Regulation of KiSS-1 Metastasis Suppressor Gene Expression in Breast Cancer Cells by Direct Interaction of Transcription Factors Activator Protein-2α and Specificity Protein-1*

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KiSS-1 has been shown to function as a tumor metastasis suppressor gene and reduce the number of metastases in different cancers. The expression of KiSS-1 or KiSS1, like other tumor suppressors, is commonly reduced or completely ablated in a variety of cancers via an unknown mechanism. Here we show that the loss of KiSS-1 expression in highly metastatic breast cancer cell lines correlates directly with the expression levels of two transcription factors, activator protein-2α (AP-2α) and specificity protein 1 (Sp1), which synergistically activate the transcriptional regulation of KiSS-1 in breast cancer cells. Although the KiSS-1 promoter contains multiple AP-2α binding elements, AP-2α-mediated regulation occurs indirectly through Sp1 sites, as determined by deletion and mutation analysis. Overexpression of AP-2α into highly metastatic breast cell lines did not alter KiSS-1 promoter-driven luciferase gene activity. However, co-transfection of AP-2α wild-type or the dominant negative form of AP-2 lacking its C-terminal DNA-binding domain, AP-2B, together with Sp1, increased KiSS-1 promoter activity dramatically, suggesting that AP-2α regulation of KiSS-1 transcription does not require direct binding to the KiSS-1 promoter. Furthermore, we demonstrated that AP-2α directly interacted with Sp1 to form transcription complexes at two tandem Sp1-binding sites of the promoter to activate KiSS-1 transcription. Together, our results indicate that AP-2α and Sp1 are strong transcriptional regulators of KiSS-1 and that loss of or decreased expression of AP-2α in breast cancer may account for the loss of tumor metastasis suppressor KiSS-1 expression and thus increased cancer metastasis.

The vast majority of breast cancer deaths result from complications caused by tumor cell metastasis rather than as a consequence of the original tumor growth. Once tumorigenic cells enter into the vascular and lymphatic systems, they travel to peripheral regions where they invade tissues and form neoplasms. Metastasis is a process requiring detachment of cancer cells from the primary site, survival of shear forces encountered in the circulation, migration to other organs, attachment to and invasion of tissues, proliferation of these cells at the secondary site, and finally the capacity to enlist neighboring capillaries to supply the tumor with nutrients as it develops (1). Interference at any one of these steps can block this metastatic cascade thereby preventing the formation of metastatic tumor growths. Consequently, there is a growing interest in researching the metastatic process to identify possible ways to inhibit its progression.

Metastasis suppressor genes, which inhibit the spread of cancers to secondary sites, have become the target of mounting clinical and basic cancer research. One such gene, KiSS-1 or KiSS1, was originally identified as a metastasis suppressor by microcell-mediated transfer in melanoma lines, by which it was found to reduce tumor cell invasive and migratory properties without affecting their tumorigenicity (2). Since then, KiSS-1 has been shown to act as a potent anti-metastatic agent either by treatment using synthesized KiSS-1 peptide or upon ectopic expression in highly metastatic cells (2–5). Loss or reduced expression of KiSS-1 has been found in a variety of tumor metastasis, including breast cancer, bladder cancer, pancreatic cancer, and esophageal squamous cell carcinoma (3, 5–7). Together, these studies suggest that KiSS-1 is a human metastasis suppressor gene and that loss of KiSS-1 and its receptor may correlate with human tumor progression and metastasis.

The KiSS-1 gene encodes a largely hydrophobic 145-amino acid protein highly expressed in the placenta (4, 8). The KiSS-1 gene product consists of a protein kinase phosphorylation domain, a secretory signal, and polyproline-rich region, and a number of potentially important motifs for post-translational modifications (8, 9, 13). Independently, three groups discovered and isolated the 54-amino acid C-terminally amidated fragment of KiSS1 protein (amino acids 68–121), termed metastin and kisspeptin, respectively (4, 10). The function of KiSS-1 peptide is mediated through interaction with the membrane-bound G-protein-coupled receptor, GPR54, a close relative of the galanin receptor (4, 8, 10–13). C-terminal amidation of the KiSS-1 peptide leads to strong binding with GPR54, initiating a series of cellular changes, including increased intracellular [Ca2+]i and inositol 1,4,5-trisphosphate release, as well as morphological changes, such as up-regulating focal adhesion and stress fiber formation (4, 8, 10, 13). Collectively, these signaling events inhibit chemotaxis and invasion, reducing the incidence of tumor metastasis (4, 5, 13). However, while much has been learned about the possible physiological effects of KiSS-1 expression in cancer cells, the mechanism controlling KiSS-1 transcriptional regulation is still unknown as is the underlying reason for its loss during metastatic progression.

As breast cancer cells become increasingly metastatic, expression levels of different genes contributing to cell cycle, tumor cell invasion, and migratory properties, are altered. Genes commonly found up-regulated...
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in breast cancer, including cyclin D1, c-myc, and matrix metalloproteinase-9, mediate behavioral and proliferative changes that stimulate oncogenesis, whereas the loss of c-Kit or p53 may also encourage an increased metastatic phenotype, because they are no longer present to block formation of neoplasms (14–19). Transcription factors that modulate the expression of oncogenes play important roles during tumorigenesis and metastasis. AP-2α, a 52-kDa transcription factor, regulates genes that are important during development and metastatic processes (20, 21). Genes under AP-2α-modulated transcriptional regulation contain the consensus palindromic sequence 5′-GCCNNNGGC-3′ and have a variety of cellular functions, such as human proenkephalin, plasminogen activator inhibitor type 1, c-erbB-2, c-Kit, thrombin receptor, and vascular endothelial growth factor (19, 22–26). Located on the short arm of chromosome 6, AP-2 protein contains a DNA-binding domain, a protein kinase A phosphorylation site, and a transactivation domain (27, 28). A naturally occurring splice variant, AP-2B, lacks the DNA-binding domain, thus enabling it to function as a dominant negative form of the AP-2α protein (29). AP-2α has been shown to regulate neoplasm development by directly and indirectly regulating gene expression. In addition to inducing or repressing activity of cancer-related genes at their promoter, AP-2α can physically interact with oncogenes, such as β-catenin, DEK, and Pax6, and is thereby directly involved in tumorigenesis and development (30–35). Loss of AP-2α is common in breast cancer and in many other cancers, resulting in the loss of regulation of multiple oncogenes and increased tumorigenesis.

Sp1 has also been shown to regulate genes involved in tumorigenesis, including the up-regulation of both hepatocyte growth factor receptor and vascular endothelial growth factor (36–39). Additionally, Sp1 can directly interact with the c-Jun transcription factor to modulate the up-regulation of vimentin, a protein commonly found misregulated in metastatic tumors (40). Sp1 itself has been shown to be a useful molecular marker is gastric cancer and Sp1 expression has been shown to be closely associated with patient survival rate (16, 41).

Here, we report that the expression of KiSS-1 metastasis suppressor gene in breast cancer cells is directly correlated with the expression of transcription factors AP-2α and Sp1, and that AP-2α and Sp1 synergistically activate the transcriptional regulation of KiSS-1 in breast cancer cells. Furthermore, we demonstrate that KiSS-1 expression is modulated by AP-2α through direct interaction with the transcription factor Sp1 at two tandem Sp1-binding sites rather than via interaction with the consensus AP-2-binding sites of KiSS-1 promoter. These results offer a mechanism for the loss of KiSS-1 gene expression commonly seen in metastatic breast cancers and provide another molecular mechanism by which AP-2α and Sp1 transcription complex modulates tumorigenesis and tumor progression.

MATERIALS AND METHODS

Cell Lines, Chemicals, Constructs, and Oligonucleotides—MCF-7, MDA-231, MDA-435, and T47D cells were obtained from the American Type Culture Collection (Manassas, VA). Dulbecco’s modified Eagle’s medium or RPMI 1640 supplemented with 10% fetal bovine serum. After 18–20 h when cells were ~60% confluent, reporter gene constructs were transfected using Lipofectamine reagent according to the manufacturer’s protocol (Invitrogen). In brief, 1 μg of total DNA was transfected into each well of a 24-well plate using a Lipofectamine to DNA ratio of 2:1 for a period of 6 h. Empty vector was used to offset the difference in DNA concentrations in reactions in which fewer test plasmids were transfected. Transfection reagent was then removed from each well, and cells were incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were harvested after 48 h, and luciferase activity of protein lysates was measured following the manufacturer’s protocol (Luciferase Assay System, Promega). To normalize for differences in cell line transfection efficiencies, all cells were transfected with pRSV-β-gal control vector (Promega). β-Galactosidase levels were then measured following the manufacturer’s protocol (Galacto-Light Plus, Bedford, MA). For electrophoretic mobility shift assay (EMSA), nuclear extracts from MCF-7 cells were harvested as described previously (38). Concentration of nuclear extracts was determined using BCA assay (Pierce). Aliquots of nuclear protein were frozen and stored at ~80 °C until used.

Western Immunoblot Analysis—Breast cell lines (2.0 × 10^7) were seeded in 100-mm Petri dishes with 10 ml of complete medium and incubated overnight. The cells were then scraped off and washed in cold phosphate-buffered saline. The cell pellet was then lysed in 0.5 ml of RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 mM NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, 100 units/ml aprotinin). Soluble proteins were then separated by centrifugation at 15,000 × g for 5 min at 4 °C. Protein concentration was determined. Samples were then diluted into loading buffer at 1 mg/ml. Following heat denaturation, samples containing 10 μg of protein were loaded onto and separated on 10% or 15% SDS-PAGE gels as needed. Proteins were then transferred electrophoretically to 0.45-μm nitrocellulose membrane (Pall Corp., Pensacola, FL). After incubating the membranes in blocking solution, primary antibody was added at 1:1,000 dilution, followed by secondary antibody incubation at 1:10,000. Proteins were detected using the SuperSignal West Pico Chemiluminescent substrate (Pierce) according to the manufacturer’s instructions.

Electrophoretic Mobility Shift Assay—KiSS-1 promoter-derived oligonucleotides were synthesized and annealed, and 5 pmol was 5′-end labeled using T4 Kinase and [γ-32P]ATP. A 30-μl EMSA reaction containing ~100 mM potassium chloride, 3 μg of crude nuclear extract, 1
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μg of poly(dI-dC) with or without unlabeled competitor oligonucleotide, and 10 fmol of labeled probe was incubated on ice for 20 min. A Sp1-specific antibody was then incubated in appropriate reactions for 20 min on ice. A separate AP-2 antibody was used for gel-shift analysis (Active Motif, Montreal, Canada). DNA-protein complexes were then resolved on 5% PAGE gel at ~120 V at room temperature for 2 h. Antibody-protein complexes were observed as supershifted or immunodepleted complexes.

ChiP Assay—Chromatin immunoprecipitation (ChiP) was performed following the protocol outlined by the ChiP assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, MCF-7 cells transfected with both AP-2α and Sp1 (2 × 10⁷ cells) were fixed with 1% formaldehyde, scraped into conical tubes, pelleted, and lysed in SDS lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A. DNA was sheared to fragments of 200–500 bp by eight 10-s sonications. The chromatin was precleared with salmon sperm DNA/protein A-agarose slurry (Upstate Biotechnology) for 1 h at 4 °C with gentle agitation. The agarose beads were pelleted, and the precleared supernatant was incubated with antibodies to IgG, AP-2α, and Sp1 overnight at 4 °C. The region between -288 and -188 of the KiSS-1 promoter was amplified from the immunoprecipitated chromatin using the following primers: sense, 5'-ATAAGCCCATTTCCAGTTG-3' and antisense, 5'-GGCCGGACTTTCTCTTTC-3'. Following PCR, the 100-bp product was resolved on a 2.5% agarose gel and stained with ethidium bromide. Samples were visualized under UV light.

Co-immunoprecipitation Analysis—Binding of AP-2α and Sp1 in transfected MCF-7 cells was examined by immunoprecipitation (IP) and by Western blot analysis. Briefly, cells were lysed with RIPA buffer containing 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 10 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 1 mg/ml leupeptin, followed by sonication with a 550 Sonic dismembrator (Fisher Scientific) and immunoprecipitated with the indicated antibodies. Anti-AP-2α and anti-Sp1 immunocomplexes were recovered by using protein A beads (Sigma). All immunoprecipitates were washed four times with lysis buffer and were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore). After incubation in TBST buffer (20 mM Tris-HCl, pH 7.5/500 mM NaCl/0.02% Tween 20) containing 0.2% bovine serum albumin and 5% dry milk powder for 2 h, the membranes were probed with the indicated antibodies and visualized with the SuperSignal West Pico detection system (Pierce).

Semi-quantitative Reverse Transcription-PCR Analysis—Total RNA was isolated from breast cancer cell lines with TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase and oligodT (Promega) according to the manufacturer’s protocol. Primer sequences used for detection of KiSS-1 transcripts were 5’-GCCCACTGAACTCAGCTG-3’ and 5’-CTGC-CGGCAGCTGGC-3’. Amplified products were ~400 bases in length. Additionally, primers for β-actin were 5’-GGCTCCCGATGTGCAAGGC-3’ and 5’-AGATTTTCTCTCACCTGTC-3’, which resulted in PCR products of ~200 bases. Optimal PCR cycles required for linear amplification for each set was determined. β-Actin required 21–23 cycles per reaction, whereas KiSS-1 required 24–28 cycles. PCR products were separated using agarose gels of appropriate concentration, visualized by EtBr staining and quantitated using Alpha Imager software (Alpha Innotech, San Leandro, CA).

RESULTS

Endogenous KiSS-1 Expression in Breast Cancer Cell Lines Correlates with AP-2α Expression Levels—We and other laboratories have reported that KiSS-1 expression is lost in highly metastatic breast cancer cells. To determine if loss of the transcription factor AP-2α and Sp1 was directly or inversely associated with loss of KiSS-1, the expression levels of AP-2α and Sp1 in both highly metastatic and non-metastatic breast cells were compared with the expression of KiSS-1 (Fig. 1A). Due to the lack of a specific and effective KiSS-1 antibody, KiSS-1 expression level in the breast cell lines was quantitated using RT-PCR and normalized to the β-actin level within each sample. The relatively non-metastatic breast cell line, MCF-7, showed dramatically higher -fold expression as compared with the more metastatic breast tumor cell lines (T47D, MDA-231, and MDA-435). Similarly, Western blot analysis demonstrated that AP-2α expression was lost or barely detectable in the metastatic cell lines, MDA-231 and MDA-435 (Fig. 1A). The expression levels of Sp1 were also examined in these cells. As shown in Fig. 1A (bottom), Sp1 expression was slightly decreased in the metastatic breast cancer cell lines (Fig. 1A). Together, these data suggest that the expression of KiSS-1 was correlated very well with the expression levels of the two transcription factors, especially AP-2α, in breast cancer cells. To further confirm that KiSS-1 expression is regulated by AP-2α and Sp1, we reintroduced both AP-2α and Sp1 into these cells (Fig. 1B). Activation of the KiSS-1 promoter by overexpression of AP-2α and Sp1 was enhanced.

![FIGURE 1. Positive correlation of KiSS-1 expression with transcription factors, AP-2α and Sp1, in breast cancer cells.](image-url)
considerably from 4- to 27-fold depending on the cell line. Although it is not clear why transfection of AP-2/H9251 and Sp1 strongly activate KiSS-1 promoter construct in MCF-7 cells where these factors are expressed at higher endogenous level, we speculate that overexpression of these two proteins have an additive effect on the activation of the KiSS-1 promoter construct with the endogenous factors. Therefore, we concluded that the loss of both AP-2/H9251 and Sp1 strongly and directly correlated with the loss of KiSS-1 expression in breast cancer cells.

Identification of the Regulatory Region of the KiSS-1 Gene by AP-2/H9251 and Sp1—Regions of sequence conservation, found between humans and mice or rats, are commonly used as an indicator of whether a sequence, such as a promoter sequence, may be maintained over the course of evolution to serve an important molecular function, such as the binding of transcription factors necessary for transcriptional regulation of that gene. To understand how AP-2 and Sp1 regulate the expression of KiSS-1 gene, we examined and analyzed the potential regulatory regions of KiSS-1 gene among different species (Fig. 2A). As sequence homology appeared to drop sharply after the first 1.2 kb, we initially focused our promoter analysis within this region, which was then cloned into a luciferase reporter vector. Sequence analysis of the highly conserved 400-bp region (~70% homology) located 5′ proximal to the KiSS-1 coding region contains three consensus AP-2α binding elements flanked by multiple Sp1 sites (Fig. 2B). Given that the KiSS-1 promoter, much like other AP-2-regulated genes, contains multiple AP-2α binding elements and a regulatory region rich in G+C content, it became a likely candidate gene regulated by AP-2/Sp1 complexes in tumor cells.

AP-2α and Sp1 Synergistically Activate KiSS-1 Transcription—To determine whether AP-2 and Sp1 were involved in transcriptional regulation of the KiSS-1 tumor metastasis suppressor gene, luciferase reporter constructs bearing 1.2-kb region of the KiSS-1 promoter were transfected into breast cell lines along with AP-2, Sp1, or both. Using β-galactosidase to normalize for transfection efficiencies, we analyzed the luciferase activity driven by the KiSS-1 promoter. Results showed that while KiSS-1-luciferase activity was only slightly increased upon overexpression of either AP-2 or Sp1 in MCF-7 cells, KiSS-1 promoter activity was increased more than 10-fold in cells co-transfected with both AP-2 and Sp1 constructs (Fig. 2C). Additionally, luciferase constructs bearing serial deletions of the KiSS-1 promoter were assayed for activation upon co-expression of AP-2/Sp1 complex and that the promoter region sensitive to
AP-2α and Sp1 control intervenes between these sites (156 and 292 bp).

**AP-2α-activated KiSS-1 Transcription Is Independent of Direct Promoter Binding but through Interaction with Sp1 at Two Consensus Sp1 Sites**—To understand the molecular mechanism of KiSS-1 gene transcriptional regulation by AP-2α and Sp1, we examined whether direct DNA binding of AP-2α and Sp1 is essential for the remarkable activation of KiSS-1 promoter. AP-2 and Sp1 constructs lacking the DNA-binding domains of each transcription factor (AP-2β and Sp1-ΔDBD) were co-transfected into MDA-435 and MCF-7 cells with the 292-bp KiSS-1 promoter luciferase construct and measured for luciferase activity (Fig. 3A). As expected, dominant negative Sp1-ΔDBD failed to acti-
vate KiSS-1 promoter-mediated luciferase activity when co-transfected with AP-2α. However, AP-2B, the truncated AP-2α lacking the C-terminal DNA-binding domain, significantly increased luciferase activity to the same degree as the wild-type form of AP-2 when co-transfected with Sp1 transcription factor. These results suggest that the DNA-binding domain of AP-2α is not necessary in modulating KiSS-1 transcriptional activity in human breast cancer cells.

To determine the discrete transcriptional binding sites essential in mediating AP-2α/Sp1 transcriptional regulation of KiSS-1, we generated additional truncation mutants of 150, 190, 230, 260, and 300 bp in the luciferase reporter plasmid, as shown in Fig. 3B. These truncated reporter plasmids were co-transfected with AP-2α and Sp1 in breast cell lines for further luciferase analysis. As shown in Fig. 3B, the promoter region between −230 and −260 demonstrated the greatest sensitivity to AP-2α/Sp1 transcription factors. Co-transfection of AP-2α and Sp1 significantly stimulates the activity of the 260-bp KiSS-1-Luc, similar to that exhibited by the 300-bp KiSS-1-Luc construct, but did not contain any putative AP-2 sites. The 260-bp KiSS-1-Luc does, however, contain two consensus Sp1 sites in tandem, identified as Sp1A and Sp1B (Fig. 3B). To further determine if these two Sp1 sites were essential in mediating the transcriptional modulation of the KiSS-1 promoter by AP-2α and Sp1, constructs of single targeted deletion (mut Sp1-A or mut Sp1-B) at these two sites as well as double deletions (mut Sp1-A/Sp1-B) at both sites of the KiSS-1 promoter were generated and tested in luciferase assay (Fig. 3C). Results showed that mutation at either Sp1 site rendered the KiSS-1 promoter insensitive to the regulation of AP-2α and Sp1 transcription factors in MCF-7 cells (Fig. 3C), suggesting that the two Sp1 sites are essential for the observed regulation of KiSS-1 promoter by AP-2α and Sp1 transcription factors.

Sp1 and AP-2α Form a Complex at the Sp1 Sites of KiSS-1 Promoter—To determine the complex formation of Sp1 and AP-2α at the two Sp1 sites in mediating AP-2α/Sp1 regulation of the KiSS-1 gene transcription, gel mobility shift analysis (EMSA) was performed using the GC-rich sequence spanning −230 to −260 of the KiSS-1 promoter (Fig. 4A). Results of EMSA using AP-2α and Sp1-transfected MCF-7 cells showed that Sp1 binds to the 30-bp γ32P-labeled probe (lanes 2–4), however this DNA-protein interaction was abrogated in the presence of unlabeled competitor (lane 5). The negative control showing labeled probe in the absence of nuclear extract is shown in lane 1. Upon addition of a Sp1-specific antibody, the migration of the DNA-protein complex was sterically hindered by the bound antibody, resulting in a supershifted third band (lane 3). However, the ability to supershift the labeled probe was specific for the Sp1 antibody, because the antibody for IgG failed to exhibit any ability to complex with this protein-bound probe (negative control, lane 4). Incubation of the labeled promoter fragment with anti-AP-2 antibody resulted in a diffusion of the bands formed by the Sp1-DNA complex, indicating that AP-2 may also bind to this complex.

To further demonstrate that AP-2α exists in the Sp1-DNA complex, we performed chromatin immunoprecipitation (ChIP) assays using sheared DNA isolated from AP-2α/Sp1 co-transfected MCF-7 cells. Overnight immunoprecipitation of chromatin-bound DNA using antibodies to Sp1, AP-2, and IgG was followed by PCR using primers that amplified the 100-bp region spanning the length of the two Sp1 sites (Sp1-A and Sp1-B) (Fig. 4B). As shown in the ChIP assays (Fig. 4C), anti-Sp1 and anti-AP-2 antibodies were capable of immunoprecipitating the KiSS-1 promoter fragment containing the two Sp1 sites (lanes 4 and 5), however, immunoprecipitation using anti-IgG failed to produce a PCR product (Fig. 4C, lane 3).

AP-2α Interacts Directly with Sp1 at an N-terminal Domain of the Protein—Our data indicate that both AP-2α and AP-2α C-terminal deletion mutant (AP-2B) lacking the DNA-binding domain synergistically enhance Sp1-mediated activation of KiSS-1, suggesting that the effect of AP-2α is through the interaction with Sp1, independent of its ability to directly bind the AP-2 consensus sites of the KiSS-1 promoter. To demonstrate the N-terminal region of AP-2α without the C-terminal DNA-binding domain directly interacts with Sp1 in the cell, MCF-7 breast cancer cells were co-transfected with Sp1, AP-2, and AP-2B or control vector. Cell lysates were incubated with antibodies specific to Sp1 as well as antibodies to the N-terminal domain of AP-2α, respec-
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Treatment of metastatic breast cancer cells with synthetic KiSS-1 peptide as well as ectopic overexpression of KiSS-1 in these cells has been shown to significantly alter overall cellular morphology and behavior, thus hindering metastasis (2, 4, 8, 10). The expression of KiSS-1, like other tumor suppressors, is commonly reduced or completely ablated in a variety of different cancers (3, 5–7). As the level of KiSS-1 expression has been correlated to patient survival and severity of metastatic development, KiSS-1 has become an effective biomarker for the metastasis of these cancers (7). In this study, we demonstrate that the expression of KiSS-1 is directly correlated with the expression level of another known tumor-suppressor, the AP-2α transcription factor. Highly metastatic breast cell lines exhibit little to no AP-2α expression, paralleling the loss of the KiSS-1 gene in these cells. Additionally, we found that AP-2α and Sp1 synergistically activate the transcriptional activation of KiSS-1 promoter in multiple breast cancer cell lines via direct interaction of these two transcription factors at two tandem Sp1-binding sites of the promoter. Although MCF-7 exhibited higher levels of endogenous AP-2α and Sp1 expression relative to the other breast lines (Fig. 1A), the significant increase in KiSS-1 promoter activation seen upon transfection of both transcription factors (Fig. 1B) is likely due to the additive effect caused by the overexpression of these proteins in the same cells transfected with the KiSS-1 promoter construct.

The KiSS-1 promoter was found to contain multiple AP-2α-binding sites, all of which were flanked by clusters of Sp1 sites; furthermore, this region was rich in G+C content, making KiSS-1 a likely candidate for regulation by AP-2α and Sp1 complex. Serial truncations of the KiSS-1 regulatory region demonstrated that the AP-2α/Sp1 regulation was mediated through a region of the promoter between −230 and −260 bp with only two putative Sp1 consensus sites. The ability of Sp1 to bind to this discrete 30-bp sequence was confirmed in EMSAs in which an Sp1-specific antibody could bind this protein-bound promoter segment as evidenced by its ability to supershift the complex. EMSA results for anti-AP-2α-antibody incubated nuclear extracts showed a diffusion of the bands formed by the Sp1-DNA complex, thus indicating that AP-2α may also bind this complex. Additional chromatin immunoprecipitation using the region of the promoter carrying the two tandem Sp1 sites demonstrated that both AP-2α and Sp1 were capable of promoter interaction. Mutation analysis at these two putative Sp1 sites further demonstrated the binding of the AP-2α/Sp1 complex at these two sites and the essential of these two sites in AP-2α- and Sp1-mediated transcriptional activation of KiSS-1. Although AP-2α is necessary to mediate transcriptional regulation of the KiSS-1 promoter, our EMSA results, luciferase-reporter assays using a dominant negative form of AP-2α that lacks the C terminus DNA-binding domain, and the co-immunoprecipitation assays demonstrated that the N-terminal domain of AP-2α forms a protein complex to regulate transcriptional activation of KiSS-1 and that the role of AP-2α is independent of its promoter-binding ability.

Taken together, our data suggest a possible model for transcriptional activation of KiSS-1 in breast cells (Fig. 6). In normal cells, in which AP-2α is regularly expressed, the N-terminal region of AP-2α interacts with Sp1, which binds to the two-consensus Sp1-binding sites. The AP-2α and Sp1 complex initiates transcription of the KiSS-1 promoter and leads to the expression of KiSS-1 gene (Fig. 6). Alternatively, when AP-2α expression is lost, as commonly found in many cancer...
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types, depletion of AP-2α/Sp1 transcription factor complex leads to a loss of KiSS-1 transcriptional activation (Fig. 6). Contrary to conventional transcriptional regulation by AP-2α, in which AP-2α recognizes and binds to its consensus DNA-binding site 5′-GCCCNNNGGC-3′ (28), our results demonstrate a novel mechanism in which AP-2α regulation is mediated through two tandem Sp1 elements located between −230 bp to −260 and requires direct protein-protein interaction of the N-terminal portion of AP-2α with Sp1 protein.

KiSS-1 was originally identified to reduce melanoma metastasis by 95% using microcell-mediated transfer of chromosome 6 (2). Although KiSS-1 was later accurately mapped to chromosome 1q32-q41, it was believed that this region of the chromosome 6 contained a key, and as yet, unidentified KiSS-1 regulator that activated KiSS-1 expression upon transfer into melanoma cells. Interestingly, AP-2α is located in the short arm of chromosome 6, the exact region believed to regulate KiSS-1; therefore, AP-2α may in fact be the missing KiSS-1 regulatory factor.

Our data strongly establish a correlation between the loss of the AP-2α transcription factor and loss of the KiSS-1 metastasis suppressor in breast cancer cell lines. Loss of AP-2α has been implicated in the development of many other cancers besides invasive breast carcinomas, including colorectal, prostate, and ovarian cancer, renal cell carcinoma, and melanoma. Because KiSS-1 expression has also been found to be reduced in many of these same cancers, it is possible that KiSS-1 expression in these tissues is similarly reliant on the interaction of AP-2α with Sp1 and that the loss of AP-2α is paralleled by the loss of KiSS-1 expression, causing such cells to effectively lose another barrier to metastatic development. For this reason, our current studies are extending these observations of the role of AP-2α in mediating KiSS-1 expression in other cancers to identify similar mechanisms underlying the loss of KiSS-1 expression in such tissues and cancers.

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