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BRMS1 negatively regulates NF-κB–dependent uPA expression following recruitment of HDAC1 to the NF-κB binding site of the uPA promoter

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Abstract

The BRMS1 metastasis suppressor was recently shown to negatively regulate NF-κB signaling and down regulate NF-κB-dependent uPA expression. Here we confirm that BRMS1 expression correlates with reduced NF-κB DNA binding activity in independently derived human melanoma C8161.9 cells stably expressing BRMS1. We show that knockdown of BRMS1 expression in these cells using small interfering RNA (siRNA) leads to the reactivation of NF-κB DNA binding activity and re-expression of uPA. Further, we confirm that BRMS1 expression does not alter IKKβ kinase activity suggesting that BRMS1-dependent uPA regulation does not occur through inhibition of the classical upstream activators of NF-κB. BRMS1 has been implicated as a corepressor of HDAC1 and consistent with this, we show that BRMS1 promotes HDAC1 recruitment to the NF-κB binding site of the uPA promoter and is associated with reduced H3 acetylation. We also confirm that BRMS1 expression stimulates disassociation of p65 from the NF-κB binding site of the uPA promoter consistent with its reduced DNA binding activity. These data suggest that BRMS1 recruits HDAC1 to the NF-κB binding site of the uPA promoter, modulates histone acetylation of p65 on the uPA promoter, leading to reduced NF-κB binding activity on its consensus sequence, and reduced transactivation of uPA expression.

INTRODUCTION

The molecular and biochemical mechanisms underlying cancer dissemination and metastasis remain poorly understood despite their obvious clinical importance. BRMS1 belongs to a growing number of metastasis suppressors that have the ability to suppress the metastatic potential of cancer cells *in vivo* without affecting tumorigenicity (1-3). This diverse group of

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genes includes NM23, KAI1, MKK4, KiSS1 and BRMS1 among others (4-8). The mechanism underlying metastasis suppression remains unknown for many of these genes. However, growing evidence suggests that metastasis suppressors may affect common signal transduction pathways including mitogen-activated protein kinases, G-protein coupled receptors and tyrosine kinase receptors (3). Recently we reported that BRMS1 suppresses metastasis at least in part through the inhibition of NF- κ B signaling (9). However the mechanisms underlying this inhibition remain to be elucidated.

NF- κ B is activated by a number of diverse signals, and the IKK complex (consisting of two related kinase subunits, IKK α and IKK β and the structural subunit IKK γ) plays a key role in the cytokine-induced activation of latent NF- κ B (10-13). Both IKK α and IKK β are required for cytokine-induced ubiquitination and degradation of the cytoplasmic inhibitors of NF- κ B (I κ Bs) (14) and the phosphorylation and activation of the p65/RelA subunit of NF- κ B that leads to the liberation and translocation of NF- κ B to the nucleus and subsequent activation of NF- κ B responsive genes (15).

Here we confirm the inhibition of NF- κ B-dependent uPA expression by BRMS1 in human C8161.9 melanoma cells stably expressing high levels of BRMS1 in independently derived cell lines. We further show that BRMS1 expression does not alter IKK β kinase activity suggesting that BRMS1 does not affect NF- κ B signaling through inhibition of the classical upstream activators of NF- κ B in these cells. Furthermore, we show that BRMS1 recruits HDAC1 to the NF- κ B consensus binding region of the uPA promoter using ChIP assays, and show reduced acetylation, suggesting that HDAC1 leads to H3 deacetylation and reduced binding of p65 at the NF- κ B site of the uPA promoter. These findings reveal important novel insight into the potential mechanisms underlying the role of BRMS1 in metastasis suppression.

MATERIALS AND METHODS

Cell culture

The amelanotic human melanoma cell line C8161 metastasizes to the lung when injected subcutaneously, intradermally or intravenously in nude mice (16). The metastatic clone C8161.9 was obtained by limiting dilution cloning of parental C8161 cells (17). C8161.9 cells and derivatives were grown in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 5% fetal calf serum, 1% L-glutamine and 1% penicillin and streptomycin, in 5% CO₂ and 95% air at 37°C. C8161.9 cells passage at 80-90% confluence using Ca²⁺/Mg²⁺-free PBS containing 2 mM EDTA. Full length, sequence verified, BRMS1-His cDNA was cloned into the mammalian constitutive expression vector pcDNA3 (Invitrogen, San Diego, CA) as described previously (9).

Electrophoretic Mobility Shift Assay (EMSA) and NF- κ B activation

To determine the effect of BRMS1 expression on activation of NF- κ B and other transcription factors, EMSA was performed as described previously (9). Ten micrograms of nuclear protein were incubated at room temperature for 20 minutes with ³²P-end-labeled nucleotide derived from a NF- κ B binding sequence (5'-AGT TGA GGG GAC TTT CCC AGG -3') from the immunoglobulin gene promoter. SMAD 3/4 (5'-AGT ATG TCT AGA CTG A-3') and OCT-1 (5'-TGT CGA ATG CAA ATC ACT AGA A-3') transcription factor binding site oligonucleotides were obtained commercially (Santa Cruz Biotechnology, Santa Cruz, CA). The complexes were resolved on 5% native polyacrylamide gels, which were dried and exposed to KODAK BioMax film at -80°C.

IKK activity assay

Kinase assays were performed as described previously (18). Cells were washed once with phosphate-buffered saline and lysed and cellular debris was removed by centrifugation at 16,000g for 15 min. For immunoprecipitations, cell extracts from C8161.9/pcDNA and C8161.9/BRMS1-His cells were incubated with 3 µg of total IKKγ antibody for 4hr, followed by incubation for 1hr with 50µl of protein A-Sepharose beads (50% suspension in dH₂O). In vitro phosphorylation was performed using ~1µg of GST-IκBα substrate peptide (residues 1-54) with the immunoprecipitated IKKβ as the kinase at 30°C for 30 min. Following the kinase reaction, phosphorylation of the GST-IκBα substrate was analyzed following SDS-PAGE and autoradiography. To determine the total amounts of IKKα, IKKβ and IKKγ in each sample, 40µg of the whole-cell extract protein were resolved on a 7.5% acrylamide gel and then electroblotted onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk protein for 1hr and incubated with anti-IKKα, anti-IKKβ and anti-IKKγ (1:1000 dilution) (Cell Signaling Technology, Beverly, MA) for 1hr. The membrane was washed and treated with horseradish peroxidase-conjugated secondary anti-mouse IgG antibody (Amersham Biosciences, Piscataway, NJ), and proteins were detected by chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Real-time quantitative PCR amplification

Total RNA was isolated from C8161.9/pcDNA and C8161.9/BRMS1-His clones using the TRIzol method (Invitrogen, San Diego, CA). cDNA was synthesized from the RNA using SuperScript II reverse transcriptase according to vendor's instructions (Invitrogen, San Diego, CA). Oligonucleotide primers for real-time quantitative PCR (RTQ-PCR) of BRMS1, uPA, and GAPDH were designed using Primer Express v.1.5 (Applied Biosystems, Foster City, CA). Amplicons for each gene were designed to span introns to prevent contamination by genomic DNA. PCR conditions were set equal for BRMS1, uPA and GAPDH genes, using Syber Green PCR Core kit (Applied Biosystems, Foster City, CA) according to vendor instructions and an ABI 7900HT (Applied Biosystems, Foster City, CA) real time PCR instrument. BRMS1 and uPA expression levels were calibrated using endogenous GAPDH expression levels (9) and confirmed with 18S as internal controls. Cycle conditions were: 95°C for 10 minutes (AmpliTaQ Gold) followed by 45 cycles of 95°C for 15 seconds (denaturation) and 60°C for 60 seconds (annealing and extension) and fluorescence was quantified as a Ct value. The differences between the mean Ct values of BRMS1, uPA, and GAPDH were denoted (Δ -Ct) and the difference between $\Delta\Delta$ -Ct and the Δ -Ct value of the GAPDH was calculated as $\Delta\Delta$ -Ct. The $\log_2(\Delta\Delta$ -Ct) gave the relative quantification value of expression. A single PCR amplified band was observed on ethidium bromide stained gels using standard PCR conditions for all genes. The following primers were used in the RTQ-PCR experiments: GAPDH (F): 5'-GAA GGT GAA GGT CGC AGT-3', GAPDH (R): 5'-GAA GAT GGT GAT GGG ATT TC-3'. uPA (F): 5'-GCC TTG CTG AAG ATC CGT TC-3', uPA (R): 5'-GGA TCG TTA TAC ATC GAG GGG CA-3'. BRMS1 (F): 5'-GCT CTG AAT GGT GGG ACG AC-3', BRMS1 (R): 5'-AAG ACC TGG AGC TGC CTC TG-3'.

Western Blot Analysis

Western blotting was performed as described previously (9). Briefly, Proteins were separated on 12% SDS-PAGE gels. Blots were incubated with the primary antibodies specific to IκBα (Santa Cruz, CA), phospho IκBα, p38, pp38, IKKα, IKKβ and IKKγ (Cell Signaling Technology, Inc, Beverly, MA), uPA (Santa Cruz Biotechnology, Santa Cruz, CA) and PIA1 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr. His antibody (Amersham Biosciences, Piscataway, NJ) specific to 6X His tag at C-terminus of BRMS1 was used to detect exogenous BRMS1-His expression. To monitor secreted uPA, cells were grown in serum free medium for 24 hr. After this time, supernatant was concentrated 10-fold using Amicon ultra 10K filters

(Millipore, Bedford, MA) at $4,000 \times g$ for 30 minutes and analyzed by Western blot analysis using antibodies specific for uPA and PAI-1 as a loading control.

siRNA inhibition of BRMS1 expression in C8161.9/BRMS1-His clones

Targeted interfering RNA (siRNA) oligonucleotides for BRMS1 (SmartPool) was purchased from Dharmacon (Lafayette, CO). A mixture of siRNA duplexes were transiently transfected into C8161.9/pcDNA cells and C8161.9/BRMS1-His clones 2-4, 4-1 and 5-1 using LipofectAMINE 2000 (Invitrogen, San Diego, CA) according to the manufacturer's instructions. To optimize conditions, a dose response increasing concentrations of siBRMS1 oligonucleotide concentrations were used for each clone and the efficiency of inhibition of BRMS1 expression was confirmed by RTQ-PCR. Transient transfections of a range of concentrations of 0 to 50 nm siBRMS1 oligonucleotides were performed in OPTI-MEM media. After 72 hours, nuclear and cytoplasmic proteins were extracted from siBRMS1-treated cells, and protein concentration quantified using PIERCE BCA protein assay (Pierce, Rockford, MA). Ten micrograms of nuclear protein were incubated at room temperature for 20 minutes with ^{32}P -end-labeled nucleotide derived from a NF- κB binding sequence as described above.

Chromatin Immunoprecipitation (ChIP)

Parental C8161.9 and BRMS1-His 2-4, BRMS1-His 4-1 and BRMS1-His 5-1 clones were fixed with 1% formaldehyde for 10 min and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1). The lysates were sonicated to reduce DNA fragments to 200-500 base pairs. The samples were precleared with protein A-agarose beads before Ab addition. 2 μg antibodies, Acetylated H3, HDAC1 and p65 (all from Santa Cruz Biotechnology, Santa Cruz, CA) were added to the chromatin samples and incubated at 4 °C overnight. 40 μl of treated beads was then added to the chromatin samples and incubated at 4 °C for 1 hr. The beads were then washed three times and immunoprecipitated. The DNA fragments were then eluted after proteinase K treatment. DNA fragments were recovered by phenol/chloroform extraction. DNA was dissolved in 20 μl of TE buffer (10 mM Tris, 1 mM, pH 8.0). In immunoprecipitated samples, the NF- κB region in the uPA promoter was analyzed by RTQ-PCR. $\Delta\Delta\text{-Ct}$ values are calculated as a ratio of immunoprecipitated product to the input product. PCR primers for uPA promoter (including the NF- κB binding site, GenBank accession number, Y11873) were as follow; uPA forward, 5'-GAG GGG GCG GAA GGG GAG AA-3', uPA reverse 5'-TGT GGT CAG TTT TGT TTG GAT TTG-3'.

RESULTS

Previously, we demonstrated that the transcription factor NF- κB was constitutively activated in C8161.9 melanoma and MDA-MB-231 breast cancer cells, but was inhibited in C8161.9 and MDA-MB-231 cells stably expressing BRMS1 compared to parental cells (9). To confirm these findings and to provide additional insight into mechanisms underlying BRMS1 suppression of NF- κB signaling, we now report the analysis of independently isolated C8161.9 cell lines stably expressing BRMS1-His for effects of BRMS1 expression on NF- κB signaling and HDAC1 recruitment and H3 deacetylation at the NF- κB binding site of the uPA promoter.

BRMS1 expression leads to the reduced expression of NF- κB regulated genes

C8161.9 cells show high basal mRNA expression levels of the NF- κB regulated gene uPA and undetectable levels of BRMS1 mRNA expression (9). Consistent with our previous findings (9), all BRMS1-His clones showed reduced NF- κB -dependent uPA mRNA and protein expression compared to C8161.9/pcDNA (Figure 1A and B). Exogenous BRMS1-His expression was determined by RTQ-PCR using BRMS1-specific primers. The expression level of BRMS1-His in C8161.9/BRMS1-His clones ranged from 5.1 fold to 11.5 fold compared to parental C8161.9/pcDNA cells (Figure 1A). We also confirmed reduced protein expression of

uPA in BRMS1-His expressing cells by Western blotting (Figure 1B). As previously shown, PAI-1 the main inhibitor of uPA was unaffected by BRMS1 expression.

Translocation of the NF- κ B p65/p50 heterodimer to the nucleus is mediated by I κ B α phosphorylation and degradation. To better understand the mechanisms underlying BRMS1 inhibition of NF- κ B signaling, we examined basal levels of I κ B α phosphorylation and degradation in C8161.9/pcDNA and C8161.9/BRMS1-His clones. All C8161.9/BRMS1-His clones consistently showed reduced constitutive levels of I κ B α phosphorylation compared to parental C8161.9/pcDNA cells (Figure 2A).

To better understand how BRMS1 may inhibit the NF- κ B pathway, we performed IKK kinase pull-down experiments. IKK kinase activity was based on phosphorylation of the IKK substrate, GST-I κ B α , in C8161.9/pcDNA and BRMS1-His clones (Figure 2B). Basal IKK kinase activity was found at similar levels in parental and the majority of BRMS1-His clones, indicating that IKK kinase activity was not altered by BRMS1 expression. These data imply that BRMS1 does not modulate the upstream classical NF- κ B pathway through IKK β .

uPA is also regulated by MEKK1 which in turn regulates both the ERK1/2 and JNK signaling pathways (19,20). The p38 mitogen activated protein kinase (MAPK) pathway is also known to activate uPA expression in many cancer cell lines (19). Published studies indicated that endogenous p38 MAPK activity increase invasiveness in breast cancer cells by increasing uPA/uPAR expression suggesting that p38 MAPK signaling pathway is involved in the regulation of uPA/uPAR expression (21). We asked if this signaling pathway was altered by exogenous BRMS1 expression in C8161.9 cells. Our data show that activity of p38 MAPK (Figure 2A) was not affected by BRMS1-His expression supporting a specific effect of BRMS1 on NF- κ B signaling.

The effect of BRMS1-His expression on NF- κ B binding activity in C8161.9 cells was examined by EMSA using a ³²P-end-labeled oligonucleotide derived from a NF- κ B consensus binding sequence (9). While there was some variability in the reduction in constitutive NF- κ B binding activity across C8161.9/BRMS1-His clones compared to C8161.9/pcDNA parental cells (Figure 3) these data were consistent with our previous observation that BRMS1 expression negatively regulates NF- κ B binding activity (9). The effect of BRMS1 expression on the binding activity of transcriptional factor OCT-1 was also examined to determine if BRMS1 had a systematic effect on other transcriptional factors (Figure 3). BRMS1 had no effect on OCT-1 binding activity.

Inhibition of exogenous BRMS1 expression by siRNA leads to re-activation of NF- κ B signaling

To confirm that reduced NF- κ B binding activity in BRMS1 expressing cells was the result of expression of BRMS1-His, we knocked down BRMS1 expression in 3 independent clones expressing BRMS1 (BRMS1-His 2-4, BRMS1-His 4-1 and BRMS1-His 5-1) using siRNA. Optimal inhibition of BRMS1 was achieved for each clone using different concentrations (25-50 nM) of siBRMS1 oligonucleotides (Figure 4A). siBRMS1 treatment did not affect OCT-1 binding activity (Figure 4A). Real-time PCR analyses demonstrated that BRMS1 inhibition by siRNA treatment led to re-expression of uPA (Figure 4B).

Reduced histone acetylation of the uPA promoter NF- κ B binding region in C8161.9/BRMS1 cells

To examine whether the observed BRMS1-dependent reduction in uPA expression was the direct result of HDAC1 targeting of the NF- κ B binding region of the uPA promoter, ChIP assays were performed using antibodies against Ac-H3, HDAC1 and p65. The

immunoprecipitated chromatin DNA fragments from parental and BRMS1 expressing clones were analyzed by RTQ-PCR of the NF- κ B region of uPA promoter. Our data show a reduced PCR amplification of the NF- κ B binding region of the uPA promoter in Ac-H3 and p65 pull-down chromatin and an increased PCR amplification in HDAC1 pull-down chromatin (Figure 5) in BRMS1-His 2-4, BRMS1-His 4-1 and BRMS1-His 5-1 clones. These results suggest that BRMS1 mediates HDAC1 recruitment to the NF- κ B binding region of the uPA promoter and diminishes histone acetylation of this region.

DISCUSSION

We previously demonstrated an inverse correlation between BRMS1 expression and NF- κ B-dependent uPA gene expression in breast and melanoma cell lines (9). In this study we confirm this observation in independently derived C8161 melanoma cells stably expressing BRMS1 and show modulation of its effect on uPA expression through siRNA knockdown studies of BRMS1. Importantly, we now demonstrate that BRMS1 potentially modulates NF- κ B-dependent uPA gene expression by functioning as a HDAC1 corepressor of NF- κ B-dependent gene transactivation.

The underlying mechanism of NF- κ B-dependent transcription regulation of uPA by BRMS1 remains to be elucidated. BRMS1 expression leads to the inhibition of I κ B α phosphorylation and degradation, however, we confirm here that the BRMS1-dependent inhibition of NF- κ B signaling occurs without affecting IKK β activity even though reduced basal levels of I κ B α phosphorylation and degradation are observed. These data imply that BRMS1 regulated uPA gene expression through an IKK β -independent mechanism.

Mitogen activated protein kinase (MAPK) p38 has been reported to activate uPA expression in many cancer cell lines including breast cancer (19,20), suggesting that uPA expression could be regulated by MAPK signaling in BRMS1-expressing cells. However, we demonstrated that the phosphorylation level p38 was not altered by BRMS1 expression suggesting that BRMS1 does not involve MAPK signaling pathway.

There is recent evidence that BRMS1 plays a role in acetylation. BRMS1 has been shown to interact with the RBP/Sin3a transcriptional factor complex (22-24), and studies have implicated BRMS1 in the repression of both basal and inducible p65 transactivation through recruitment as a corepressor for HDAC1 (25). A direct interaction between BRMS1 and the ARID-4 transcriptional factor within the Sin3A/HDAC complex has been reported, however disruption of binding between ARID-4 and BRMS1 did not change the metastatic suppression ability of BRMS1 (26). Here, we demonstrate that BRMS1 significantly reduces NF- κ B-dependent uPA expression in C8161 human melanoma cells by recruiting HDAC1 to the NF- κ B site of the uPA promoter in association with deacetylation and reduced p65 levels. We found reduced levels of Ac-H3 and p65, but increased levels of HDAC1 associated with the NF- κ B binding region of the uPA promoter in BRMS1 expressing cells using ChIP experiments. These data imply that BRMS1 is recruited to the uPA NF- κ B promoter binding region where it acts as a HDAC1 corepressor leading to chromatin deacetylation of p65 and reduced transactivation by NF- κ B.

Recent studies have suggested the importance of acetylation regulation in BRMS1-dependent gene regulation of NF- κ B transactivation. The p65 subunit of NF- κ B is acetylated by coactivators p300, CBP and p/CAF at several lysine residues with K310 playing the prominent role in p65 transcriptional regulation. BRMS1 was recently shown to abolish TNF-dependent acetylation of p65 on K310, resulting in a substantial decrease in p65 transactivation potential (25). BRMS1 promoted HDAC1 binding to the RelA/p65 subunit of NF- κ B where HDAC1 deacetylated lysine K310 on p65 at NF- κ B binding sites of promoters of cIAP2 and Bfl-1/A1

leading to the loss of NF- κ B-dependent transcriptional activation (25). Interestingly, Samant et al also recently showed that BRMS1 recruited HDAC3 to the NF- κ B binding site of the OPN promoter in MDA-MB-435 breast cancer cells leading to deacetylation of p65 (27), suggesting a complex role for BRMS1 in regulation of acetylation. Indeed, BRMS1 has been shown to interact with the RBP/Sin3a transcriptional factor complex (22-24), and a role for this complex in BRMS1-dependent regulation of uPA cannot be ruled out.

In summary, we demonstrate that BRMS1 expression leads to the recruitment of HDAC1 to the NF- κ B binding site of the uPA promoter and correlates with reduced acetylated H3 and dissociation of NF- κ B from its consensus DNA sequence on the uPA promoter. These findings suggest that BRMS1 regulates NF- κ B-dependent transactivation of uPA at least in part through acting as a corepressor for HDAC1-mediated deacetylation of p65.

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Figure 1.

A. Relative expression of BRMS1 and uPA mRNA was analyzed in stably expressing C8161.9/BRMS1-His clones by real time quantitative PCR. BRMS1-His clones showed reduced uPA mRNA expression levels and protein expression compared to C8161.9/pcDNA cells. **B.** Total secreted endogenous uPA protein was determined by Western blot analysis in BRMS1-His expressing clones. The uPA inhibitor PAI-1 was used as a control.

Figure 2.

A. IkBα phosphorylation was inhibited by BRMS1 expression. Cytosolic protein fractions C8161.9/pcDNA, and corresponding BRMS1 clones were extracted from exponentially growing cells and analyzed by Western blot using antibodies specific for IkBα, phospho-IkBα (Ser³²), p38 and phospho-p38 in BRMS1-His clones. Equal protein loading was confirmed using β-actin antibody. IkBα phosphorylation was substantially reduced in BRMS1-expressing C8161.9 clones, however phospho-p38 activity remained unaltered in BRMS1-His clones suggesting that BRMS1-His expression had no effect on MAPK signaling. **B.** In vitro kinase activity was performed using ~1μg of GST-IkBα substrate peptide (residues 1-54) with the immunoprecipitated IKKα, IKKβ as the kinase. Following the kinase reaction, phosphorylation of the GST-IkBα substrate was analyzed following SDS-PAGE and autoradiography. To determine the total amounts of IKKγ, IKKα, IKKβ in each sample, 40μg of the whole-cell extract protein were resolved on a 7.5% acrylamide gel and then electroblotted onto a nitrocellulose membrane.

Figure 3.

A, NF-κB activity was inhibited in C8161.9 BRMS1-His expressing clones. Nuclear proteins were extracted from exponentially growing C8161.9/pCDNA, and corresponding BRMS1 clones and 10 μg nuclear proteins were incubated with ³²P-end-labeled NF-κB oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGG-3') from the immunoglobulin gene promoter. NF-κB DNA binding activity was substantially reduced in C8161.9/BRMS1 cells. The arrow indicates the p50/p50 homodimer that shows intense activity, not the p65/p50 heterodimer. **B**, 10 μg nuclear proteins were also incubated with ³²P-end-labeled OCT-1 consensus oligonucleotides as a control.

A **B** **Figure 4.**

A, Inhibition of BRMS1 expression by siRNA treatment. Relative expression of BRMS1 was analyzed in siRNA/BRMS1 treated randomly selected BRMS1-His 2-4, BRMS1-His 4-1 and BRMS1-His 5-1 clones by RTQ-PCR. siRNA treatment against BRMS1 significantly reduced the BRMS1 mRNA expression and correlated with increased uPA expression. **B**. Activation of NF- κ B binding by BRMS1 inhibition. In parallel, NF- κ B activity was activated in corresponding clones with increasing siRNA concentration. OCT-1 binding activity was not changed following knock down of BRMS1 expression following siRNA treatment.

Figure 5.

ChIP assays were performed in C8161.9/pCDNA and BRMS1-His 2-4, BRMS1-His 4-1 and BRMS1-His 5-1 clones using antibodies against HDAC1, ACh3 and p65. The NF- κ B region in the uPA promoter was amplified using uPA promoter specific primers. Immunoprecipitated promoter fragments were analyzed by RTQ-PCR. Results are presented as a ratio of immunoprecipitated product to the input product.