KiSS-1 Represses 92-kDa Type IV Collagenase Expression by Down-regulating NF-κB Binding to the Promoter as a Consequence of IκBα-induced Block of p65/p50 Nuclear Translocation*

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The 92-kDa type IV collagenase (MMP-9) plays a critical role in tissue remodeling. We undertook a study to determine whether the KiSS-1 gene, previously shown to suppress cancer spread (metastases), negatively regulates MMP-9 expression. Six cell lines positive for MMP-9 mRNA were deficient in KiSS-1 mRNA. One of these cell lines, HT-1080, stably transfected with a KiSS-1 expression construct, demonstrated substantially lower MMP-9 enzyme activity/protein and in vitro invasiveness. The lower MMP-9 enzyme activity reflected reduced steady-state mRNA levels which, in turn, was due to attenuated transcription. Activation of ERKs and JNKs by phorbol 12-myristate 13-acetate and tumor necrosis factor α, respectively, leading to increased MMP-9 amounts was not antagonized by KiSS-1 expression, suggesting that MAPK pathways modulating MMP-9 synthesis are not the target of KiSS-1. Although MMP-9 expression is regulated by AP-1, Sp1, and Ets transcription factors, KiSS-1 did not alter the binding of these factors to the MMP-9 promoter. However, NF-κB binding to the MMP-9 promoter required for expression of this collagenase was reduced by KiSS-1 expression. Diminished NF-κB binding reflected less p50/p65 in the nuclear secondary to increased IκBα levels in the cytosols of the KiSS-1 transfectants. Thus, KiSS-1 diminishes MMP-9 expression by effecting reduced NF-κB binding to the promoter.

Tissue remodeling in physiological and pathological conditions such as trophoblast implantation, bone development, angiogenesis (1–3), and the spread of cancer (invasion/metastases) (4–6) requires proteolytic activity to degrade the surrounding extracellular matrix and to activate cytokines such as tumor growth factor β and interleukin 1β (2, 3). There is now compelling evidence implicating the type IV collagenase-degrading 92-kDa type IV collagenase (MMP-9) (4, 7, 8) in these processes. Thus, mice null for the MMP-9 gene exhibited an abnormal pattern of skeletal growth plate vascularization (1). Additionally, MMP-9 was shown to be required for human bronchial epithelial cell migration and spreading following injury (9). In cancer, MMP-9 mRNA/protein is produced in both cancer and normal cells (10–12), and there is strong evidence implicating this type IV collagenase in the spread of the disease. Thus, Bernhard et al. (7) reported that the overexpression of this metalloproteinase in nonmetastatic rat embryo cells conferred a metastatic phenotype upon these cells. In contrast, inhibition of MMP-9 expression by a ribozyme blocked metastasis of rat sarcoma cells (6).

The MMP-9 gene, located on chromosome 20 (13), covers 13 exons spanning 7.7 kilobases. Transcription gives rise to a 2.5-kilobase mRNA (14, 15). Translation of the message produces a 92-kDa precursor that is subsequently processed by the proteolytic removal of 73 amino acids at the amino terminus of the metalloproteinase (8, 16–18). The activity of the enzyme is controlled by the levels of physiological inhibitors including tissue inhibitor of metalloproteinases 1 and 2, which form noncovalent bonds with the enzyme (19, 20).

Regulation of MMP-9 protein levels has been ascribed to transcriptional activation of the gene (21) and to reduced mRNA turnover (22). The 5′ flanking sequence contains putative binding sites for AP-1, NF-κB, Sp1, and Ets transcription factors within the first 670 base pairs, and these have been implicated in the induction of MMP-9 gene expression by TNF-α, v-Src, c-Ha-Ras, Tat, and contact inhibition using transient transfection of cultured cells (15, 21, 23–26). In addition, studies with transgenic mice have demonstrated the requirement of regions −522/+19 and −2722/−7745 for developmental regulation in mice (27) and for tissue-specific expression in osteoclasts and migrating keratinocytes, respectively (27, 28).

Although MMP-9 has been implicated in the invasive/metastatic phenotype of many cancers, how its expression is regulated is still poorly understood. In the last 5 years, a great deal of interest has been focused on a group of genes collectively referred to as metastases-associated genes, which modulate cancer metastases but not tumorigenesis (29, 30). One of these is the KiSS-1 gene, which was discovered by subtractive hybridization and is reduced in its expression in metastatic cancer (31). Further, its enforced expression in MDA-MB-435 breast cancer cells and melanoma suppressed the metastatic potential of these cells by 95% without affecting their tumorigenicity (31, 32). The KiSS-1 gene is comprised of 4 exons, the first two of which are not translated. The third exon contains 38 noncoding base pairs at the 5′ end followed by 100 base pairs of translated sequence. The terminal exon contains 332 base pairs of translated sequence. The gene maps to chromosome 1q32 (33), and its sequence predicts a unique hydrophilic 145-amino acid protein with a leader sequence and a predicted

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1 The abbreviations used are: TNF-α, tumor necrosis factor-α; PCR, polymerase chain reaction; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; JNK(s), c-Jun NH2-terminal kinase(s); ERK(s), extracellular signal-regulated kinase(s); RT, reverse transcriptase; bp, base pairs; PMA, phorbol 12-myristate 13-acetate; MAPK, mitogen-activated protein kinase.
molecular mass of 15.4 kDa (34). However, the mechanism by which KiSS-1 represses the metastatic phenotype is unknown. Because MMP-9 has a well-established role in tumor cell invasion and metastases, we undertook the present study to determine whether the expression of this collagenase is regulated by KiSS-1.

**EXPERIMENTAL PROCEDURES**

Cell Culture and Stable Transfections—HT-1080 cells were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum. For stable transfections, cells were transfected at ~60% confluence using polyethyleneimine as described previously (35). Essentially, the cells were incubated with 20 μg of DNA and 9.3 μg of poly-L-ornithine (Sigma) for 6 h and then shocked for 4 min with 25% glycerol. Cells were incubated for 2 days and then selected with 600 μg/ml of G418 (active concentration). Clones were isolated, expanded, and screened for KiSS-1 cDNA integration by PCR.

Invasion Assays—These were carried out as described previously but with minor modifications (36). Briefly, Matrigel® was diluted in cold serum-free medium, and 25 μg of the preparation was added to the porous filters (pore size, 8 μm) and allowed to gel at 37 °C. Cells are dispersed with CaCl2/MgCl2-free medium containing 3 mM EDTA, and 250,000 cells dispensed into the Transwell®. The Transwell® was subsequently inserted into a well, with the latter also containing culture medium. The cells were incubated at 37 °C for 6 h. After this time, cells on both the upper and lower aspects of the membrane were stained with Diff-Quik (Baxter Scientific Products, McGaw Park, IL) and enumerated.

Northern Blotting—The level of steady state mRNAs was determined by Northern blot analysis (37). Total cellular RNA was extracted from 90% confluent cultures using 5.0 μl guanidinium isothiocyanate and purified on a cesium chloride cushion (5.7 M) by centrifugation at 150,000 × g for 20 h. Purified RNA was electrophoresed in a 1.5% agarose-formaldehyde gel and transferred to Nytran-modified nylon by capillary action using 10× SSC. The Northern blot was probed at 42 °C with random-primed radiolabeled cDNAs (38). The KiSS-1 cDNA coding sequence was generated by restriction digestion of the KiSS-1 expression plasmid with XhoI/BamHI, yielding a 0.7-kilobase fragment. The blots were then washed at 65 °C using 0.25× SSC in the presence of 0.75% SDS. Loading efficiencies were checked by reblotting the blot with a radioactive GAPDH cDNA.

Nuclear Run-on Experiments—Nuclear run-on experiments were as described previously (35). Nuclei from ~6 × 10^6 cells were isolated and incubated in the presence of [α^32P]UTP in transcription buffer (150 mM KCl, 5 mM MgCl_2, 1 mM MnCl_2, 20 mM Hepes, pH 7.9, 10% glycerol, 5 mM DTT). The nuclei were next treated with DNase I and proteinase K, and the RNA was extracted with phenol/chloroform and precipitated. Radioactive RNA (6.6 × 10^6 cpm) was hybridized to nylon-immobilized cDNAs corresponding to MMP-9 (38), MMP-2 (40), and GAPDH. Quantitation of the data was accomplished using Quantity-One software (Bio-Rad). One unit of RT mixture (10 μg total RNA, 0.1 μg μM oligo(dT)~18, and 50 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI)) was used for the detection of MMP-9. All the other antibodies were purchased from Santa Cruz Biotechnology. After being washed with TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) buffer, the blot was incubated with an horseradish peroxidase-conjugated secondary antibody, and proteins were visualized with ECL reagents (PerkinElmer Life Sciences) according to the manufacturer’s recommendation.

PCR—To verify the insertion of KiSS-1 cDNA into the genome, PCR was performed using genomic DNA as the template. To prepare the genomic DNA, the cells were lysed in a solution containing 10 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0, 20 μg/ml RNase A, and 0.5% SDS at 37 °C for 1 h. The cell lysates were then treated with 100 μg/ml of proteinase K for 2 h at 50 °C, although this procedure is used twice with phenol/chloroform (1:1). DNA was precipitated with ammonium acetate/ethanol. The PCR reaction mixture contained 50 ng of genomic DNA, 1.5 mM MgCl_2, 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 0.5 μM of each of the primers, and 1× Q solution (Qiagen). The upstream primer (5'-CCACTGCTTACTGGCTTATCG-3') and the downstream primer (5'-GCATGCTCTGACTCCTTTGGG-3') were complementary to the KiSS-1 cDNA sequence (nucleotides 376 to 395). After the template genomic DNA was denatured at 95 °C, the PCR reaction was initiated by adding 1 unit of Taq DNA polymerase (Qiagen). The template was amplified for 32 cycles. Each PCR cycle consisted of denaturation at 95 °C, annealing at 65 °C, and extension at 72 °C (1 min, 1 min, and 40 s, respectively). The PCR cycles were terminated by extending at 72 °C for 7 min. The PCR products were resolved in a 2% agarose gel.

RT-PCR—Total RNA was isolated from cultured cells using the TRIZOL reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. After denaturation at 65 °C for 5 min, RNA (2 μg) was added to 20 μl of RT mixture (10 μg/ml oligo(dT); 1× RT buffer (Promega, Madison, WI), 0.5 mM each of four deoxynucleosides, 1 unit/ml RNasein (Promega, Madison, WI, USA), and 10 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). The RNA was reverse transcribed at 42 °C for 60 min, and 2 μl of the products were used as the template for multiplex PCR, which contains both the target KiSS-1, MMP-9 or MMP-2 primers, and β-actin primers amplification. The primer sets were primed with 16–20 cycles at the following thermal parameters: at 120 bp, 5'-TGGTTTCACAATATCAGACCGCAGCT-3' and 5'-CATAGT-CACCTAGAGCCCTTTGCTTC-3'; MMP-2 (PCR product size, 447 bp), 5'-ACCCTGAGTCCTGCTGACAC-3' and 5'-ATGGCGATTTCTGCTCCTTT-3'; KiSS-1 (PCR product size, 389 bp), 5'-ATTCTACTGACCCAGGGCA-3' and 5'-GCTATCTGCTGCTGCT-3'; and β-actin (product size, 621 bp), 5'-ACACTCTGACCCTCACCTTACGAGG-3' and 5'-AGGAGCGAGAGAGACTCTAC-3'. The PCR conditions were
Chromatin Immunoprecipitation Assays—Cells (2–3 \times 10^6) were treated with 1% formaldehyde in fresh medium at 37 °C for 10 min followed by the addition of 0.125 mM glycine. After rinsing with ice-cold phosphate-buffered saline, the cells were resuspended in 200 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris- HCl, pH 8.1) and incubated for 10 min on ice. The cell lysates were then sonicated with a Sonic Disembrator (Fisher) at 50% maximum power for six 20-s pulses on ice. After removing cell debris by centrifugation, the cell lysates were diluted 10-fold in IP buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) supplemented with 1 mM PMSF and protease inhibitor mixture (Roche Molecular Biochemicals). At this step, 1% of the diluted chromatin solutions was saved to control (input DNA) for the total DNA amounts of each sample.

After being treated with proteinase K, the DNAs in the samples were precipitated and subjected to RT-PCR. HT-1080 cells transfected with the empty vector (clones 1 and 3) showed no amplified product using primers corresponding to the KiSS-1 coding sequence (Fig. 1B). These data indicate that the expression of KiSS-1 mRNA (endogenous) in HT-1080 cells transfected with the pcDNA3 vector is below the detection limit of RT-PCR. In contrast, an amplified product (of the predicted size, 389 base pairs) corresponding to the KiSS-1 mRNA was readily detected in HT-1080 clones 4, 5, 7, and 10, which were positive for genomic integration of the exogenous KiSS-1 plasmid.

To corroborate the earlier findings (31, 32) that KiSS-1 functions as a metastases suppressor gene, in vitro invasion assays were performed. In these assays, cells are plated on an extracellular matrix-coated porous filter, and invasion is defined as the percent of cells penetrating this impervious barrier. Expectedly, HT-1080 cells bearing the vector alone (clone 3-V3) (Fig. 1C) penetrated the extracellular matrix-coated porous filter (32 ± 3% invaded). In contrast, a representative KiSS-1-expressing clone (clone 7-K7) showed over a 65% reduction in invasiveness (Fig. 1C). This reduced invasiveness was not due to diminished adhesion. Thus, KiSS-1 clone 7 demonstrated an adhesion rate (percent of cells attached 1 h post-plating) of 44 ± 2 compared with 46 ± 4% for the vector clone 3. Thus, these data illustrate that in HT-1080 cells, KiSS-1 represses invasion, which is a crucial component of the metastatic phenotype.

Because the ability of cells to demonstrate an invasive/metastatic phenotype requires proteolysis, we hypothesized that KiSS-1 negatively regulates MMP-9 expression. To answer this question, conditioned medium from clones positive for KiSS-1 expression or the vector only were assayed for MMP-9 enzyme activity by zymography (Fig. 2A). Conditioned medium from HT-1080 cells harboring the empty vector (pcDNA3) contained a collagenase activity that was indistinguishable in size (92 kDa) from MMP-9. The amount of this enzyme was greatly reduced in the KiSS-1-transfected cells and below the detection limit of this assay for clones 4 and 7. In contrast, the activity of a metalloproteinase, whose size was identical to that of the 72-kDa type IV collagenase MMP-2 (44, 45), was not reduced in the KiSS-1 transfecteds.

Because the reduced MMP-9 enzyme activity evident by zymography could arguably be due to the increased presence of a physiological inhibitor such as tissue inhibitor of metalloproteinases families member (20), we also assayed the conditioned medium for MMP-9 protein amounts by Western blotting (Fig. 2B). Similar to the zymography data, immunoblotting of conditioned medium from the KiSS-1-transfected HT-1080 clones showed a marked reduction in the amount of this metalloproteinase when compared with the empty vector (pcDNA3) clones. Thus, it is likely that the reduced MMP-9 enzyme activity is due to a diminished amount of the protein.

If KiSS-1 is indeed a negative regulator of MMP-9 protein levels, we would predict that other cell lines or resected cancer that secrete this collagenase would be deficient in KiSS-1 expression. To address this question, 5 squamous cell carcinoma cell lines from the oral cavity (UM-SCC-1, Tu159, Tu167, Tu177, and Tu684) and two resected tumors, which all secrete MMP-9, were analyzed for KiSS-1 mRNA levels by RT-PCR (Fig. 2C). The level of KiSS-1 mRNA was below the detection limit of RT-PCR, whereas amplified fragments corresponding to the β-actin and MMP-9 transcripts were evident with these 5 different oral cancer cell lines and the resected tumors. In contrast, a cell line positive for KiSS-1 expression (MDA 231) had a level of MMP-9 mRNA barely detectable by RT-PCR. Thus, in summary, various squamous cell carcinoma cell lines and two resected oral cancers characterized by the synthesis of identical to those described above, with the exception that the annealing temperature was 62 and 68 °C for MMP-9 and KiSS-1, respectively. The concentration ratio of target cDNA primers to β-actin primers and the PCR cycle number was optimized for each reaction.

For amplification of KiSS-1 cDNA, the reaction mixture contained 0.5 μM KiSS-1 primers and 0.05 μM β-actin primer, whereas the cycle number was 30. For MMP-9 amplification, the concentration of β-actin primers was 0.025 μM, and the cycle number was 34. For MMP-2 amplification, the concentration of β-actin primers was 0.05 μM, and the cycle number was 32. The PCR products were resolved on 2% agarose gel.

RESULTS

Expression of KiSS-1 in HT-1080 Cells Decreases MMP-9 Protein/mRNA—Although KiSS-1 has been shown to dramatically attenuate the metastatic capacity of cancer cells, the mechanism as to how this is achieved is not known. We hypothesized that this may be due, at least in part, to a suppression of the expression of MMP-9, which degrades the extracellular matrix. To test this hypothesis, MMP-9-producing HT-1080 cells transfected with the pcDNA3 plasmid.
KiSS-1 Expression Reduces the Transcription of the MMP-9 Gene—To determine whether the reduced amount of MMP-9 activity was a consequence of less mRNA encoding this collagenase, we then compared MMP-9 mRNA levels in the HT-1080 clones expressing the KiSS-1 or the empty vector. An amplified fragment of the predicted size (120 base pairs) was readily detected in HT-1080 cells and two clones bearing the empty pcDNA3 vector (Fig. 3) by RT-PCR. However, in all four of the KiSS-1-expressing clones examined, the amount of MMP-9 mRNA determined by RT-PCR was substantially less than that evident for the untransfected HT-1080 or HT-1080 cells harboring the pcDNA3 vector (Fig. 3). In contrast to MMP-9, KiSS-1 expression in HT-1080 cells did not affect the levels of MMP-2 mRNA, a metalloproteinase encoded by a separate gene (45). Thus, although a fragment of the correct size (447 base pairs) was easily detected in HT-1080 cells, the amount of this amplified product was unchanged in HT-1080 cells stably expressing the KiSS-1 cDNA (data not shown).

Although the RT-PCR indicated that steady-state MMP-9 mRNA levels were lower in the KiSS-1-transfected HT-1080 cells, it was not clear whether this was because of decreased transcription or increased mRNA degradation. Therefore, to determine the contribution of reduced MMP-9 mRNA synthesis to the diminished levels of mRNA evident in the KiSS-1-transfectants, nuclear run-on experiments were carried out. Nuclei from KiSS-1 transfectants, verified by Northern blotting to be overexpressing KiSS-1 (Fig. 4A), and from vector-only clones were isolated. Nuclei were then incubated with radioactive dUTP, and RNA was isolated and hybridized to various cDNAs. It is apparent from Fig. 4B that the KiSS-1-expressing HT-1080 clones (numbers 4 and 7) have a lower rate of MMP-9 mRNA synthesis when compared with the pcDNA3 vector pool. Thus, for the pcDNA3 vector pool, the ratio of MMP-9/GADPH mRNA synthesis was 0.98, whereas for representative KiSS-1-expressing clones (numbers 4 and 7), it was reduced by 75% to 0.24 and 0.23. In contrast, the nuclear run-on experiments indicated that the mRNA for MMP-2 was transcribed essentially at the same rate in HT-1080 cells expressing the vector or the KiSS-1. These findings suggest that the decreased steady-state MMP-9 mRNA observed in the KiSS-1 transfectants is due largely to a reduced rate of synthesis. Further, the attenuated MMP-9 transcription is not a consequence of a generalized shut-down of gene transcription.

Because MMP-9 gene expression is known to be growth-associated (46–48), we considered the possibility that the diminished metalloproteinase synthesis evident in the KiSS-1 transfectants was secondary to a slower proliferation rate. To address this possibility, HT-1080 cells expressing either the KiSS-1 or the vector were assayed for proliferation. The growth rates of HT-1080 KiSS-1 clones 4, 5, 7, and 10, once established
in logarithmic phase, were unchanged relative to HT-1080 vector clones 1 and 3 (data not shown). Thus, the reduced expression of MMP-9 evident in KiSS-1-transfected HT-1080 cells cannot be due to a slower proliferative rate.

Stimulation of MMP-9 Activity by Agents That Increase ERK

MMP-9 expression is regulated partly by ERK- and JNK-dependent signaling pathways (49, 50). We therefore determined whether stimulation of these MAPK signaling cascades leading to increased MMP-9 expression was countered by KiSS-1 expression. To address this question, we employed the phorbol ester, PMA, and TNF-α for activating the ERKs (51–53) and JNKs (54), respectively.

HT-1080 cells expressing either the empty vector pcDNA3 or KiSS-1 were treated with PMA, and conditioned medium was collected and analyzed for metalloproteinase activity (Fig. 5A). Expectedly, PMA treatment strongly elevated MMP-9 (but not MMP-2) activity in HT-1080 cells harboring the vector only (clones 1 and 3). However, although the basal MMP-9 activity was reduced in all KiSS-1 clones, these clones all responded to the phorbol ester with a strong increase in the activity of this collagenase. Expectedly, PMA increased the amount of phospho-ERK in HT-1080 cells (Fig. 5B) bearing either the empty vector (pcDNA3) or the KiSS-1 expression construct, confirming that the phorbol ester was indeed activating the ERK

![Image](image_url)

**FIG. 2.** Reduced MMP-9 activity/protein in HT-1080 cells made to express KiSS-1. At 80% confluence, the indicated HT-1080 clones were changed to serum-free medium and incubated for 2 days. After this time, conditioned medium was collected and clarified by centrifugation, and cells were counted. Aliquots of conditioned medium, corrected for differences in cell number, was subjected to zymography (A) using an acrylamide gel containing gelatin or to immunoblotting for MMP-9 protein (B). The data are representative of at least duplicate experiments. C, RNA (2 mg) extracted from the various cell lines or resected tumors was reverse transcribed and subjected to multiplex RT-PCR using primers to amplify the MMP-9, β-actin, and KiSS-1 transcripts. The KiSS-1 and MMP-9 plasmids used as positive controls corresponded to 50 ng of DNA and were amplified without the reverse transcription step.

**FIG. 3.** Diminished MMP-9 mRNA levels in HT-1080 clones stably expressing an exogenous KiSS-1. At 90% confluence, HT-1080 cells expressing pcDNA3 or the KiSS-1 construct were harvested, and RNA was extracted. After reverse transcription, RNA was subjected to multiplex PCR using primers specific for MMP-9 and β-actin. As a positive control, the MMP-9 plasmid (50 ng) was amplified. For the negative control, RT-PCR was performed using buffer only.

![Image](image_url)

**FIG. 4.** Reduced MMP-9 transcription in HT-1080 cells expressing KiSS-1. A, total RNA was extracted and purified from 90% confluent HT-1080 cells or clones harboring pcDNA3 or KiSS-1. Purified RNA (20 μg) was resolved by electrophoresis and then transferred to a nylon membrane. The filter was subsequently probed with multiprime-labeled cDNAs specific for KiSS-1 or GAPDH. B, nuclei from 90% confluent cells were isolated and incubated with [α-32P]-labeled UTP. Radioactive mRNA was subsequently purified and hybridized with nylon filter-immobilized cDNAs specific for MMP-9, MMP-2, or GAPDH. The intensity of the MMP-9 and GADPH signals was measured using QuantityOne® software. The data are typical of duplicate experiments.
KiSS-1 did not decrease the amount of activated ERKs in the untreated HT-1080 cells. Similarly, TNF-α was an effective stimulant for MMP-9 activity and JNK activation (Fig. 5, C and D) in both the vector clone and the KiSS-1 transfectants. Furthermore, the amount of activated JNKs (phosphorylated p54 and p46) in untreated HT-1080 cells was undetectable by immunoblotting. Taken together, these data argue against the possibility that KiSS-1 represses MMP-9 expression by interfering with either ERK or JNK activation.

KiSS-1 Does Not Affect AP-1 Binding to the MMP-9 Promoter—Several studies from ours and other laboratories (15, 21, 24, 48) have reported on the trans-acting factors regulating the expression of MMP-9. These include transcription factors in the AP-1, NF-κB, Sp1, and Ets families (15, 21, 24, 48). We therefore speculated that KiSS-1 reduces MMP-9 expression by modulating the binding of one, or more, of these DNA-binding proteins to the MMP-9 promoter. Toward this end, we first investigated the possibility that KiSS-1 represses MMP-9 expression by interfering with either ERK or JNK activation.

KiSS-1 Reduces NF-κB Binding to the MMP-9 Promoter Subsequent to Cytosolic “Trapping” of p65/p50 by IkB—We next tested the possibility that KiSS-1 effects a reduction in the binding of NF-κB to the MMP-9 promoter sequence, because (a)
constitutive expression of this metalloproteinase in HT-1080 cells is achieved partly via trans-activation of an NF-κB motif (at −600) in the MMP-9 promoter (21), and (b) our own studies have shown that mutation of this NF-κB motif in the MMP-9 promoter results in up to a 75% reduction in promoter activity. Nuclear extract from untransfected HT-1080 cells bound to an oligonucleotide spanning the NF-κB motif at −600, as evident by a shifted band (parenthesis) in EMSA (Fig. 6A) that could be competed by an excess of an oligonucleotide bearing a consensus NF-κB motif (lane 3) but not an AP-1 consensus site (lane 4). Interestingly, the amount of the shifted band was greatly reduced (Fig. 6A), with nuclear extract generated from representative KiSS-1 clones (numbers 4 and 7) (lanes 7 and 8) when compared with nuclear extract (equal protein) from the pcDNA3 vector clones (numbers 1 and 3) (lanes 5 and 6). Note, however, that the amount of a nonspecific band (marked with an asterisk) bound to the oligonucleotide was not decreased in nuclear extract from the KiSS-1 transfectants.

To confirm the identity of NF-κB factors bound to the MMP-9 oligonucleotide, the following experiments were carried out. First, we tested the ability of an oligonucleotide bearing a mutated NF-κB binding site to compete for the shifted band in EMSA using HT-1080 nuclear extract (Fig. 6B). The oligonucleotide harboring nucleotide substitutions at the NF-κB motif, rendering it incapable of binding NF-κB, failed to compete for the shifted band in gel retardation assays (Fig. 6B, lane 4). Second, the identity of the transcription factors in the slower-migrating band (marked with a parenthesis) was determined using antibodies specific for p65 or p50 NF-κB family members. Competitors included oligonucleotides spanning the NF-κB binding site (−600) and the AP-1 motif at −600, as evident by a shifted band (marked with a parenthesis) in EMSA (Fig. 6B). Also, antibodies to Ets-1 and the p65 and p50 NF-κB family members were included in the reaction mixture where shown. wt, wild type

regulates MMP-9 expression, is bound in reduced amount to this promoter.

To corroborate the data generated by the EMSA, which is an in vitro assay that does not address the contribution of the in vivo environment of the promoter (DNA wrapped around a histone protein core) to transcription factor binding, we performed chromatin immunoprecipitation assays. Cultured HT-1080 cells bearing the pcDNA3 vector (clone 3) or clones harboring the KiSS-1 expression construct (clones 4 and 7) were treated with formaldehyde to cross-link transcription factors to the DNA in vivo. Subsequently, cells were lysed, and DNA was fragmented by sonication and subjected to immunoprecipitation with the anti-p65 antibody. The immunoprecipitated DNA, after reversal of cross-links, was purified and hybridized with a MMP-9 promoter-specific probe (Fig. 7A). For the empty vector clone (number 3), the signal ratio (p65 precipitable/input amount) was 0.69 as determined using QuantityOne® software (Bio-Rad). This ratio was reduced (to 0.34 and 0.13) with chromatin derived from the two KiSS-1-expressing clones.

Western blotting revealed that the decreased amount of NF-κB proteins bound to the MMP-9 promoter in the KiSS-1 transfectants is due, in part, to lower amounts of these proteins in the nuclear compartment with an accompanying increase in the cytosolic subcellular fraction (Fig. 7B). Because the ankyrin repeat-containing IκB proteins mask the nuclear localization signal of the NF-κB proteins located at the carboxyl terminus of the Rel homology domain (55), we speculated that the reduced presence of p65/p50 in the nuclear fractions of the KiSS-1 transfectants was a consequence of higher amounts of IκB. Indeed, immunoblotting (Fig. 7C) revealed larger amounts of IκBα in the cytosols of the KiSS-1-transfected HT-1080 cells. Thus, this observation would suggest that the diminished amounts of p65/p50 in the nuclear compartment of the KiSS-1 transfectants is due to an inhibition of nuclear translocation of the NF-κB proteins by IκBα. Thus, for the KiSS-1 transfectants, the MMP-9 promoter is bound with less NF-κB as a result of blockade of p65/p50 nuclear migration because of the increased IκBα cytosolic amounts.
KiSS-1 Represses MMP-9 Transcription via Reduced NF-κB Binding

The MMP-9 collagenase plays a critical role in tissue remodeling in both physiology and pathology. Thus, this metalloproteinase is a key regulator of growth plate angiogenesis (1), and cell migration required for respiratory epithelium repair has been attributed to this enzyme (9). In cancer, receptor-bound MMP-9 is required for tumor invasion and angiogenesis (2). We undertook the current study to determine whether KiSS-1, a gene that suppresses the invasive/mетastatic phenotype of divergent tumors (31, 32), regulates MMP-9 expression. Our finding that MMP-9 expression is down-regulated by KiSS-1 provides the first explanation as to how this metastases suppressor gene diminishes the spread of cancer. It should be emphasized, however, that the reduction in MMP-9 expression is probably one of multiple mechanisms responsible for diminished metastases achieved with KiSS-1. On the other hand, it is clear that KiSS-1 expression does not have a global effect on collagenase synthesis. Thus, the expression of a separate metalloproteinase (the 72-kDa type IV collagenase, MMP-2) was not reduced by this metastases suppressor gene, and this observation might partly account for the incomplete inhibition of \textit{in vitro} invasion by \textit{KiSS-1} expression.

For \textit{KiSS-1}, the down-regulation of MMP-9 enzyme activity is largely due to altered transcription as occurs with v-Src and c-Ha-Ras (21, 24). Further, our data suggest that the diminished synthesis of MMP-9 in the \textit{KiSS-1} transfectants can be partly accounted for by decreased promoter binding of NF-κB.

\textbf{DISCUSSION}

The MMP-9 collagenase activity is largely due to altered transcription as occurs with v-Src and c-Ha-Ras (21, 24). Further, our data suggest that the diminished expression of MMP-9 in the \textit{KiSS-1} transfectants can be partly accounted for by decreased promoter binding of NF-κB. 

\textbf{trans}–activation of gene expression by NF-κB has been ascribed to multiple mechanisms including the degradation of the IκBα, increased DNA binding affinity, and increased \textbf{trans}-

acting potential (55–58). The findings, herein, point to a decreased binding of the p65 and p50 NF-κB proteins to the MMP-9 promoter, although reduced \textbf{trans}–activation potential of the bound transcription factor, as previously reported for c-Rel or p65, cannot be ruled out at this stage (56). The attenuated binding of the p65/p50 evident in our study could potentially be due to a lower affinity for its MMP-9 binding site, reduced production of the transcription factor themselves, or a consequence of increased IκBα synthesis as occurs in interleukin 10-treated cells (57). However, we found no evidence that the total amount of p65/p50 (i.e. whole cell lysates) differed between the vector control and the \textit{KiSS-1} transfections. On the other hand, the observation of higher amounts of IκBα in the \textit{KiSS-1} transfectants is consistent with the contention of increased synthesis (or decreased degradation), thereby preventing the nuclear localization of p65/p50 and thus diminishing activation of the MMP-9 expression by these NF-κB proteins.

How \textit{KiSS-1} regulates IκBα amounts thereby sequestering the p65/p50 NF-κB proteins in the cytosolic compartment is currently unknown. The \textit{KiSS-1} sequence predicts a 15.4-kDa protein (34) lacking any signature transcriptional domains arguing against the possibility that it acts directly at the level of MMP-9 gene transcription. On the other hand, the presence of a putative signal peptide is consistent with the notion that the molecule is secreted. If \textit{KiSS-1} is indeed secreted, it could potentially interfere with a variety of growth factors/cytokines previously shown to up-regulate MMP-9 expression (47, 49, 59) or, alternatively, generate a suppressive signal via an autoimmune mechanism. However, preliminary findings (data not shown) have failed to show a repression of MMP-9 mRNA levels in untransfected HT-1080 cells incubated with conditioned medium from \textit{KiSS-1}-transfected HT-1080 cells arguing against the above-mentioned scenario.

Another possibility is that \textit{KiSS-1} interferes with MAPKs that connect the cell surface to the nuclear transcriptional machinery. However, our observations again would argue against this possibility. First, \textit{KiSS-1} expression did not decrease the amount of the phosphorylated ERKs, and the activity of the JNKs was undetectable in untransfected HT-1080 cells. Second, the ability of the phorbol ester, PMA (activating the JNKs) was undetectable in untransfected HT-1080 cells. Alternatively, other signaling events culminating in the activation of either the ERKs or the JNKs. However, these observations do not rule out the possibility that \textit{KiSS-1} interferes with these signaling cascades downstream of ERK or JNK activation. Alternatively, other signaling cascades, not examined in the current study, may themselves be sensitive to \textit{KiSS-1} expression. For example, activation of NF-κB by the interaction of atypical protein kinase C ζ (60, 61) with p62 and RIP, culminating in the activation of IκBα kinase β and phosphorylation of IκB, may represent a \textit{KiSS-1} target.

Interestingly, although the regulation of MMP-9 expression by the \textit{KiSS-1} metastasis suppressor is unique to date, this collagenase has recently been shown to physically interact with the metastases-promoting CD44 gene product (62). In pioneering work, CD44, which encodes a membrane glycoprotein, was reported to act as a receptor for proteolytically active MMP-9 thereby promoting tumor invasion and angiogenesis possibly via activation of transforming growth factor β (2). Thus, for CD44 the interaction with MMP-9 is a physical one and obviously separate from that of \textit{KiSS-1}, which regulates MMP-9 synthesis by interfering with the \textbf{trans}-activation of MMP-9 gene expression by NF-κB. Another gene modulating the

\textbf{FIG. 7}. Reduced \textit{in vivo} binding of NF-κB to the MMP-9 promoter is associated with higher IκBα amounts and reduced nuclear localization of p65/p50. A, cell lysates were sonicated and chromatin incubated without or with an anti-p65 antibody and protein A-agarose. DNA in immuno-complexes was recovered by proteinase K treatment and phenol/chloroform extraction and was hybridized to a radioactive MMP-9 promoter probe. As a control, a fixed portion (1%) of the total cell lysate was also hybridized to the radioactive MMP-9 promoter probe (Input). Quantitation of the bands was accomplished using QuantityOne® software. The background detected in the absence of an immunoprecipitating antibody (No Ab) was subtracted from all quantitations. B, subcellular fractions (equal protein) from the indicated clones was subjected to Western blotting for the p50 and p65 NF-κB proteins. C, the cytosolic fraction was analyzed for IκBα protein by Western blotting (Santa Cruz Biotechnology antibody sc-371). The blot was reprobed with an antibody to β-actin.
spread of cancer is KAI-1 (29) which, like KiSS-1, is a potent suppressor of metastasis (29, 63). However, the mechanism by which KAI-1 suppresses tumor cell invasion is also distinct from that of KiSS-1 and is related to its effects on cell-cell aggregation and binding to fibronectin substrate (64) rather than any change in the activity and/or amount of any collagenase (or, for that matter, protease).

Thus, in conclusion, we report for the first time that KiSS-1 down-regulates MMP-9 expression, a finding that explains, at least partly, how KiSS-1 attenuates the invasive/metastatic phenotype of different cancers (31, 32). Equally important, we have also demonstrated that the suppression of MMP-9 synthesis by KiSS-1 can be partly accounted for by reduced binding of NF-κB to the promoter secondary to the cytosolic sequestration of the p50/p65 NF-κB proteins by IκBα.

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