, where the authors determined that the homeobox domain of Nanog binds preferentially to the TAAT(G/T)ATT(G/T) site (3). Another group demonstrates that the minimal sequence for Nanog binding is an ATT(A/T)AAT sequence in the fetal liver kinase-1 (FLK1) promoter (38). Binding of Nanog transcription factor to the TAAT sequence of Cdc-2 promoter was also demonstrated by another group (39). Thus, we identified the four Nanog-binding sites in the FAK promoter and showed by Dual-Luciferase assay and by EMSA that Nanog binds to four sites in the FAK promoter, which is critical for regulation of FAK expression in cancer cells. We also show binding of Nanog to FAK promoter chromatin by ChIP assay, which confirms Dual-Luciferase and EMSAs. Interestingly, p53 was shown to bind the Nanog promoter, to suppress Nanog expression, and to induce differentiation of embryonic stem cells (40). p53 was shown to bind the FAK promoter and inhibit its activity in cancer cells (14, 26, 27, 41). The link between p53-Nanog-FAK promoter regulation in cancer stem cells will be important to study in the future. We detected that Nanog overexpression up-regulated FAK expression and increased filopodia and lamellipodia formation and increased cancer cell invasion that was inhibited by FAK inhibitors. Induction of FAK expression by Nanog and increased cell invasion are consistent with data on the role of FAK in cancer cell motility, metastasis, and angiogenesis (22, 42). We detected that two Nanog siRNAs decreased FAK expression and decreased cancer cell growth that was reversed by FAK overexpression. These data are consistent with the recent data on increased tumorigenicity of Nanog-overexpressing cells with up-regulated FAK (24) and with data that tumor-initiating stem cells of squamous cell carcinoma are controlled by FAK-integrin signaling (43).

The link between HA-induced Nanog and FAK expression is also important, because hyaluronan and FAK were shown to be linked in cancer cells, where hyaluronan activated FAK and increased invasion in glioma cells (44). Nanog has been shown also to be essential for glioblastoma tumorigenicity (45) and was regulated by the Hedgehog-GLI pathway, required for glialblasta tumor growth (46). Recently, Oct3/4 overexpression increased FAK and Src expression in glioblastoma cells, which resulted in increased cell invasion (25). Hyaluronan is the major glycosaminoglycan in the extracellular matrix and its expression regulates tumorigenesis (47). HA has been shown to stabilize focal adhesions in esophageal squamous cell carcinoma (48). Induction of Nanog by hyaluronan induced interaction of CD44 (HA receptor) and Nanog protein in breast cancer cells and Nanog overexpression regulated drug resistance by affecting cytoskeletal protein ankyrin and the efflux of chemotherapy drugs (34). The up-regulation of Nanog by HA and up-regulation of FAK promoter activity and FAK expression are important for cancer stem biology and tumorigenesis. Thus, up-regulation of FAK promoter activity and expression by HA-induced Nanog provide a novel mechanism of regulating cancer cell growth and tumorigenesis.

In addition, we show that Nanog and FAK proteins directly interact by pulldown and immunoprecipitation assays in vitro and in different cancer cells in vivo. The N-terminal domain of FAK interacts with Nanog, whereas the kinase and C-terminal domains do not interact with Nanog protein. The binding of the N-terminal domain of FAK with Nanog is consistent with data on the nuclear localization of the N-terminal domain of FAK (49) and with binding with other transcription factors and proteins, such as p53 (16, 50, 51) and Mdm-2 (17). This indicates an
additional critical function of the N terminus-containing FERM domain in carcinogenesis that was reviewed recently (52). The authors indicate that many FERM domain proteins shared nuclear export signals and nuclear localization signals, suggesting that the FERM domain mediates signaling between the nucleus and the cytoplasm. Using confocal laser scanning microscopy, we also detected co-localization of Nanog and FAK proteins in the nuclear and perinuclear cytoplasmic areas. The data on co-localization of Nanog and FAK suggest the nucleo-cytoplasmic shuffling of both proteins. In fact, both proteins were reported to be localized in the cytoplasm and the nucleus (16, 53). Nanog was reported to be localized in the nucleus in embryonic stem cells (54) and was found in the nucleus and cytoplasm in different cancer cells and tumors (10). Recently, nuclear and cytoplasmic Nanog was detected by immunohistochemical staining in human glioma tumors (55). Nanog protein was demonstrated to have in addition to the nuclear localization signal one nuclear export signal, suggesting a nucleo-cytoplasmic shuffling of Nanog (56). The mechanism of nuclear export of Nanog remains to be determined, but it is consistent with data on cytoplasmic localization of Nanog in cancer cells and tumors (10, 35). The co-localization of FAK and Nanog in the nucleus and perinuclear areas suggests the important role of this complex in the nuclear and cytoplasmic signaling.

In this report, we show that FAK directly tyrosine-phosphorylates Nanog in a dose-dependent manner by in vitro kinase assay and by immunoprecipitation and Western blotting with phosphotyrosine-specific antibody in vivo. In addition, we show that endogenous Nanog is tyrosine-phosphorylated in different cancer cells: SW620 colon cancer and teratocarcinoma NCCIT and N-TERA-2 colon cancer cells. In addition, we show that FAK inhibitor decreases Nanog phosphorylation in cancer cells. Nanog is a phosphoprotein, and the mechanisms of its post-translational modification and phosphorylation remain unknown (57). The first report demonstrated phosphorylation of Nanog at multiple serine/threonine/proline motifs (57). The authors showed that Ser/Thr/Pro phosphoprotein phosphorylated Nanog interaction with prolly isomerase Pin1 that stabilized Nanog, which was critical for self-renewal and the teratoma-forming potential of embryonic stem cells (57). Our report shows Nanog tyrosine phosphorylation by FAK using in vitro kinase assay and by immunoprecipitation assays in cancer cell lines. The detailed analyses by mass spectrometry and other approaches of all tyrosine phosphorylation sites of Nanog in cancer cells will be performed in future studies. The data on direct tyrosine phosphorylation of Nanog by FAK are linked to the PKC and HA pathways. The PKCε was reported to phosphorylate serines of Nanog in cancer cells (53). Unphosphorylated Nanog was localized in the cytoplasm in MCF-7 breast cancer cells, and after treatment with HA that activated PKC, Nanog was translocated to the nucleus. It is known that HA activates the FAK pathway, stabilizes focal adhesion and filopodia, and induces proliferation (48). PKC and FAK phosphorylation were shown to be linked and were involved in the regulation of cell motility and cytoskeletal changes in cancer cells (58). Thus, understanding the direct FAK-Nanog interaction and its cross-linked regulation by PKC and HA signaling is very important for the cancer cell biology field.

We show that site-directed mutagenesis of tyrosine 35 and tyrosine 174 with the highest predicted scores phosphorylated by NetPhos 2.0 software decreased binding and phosphorylation by FAK and affected cancer cell filopodia and lamellipodia formation and invasion. This is the first report on tyrosine phosphorylation of Nanog showing a novel function of Nanog protein dependent on FAK signaling that will be studied in detail in a future report. Interestingly, we show that c-Src can also tyrosine-phosphorylate Nanog. It is known that FAK and Src bind and are involved in survival signaling (15). The phosphorylation of Nanog by FAK and Src is consistent with a recent report (59) where inhibitors of FAK and Src suppressed cell growth and impacted the derivation of beta cells from the human pluripotent stem cells. Thus, FAK-Src-Nanog-linked signaling and Nanog tyrosine phosphorylation by these proteins can play a significant role in tumor cell survival. Pyk-2, a highly homologous kinase to FAK, is also able to phosphorylate Nanog. These data add a new mechanism of post-translational intracellular regulation of Nanog by FAK, Pyk-2, and Src proteins, which are cross-linked and are critical for cancer cell signaling and survival. We also detected direct phosphorylation of Nanog by PKC (data not shown) that was reported recently (53). The authors detected Nanog serine phosphorylation by PKCe. The HA-CD44 activated PKCe-phosphorylated Nanog, stimulated breast tumorigenesis, and increased survival signaling (53). PKC signaling is also connected with FAK signaling. In one report, the PKC epsilon/− fibroblasts were shown to express reduced FAK activity (60). In another report, FAK-overexpressing cells activated PKC-α, -β, and -ε isoforms (61). PKC signaling is also connected to the Src pathway (62). Thus, PKC-FAK-Src and Nanog cross-talk can mediate survival signaling in cancer cells. This report demonstrates that FAK directly phosphorylates Nanog and also shows that inhibition of FAK with PF-228 inhibitor from Pfizer or with Y15 or TAE-226 blocks Nanog phosphorylation, which is critical for blocking cancer cell invasion and cell growth.

Moreover, Nanog siRNA decreased cancer cell growth, and overexpression of FAK reversed this inhibition. In addition, FAK phosphorylation inhibitor Y15 decreased cell growth in a dose-dependent manner. The down-regulation of Nanog decreased FAK promoter activity and FAK expression and decreased cell growth, whereas FAK overexpression reversed this inhibition. Thus, the positive loop regulation between Nanog and FAK is critical for cancer cell growth. These data are consistent with recent data on increased FAK expression by Nanog in 293 cells (24) and increased FAK by Oct3/4 in glioblastoma (25). The up-regulation of cancer growth by Nanog was reported in prostate (11), breast (53), kidney (24), and other types of cancer. This report reveals a new mechanism of cross-regulation of FAK and Nanog in cancer cells and detects a new signaling pathway in cancer cell biology.

In summary, this is the first report to identify the four binding sites of Nanog in the FAK promoter sequence, to show that Nanog regulates FAK promoter activity, to demonstrate binding of Nanog transcription factor to the four Nanog-binding sites in the FAK promoter by site-directed mutagenesis, Dual-
Nanog and FAK Interaction in Cancer Cells

Luciferase assay, EMSA and ChIP assays, and to find direct binding of proteins by pulldown assay and immunoprecipitation analyses and co-localization with confocal laser scanning microscopy, and to show that the N-terminal domain of FAK binds Nanog protein. In addition, this report shows by in vitro kinase assay and immunoprecipitation that FAK directly phosphorylates Nanog protein in a dose-dependent manner and that phosphorylation of Nanog is blocked by a FAK phosphorylation inhibitor. By site-directed mutagenesis, we show that mutation of tyrosines Y35F and Y174F abolished Nanog tyrosine phosphorylation by FAK and blocked Nanog binding with FAK both in vitro and in vivo. We show that overexpression of wild type Nanog but not Y35F and Y174F Nanog increased lamellipodia/filopodia formation and cell polarization. We show that wild type Nanog increased cell invasion, and Nanog-induced invasion was inhibited by FAK phosphorylation inhibitor. Moreover, FAK overexpression significantly increased cell invasion in the case of wild type Nanog but less significantly in case of the mutant Y35F and Y174F Nanog. Down-regulation of Nanog blocked cancer cell growth, and this inhibition was reversed by FAK overexpression. Taken together, the data demonstrate for the first time binding of Nanog to the FAK promoter to four binding sites, induction of FAK expression by Nanog, direct binding of FAK and tyrosine phosphorylation of Nanog and show functional significance of Nanog Tyr-35 and Tyr-174 sites in cell morphology and invasion that are critical for cancer and stem cell biology and signaling.

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