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Human KIT ligand promoter is positively regulated by HMGA1 in breast and ovarian cancer cells

Nathan R Treff[1,3], Gregory A Dement[1], Jennifer E Adair[1], Rachel L Britt[1], Rong Nie[1], James E Shima[1], Wayne E Taylor[2] and Raymond Reeves*[,1]

[1]*School of Molecular Biosciences, Washington State University, PO Box 644660, Pullman, WA 99164-4660, USA;* [2]*Division of Endocrinology, Metabolism, and Molecular Medicine, Charles R Drew University of Medicine and Science, Los Angeles, CA 90059, USA*

KIT ligand (KL) and its receptor, c-kit, are coexpressed in many types of cancer cells and have been implicated in tumor growth and angiogenesis. While Sertoli cell-specific regulation of the *KL* promoter has been well characterized, regulation in cancer cells remains to be elucidated. We recently reported microarray results demonstrating that increased high-mobility group (HMG) A1a protein expression correlates with increased *KL* transcription in MCF-7 human breast cancer cells. Sequence analysis indicates a potential for multiple HMGA1 binding sites within the human *KL* promoter. In order to better define the underlying molecular mechanisms that HMGA1 uses to facilitate malignant transformation of cancer cells, we have used a variety of methods to determine whether HMGA1a directly regulates the human *KL* promoter in breast and ovarian cancer cells. Our results indicate that: (i) *KL* promoter activity is significantly higher in MCF-7 cells overexpressing HMGA1a; (ii) HMGA1a protein binds to AT-rich regions of the *KL* promoter DNA both *in vitro* and *in vivo*; (iii) mutation of the AT-rich regions inhibits HMGA1a binding *in vitro*; and (iv) HMGA1a-specific inhibition significantly decreases transcription of *KL* in OCC1 human ovarian cancer cells. In addition, MCF-7 cells with transgenic HMGA1 overexpression stained positive for the KL protein by immunocytochemistry and immunohistochemistry, and were growth-inhibited by KL neutralization. The cumulative evidence indicates that HMGA1 positively regulates the human *KL* promoter in breast and ovarian cancer cells and implicates serum KL as a diagnostic marker for HMGA1-positive carcinomas.
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Introduction

KIT ligand (KL) is a secreted growth factor (also referred to as stem cell factor, mast cell growth factor, or steel-factor) important for hematopoiesis, melanogenesis, and gametogenesis (Geissler *et al*., 1998), and has been implicated in the malignant progression of many cancers. KL overexpression increases mammary tumor growth and angiogenesis (Zhang *et al*., 2000), exogenous KL stimulates anchorage independent growth of colon carcinoma cells (Bellone *et al*., 2001), and binding of KL to its receptor, encoded by the proto-oncogene c-kit, activates the Ras/ERK signaling pathway (Lennartsson *et al*., 1999). KL is also implicated in regulating density-dependent growth of cervical cancer and leukemic cells (Caceres-Cortes *et al*., 2001), and its expression has been observed in ovarian, breast, prostate, testicular, colon, small-cell lung, and gastric cancers (Hibi *et al*., 1991; Toyota *et al*., 1993; Hines *et al*., 1995, 1999; Bokemeyer *et al*., 1996; Hassan *et al*., 1998; Parrott *et al*., 2000; Simak *et al*., 2000, respectively). Evidence also implicates KL in the formation of Schwann cell neoplasia (Ryan *et al*., 1994), neuroblastomas (Timeus *et al*., 1997), and gynecological tumors (Inoue *et al*., 1994). Despite the growing body of evidence suggesting a role for KL in cancer biology, little is known about the factors that regulate its expression in cancer cells.

We recently discovered that increased levels of the high-mobility group (HMG)A1a protein (formerly called HMG-I(Y); Bustin, 2001) are correlated with increased transcription of the *KL* gene in MCF-7 human breast cancer cells (Treff *et al*., 2004). HMGA1a has already been shown to regulate negatively the expression of BRCA1, which may account for reduced BRCA1 expression in sporadic breast cancer (Baldassarre *et al*., 2003). In addition, increased levels of HMGA1 have been associated with a poor prognosis (Langelotz *et al*., 2003) and an increased tumor grade (Flohr *et al*., 2003) in human breast cancer.

HMGA1a is an architectural transcription factor that belongs to the structurally distinct HMGA family of proteins that contain AT-hook DNA binding motifs that recognize structure, rather than nucleotide sequence, and preferentially bind to the minor groove of AT-rich stretches of DNA (reviewed in Bustin and

*Correspondence: R Reeves; E-mail: reevesr@mail.wsu.edu
[3]Current address: Department of Surgery, University of Wisconsin-Madison School of Medicine, 600 Highland Ave., Madison, WI 53792, USA
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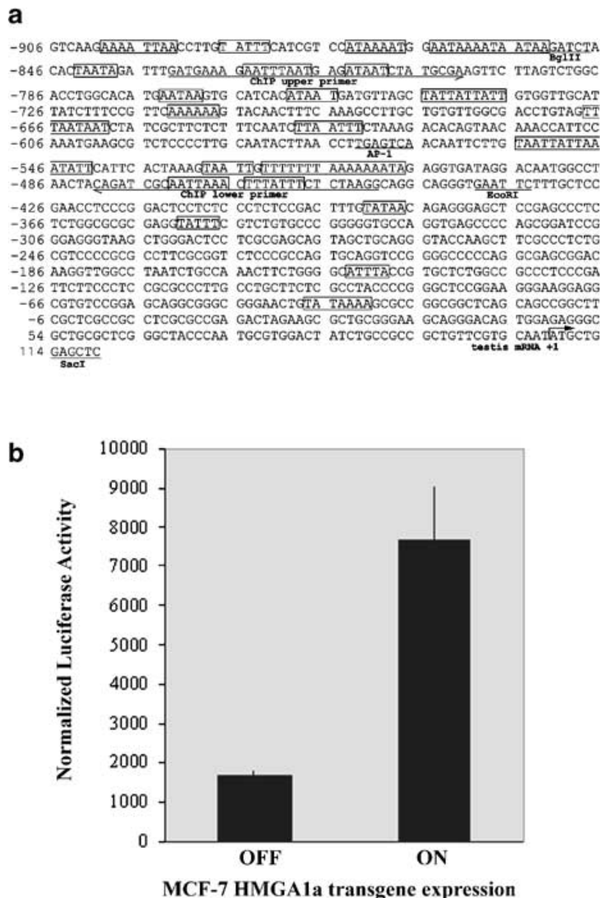


Figure 1 Human *KL* promoter (**a**) has multiple sites of potential HMGA1 binding (boxes) and is more active, based on luciferase activity, in MCF-7 cells overexpressing HMGA1a (**b**). The pGL3-Basic vector (Promega, Madison, WI, USA) was used to generate the plasmid pGL3-KL by subcloning the *Bgl*II/*Sac*I fragment of the human KL promoter from pKL-luc 2185 (Taylor *et al*., 1996). MCF-7 human mammary adenocarcinoma cells (parental or OFF) and transgenic MCF-7 HA7CCs cells (ON) were maintained as described in Reeves *et al*. (2001). Parental MCF-7 cells were used rather than the transgenic tetracycline-treated MCF-7 cells as the control cell line (HMGA1a-OFF), in order to avoid any tetracycline-dependent results. MCF-7 cells were seeded to 80% confluency in 60 mm tissue culture dishes. Cells were transfected with pSV-β-galactosidase plasmid DNA, plus either pGL3-Basic or pGL3-KL DNA, using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the supplier. Cells were lysed using 1 × Reporter Lysis Buffer (Promega) as recommended by the supplier. β-Galactosidase activity was determined using ONPG substrate and absorbance at 410 nm. Luciferase activity (**b**) was determined using luciferase substrate (Promega) and a 96-well plate luminometer (Wallac Victor-2) for both HMGA1a-ON and -OFF cells. Luciferase activity was normalized to β-galactosidase activity to control for transfection efficiency and recorded in arbitrary units of activity as the average of three independent experiments

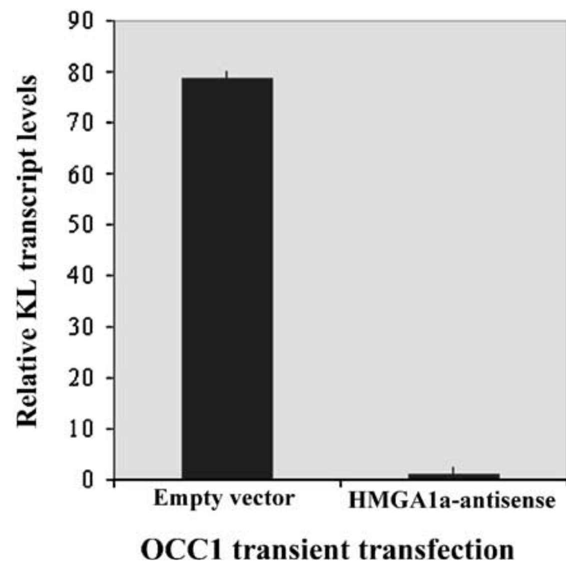


Figure 2 HMGA1a regulates KL expression in OCC1 human ovarian cancer cells. OCC1 human ovarian cancer cells were a gift from Michael Skinner (Washington State University) and were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. Quantitative reverse-transcriptase real-time polymerase chain reaction (QPCR) was used to characterize the influence of HMGA1-antisense (Himes *et al*., 1996) expression on the levels of KL in OCC1 human ovarian cancer cells. MCF-7-ON and -OFF cells were seeded in 60 mm culture plates at 80% confluency. Empty vector pRc-CMV DNA or HMGA1-antisense vector DNA (RcCMVIGMH; Himes *et al*., 1996) (2 μg) were used to transfect cells using Lipofectamine reagent (Invitrogen) following the manufacturer's recommended protocol. At 48 h following the transfection, cells were harvested for total RNA and protein using TRIzol reagent as recommended by the supplier (Invitrogen). Total RNA (2 μg) were reverse transcribed into cDNA using a TaqMan Gold RT–PCR Kit (Applied Biosystems, Foster City, CA, USA). First-strand synthesis reaction (2 μl) were used with TaqMan Universal Master Mix, and either HPRT1 (assay ID-Hs99999909_m1) or KITLG (assay ID-Hs00241497) assays-on-demand solutions as recommended by the supplier (Applied Biosystems). Relative quantitation was carried out using the comparative C_T method as described in User Bulletin #2: Relative Quantitation of Gene Expression (P/N 4303859) (Applied Biosystems)

Reeves, 1996; Reeves, 2001). HMGA1a and A1b are encoded by the same gene and differ only by the deletion of 11 internal amino acids as a result of alternative messenger RNA splicing. Binding of both the HMGA1a and HMGA1b proteins has been demonstrated to alter the structure of DNA and chromatin, thereby influencing the formation of stereospecific enhancesome complexes on the promoter regions of genes during their transcriptional activation. As shown in Figure 1a, analysis of the human *KL* promoter reveals the presence of multiple AT-rich stretches representing potential HMGA1a binding sites, suggesting its potential transcriptional regulation by this protein. Also, consistent with this idea is the observation that the transcript levels of HMGA1a parallel those of KL at different stages of testis development (data not shown). Regulation of the *KL* promoter DNA has been best characterized in Sertoli cells of the testis because of its demonstrated role in fertility (Taylor *et al*., 1996; Jiang *et al*., 1997; Grimaldi *et al*., 2003). Interestingly, the recently published phenotype of an HMGA1-heterozygous knockout mouse is that of infertility (Liu *et al*., 2003), suggesting a possible role for HMGA1 regulation of *KL* gene expression in the testis. Furthermore, both the

human and mouse *HMGA1* promoter sequences have a conserved consensus SRY response element (Pedulla *et al.*, 2001), which could contribute to testis-specific HMGA1 protein expression. This information led us to test the hypothesis that the HMGA1a protein directly regulates the human *KL* promoter *in vivo*. We have used a number of independent techniques to analyse the regulation of KL by HMGA1a in both MCF-7 human breast cancer cells and OCC1 human ovarian cancer cells.

As shown in Figure 1b, transfection experiments demonstrated that transcription from the human *KL* promoter was significantly higher in cells overexpressing transgenic HMGA1a protein than in cells without HMGA1a transgene expression ($P<0.0001$, $n=3$). In these experiments, the human *KL* promoter (nucleotides −853 to +120 using the nucleotide position designated in Taylor *et al.*, 1996) was cloned upstream of the luciferase reporter gene in the pGL3-Basic vector and transiently transfected into MCF-7 cells that were either overexpressing transgenic HMGA1a protein or into parental MCF-7 cells that were not. *KL* promoter activity was 4.6 ± 0.8-fold more active in cells overexpressing HMGA1a (Figure 1b). This value is very close to the reported 4.2 ± 0.3-fold change in *KL* transcription in MCF-7 cells overexpressing HMGA1a, as determined by oligonucleotide microarray analysis (Treff *et al.*, 2004). This indicates that the effects of HMGA1a on KL expression are at the transcriptional level.

In order to demonstrate that the effects of HMGA1a on KL expression were not limited to the MCF-7 cancer cell line, we expanded our analysis to include the OCC1 human ovarian cancer cells. We used quantitative real-time PCR to characterize the levels of *KL* transcript in response to ectopic HMGA1a-antisense expression (Himes *et al.*, 1996). The results of these experiments demonstrate that antisense HMGA1 expression in OCC1 ovarian cancer cells causes significant inhibition of *KL* gene transcription (Figure 2). These results indicate that the influence of HMGA1a on KL expression is not limited to one specific cell type.

To demonstrate the ability of HMGA1a to bind *KL* promoter DNA *in vitro*, purified recombinant HMGA1a protein was used in electrophoretic mobility shift assays (EMSAs). These analyses revealed that there are at least

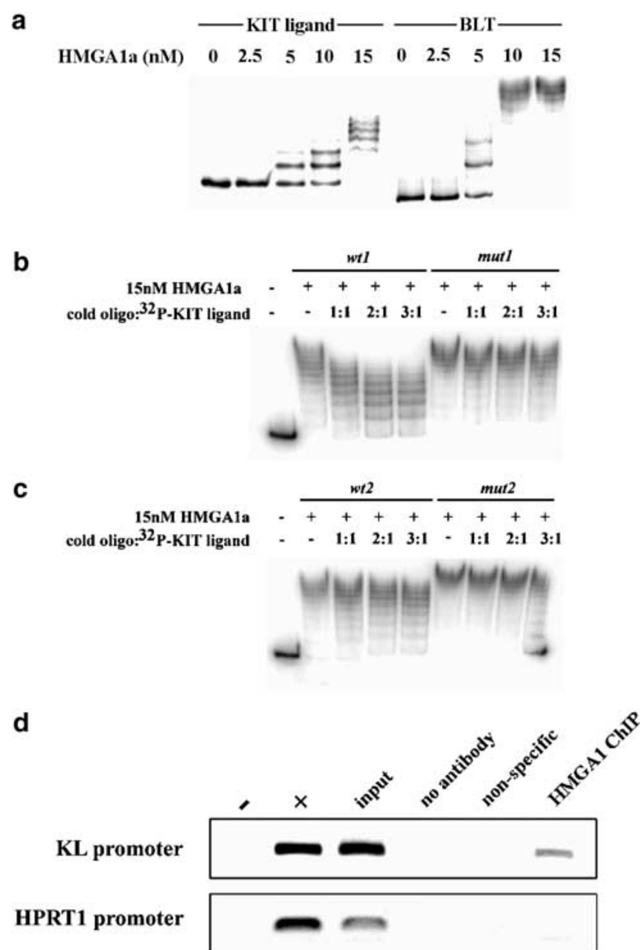
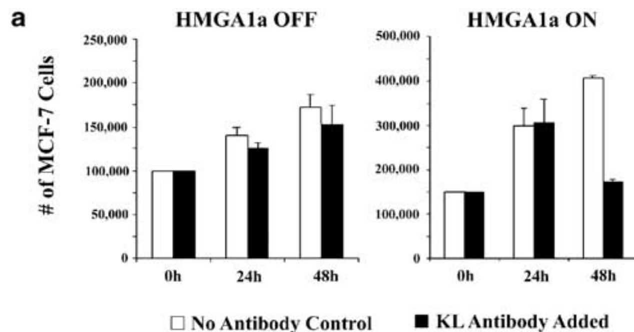


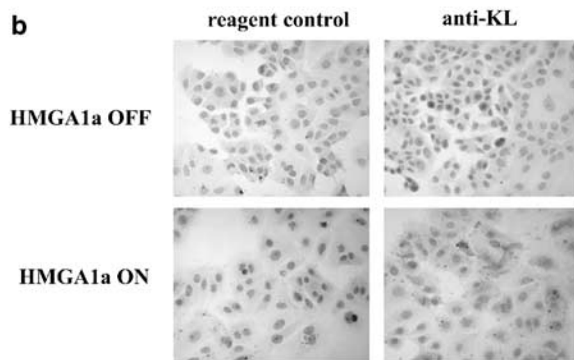
Figure 3 HMGA1 binds to KL promoter DNA *in vitro* (**a**, **b**, **c**) and *in vivo* (**d**). Panel a shows an EMSA that was performed using purified recombinant HMGA1a protein and ^{32}P end-labeled KL promoter DNA (*Bgl*II/*Eco*RI fragment, nucleotides −853 to −434). The labeling reaction was carried out on ice for 40 min using [α-^{32}P]ATP (Perkin-Elmer, Boston, MA, USA) and the large fragment of DNA polymerase I (Invitrogen). The binding reaction between KL promoter and HMGA1a protein took place at room temperature for 30 min. The binding buffer contained: 50 mM Tris (pH 7.8), 125 mM NaCl, 5 mM EDTA, 200 μg/μl BSA, and 0.15 μg/μl poly(dG–dC) DNA (Pharmacia). Samples were loaded onto a 6.5% nondenaturing polyacrylamide gel and electrophoresed at 100 V for 2 h and 30 min at 4°C. The reaction conditions for the EMSA carried out with BLT DNA were identical to the conditions described for the KL DNA. BLT, which is known to have HMGA1a binding sites, is used here as positive control. Panels b and c show analyses of HMGA1a protein binding to the KL promoter DNA (as in panel a), but with the addition of cold competitor DNA (cold oligos) representing potential HMGA1a binding sites. These DNA molecules were synthesized (Qiagen) to maintain either the wild-type KL sequence (wt) or to contain mutated KL sequence (mut). For wt1 and mut1, the DNA sequences were as follows: 5′-TTGATGAAAGAATTTAATGAGATAATCTATGCGA-3′; and 5′-TTGATGAAAGAATggAATGAGATgATCTATGCGA-3′, respectively. For wt2 and mut2, the DNA sequences were as follows: 5′-TTATGTTAGCTATTATTATTGTGGTTGC-3′; and 5′-TTATGTTAGCTAgTAgTAgTGTGGTTGC-3′, respectively. Panel d shows a ChIP assay that was carried out with HMGA1a-ON cells following Kuo and Allis (1999) and using KL-specific primers (Figure 1a); upper, 5′-TTGATGAAAGAATTTAAT GAGATAATC-TAT AATCTATGCGA-3′ and lower, 5′-TTCCTTAGAGAAA-TAAAGTTTAATTGCGATCTG-3′. Negative controls included no DNA template (−), DNA from preimmune serum (nonspecific), and immunoprecipitations without antibody (no antibody). Positive controls included pGL3-KL DNA as template (+), and input material before immunoprecipitation as template (input). A negative control ChIP assay was performed with the same cells utilizing human hypoxanthine–guanine phosphoribosyltransferase (HPRT1) promoter (NCBI Accession #M12452) specific primers; upper, 5′-GGTAGGTTTGGGAATCAGG-3′ (nt-365) and lower, 5′-TTTGCAGGCTCACTAGGTAG-3′ (nt-241). Negative controls were the same used for the KL-specific ChIP assay. Positive controls included whole-cell DNA (+) and input material before immunoprecipitation. Immunoprecipitation for HMGA1/DNA complexes was carried out with HMGA1 MR19 antiserum (HMGA1) (Reeves and Nissen, 1999)

five discrete *in vitro* HMGA1a binding sites between nucleotides −853 and −434 of the *KL* promoter (Figure 3a). EMSA results also demonstrated that HMGA1a binds with a high affinity to the promoter with the first binding site appearing between 2.5 and 5 nM of purified HMGA1a. As a positive control, the same experiment was carried out using the 3′-untranslated region of bovine interleukin-2 (BLT) DNA as the binding substrate. BLT, like the *KL* promoter, has several stretches of AT-rich DNA, and HMGA1a binding to these stretches has already been characterized via DNase I footprinting (Elton *et al*., 1987). To further confirm the specificity of binding of HMGA1a proteins to the AT-rich regions of the *KL* promoter, these regions were mutated to eliminate the AT-rich sequences within potential HMGA1a binding sites. While unlabeled wild-type oligonucleotides were able to significantly reduce the number of shifting bands of labeled *KL* promoter DNA, mutant oligonucleotides were not, indicating that HMGA1a does specifically bind to the AT-rich stretches within the *KL* promoter (Figure 3b and c).

To further demonstrate the ability of HMGA1a to bind the human *KL* promoter *in vivo*, we used a chromatin immunoprecipitation (ChIP) assay (Kuo and Allis, 1999). This technique allowed for the identification of a biologically functional interaction between the HMGA1a protein and *KL* promoter DNA inside living cells. Briefly, the ChIP assay involved generating formaldehyde-induced protein–protein and protein–DNA crosslinks in MCF-7 cells in culture. The protein–DNA complexes were then fragmented to ~500 bp by sonication and immunoprecipitated using HMGA1a-specific antiserum. The resulting immunoprecipitated DNA was then used as template in a PCR amplification reaction to probe for the presence of the *KL* promoter DNA region between −833 and −450, and as a negative control, the *HPRT1* promoter (between – and –, GenBank #). Results from these ChIP assays with HMGA1a-ON cells demonstrate that HMGA1a does, in fact, bind to *KL* promoter DNA *in vivo* (Figure 3d) and supports the *in vitro* observations of HMGA1a protein binding to the *KL* promoter obtained by EMSA analyses (Figure 3a). The results also demonstrate that in these same cells the promoter region of HPRT1, a gene that is not regulated by HMGA1 (Reeves *et al*., 2001; Treff *et al*., 2004) and therefore serves as a negative control, does not bind HMGA1a *in vivo* (Figure 3d).

In addition, we wanted to determine if MCF-7 cells overexpressing HMGA1a were more sensitive to growth





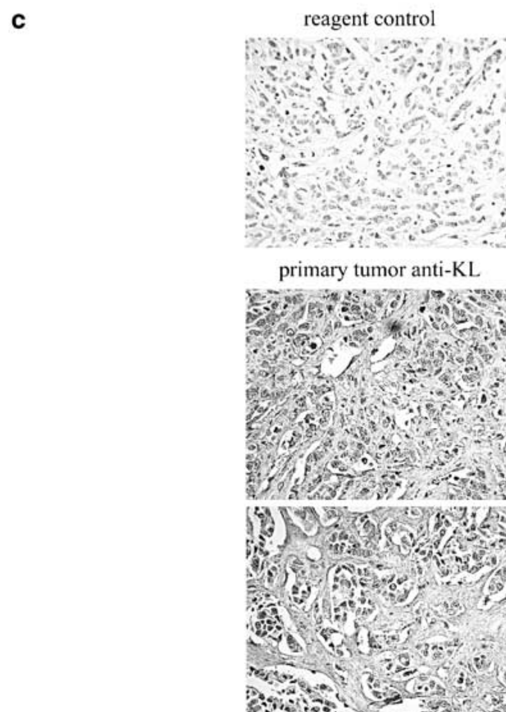


Figure 4 (**a**) HMGA1a overexpressing cells are more sensitive to growth inhibition by KL neutralization. A measure of 5 μg/ml of KL antibody (sc-1302, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to neutralize the bioactivity of KL in the culture medium of both MCF-7 parental (HMGA1a-OFF) and MCF-7 HA7CCs (HMGA1a-ON) cells. Cells were treated for 3 days with (black bars) or without (white bars) the KL antibody and counts of viable cells were made by trypan blue exclusion every 24 h. A significant difference between ON and OFF growth inhibition by KL neutralization ($P<0.0001$, $n=3$) was determined using Student's t-test. Error bars indicate 1 s.d. HMGA1a-ON but not OFF (**b**) and tissue sections from primary tumors derived from HMGA1-ON MCF-7 cells after injection into the mammary fat pad of nude mice (**c**) (Reeves *et al*., 2001) stained positive for human KL using anti-KL antibody 1 : 100 (Santa Cruz Biotechnology). Avidin–biotin complex (ABC Kit; Vector Laboratories, Burlingame, CA, USA) and the diaminobenzidine (DAB) chromogen were used for visualization. Hematoxylin (Sigma, St Louis, MO, USA) was applied as a counterstain

inhibition by KL neutralization than cells without transgenic HMGA1a overexpression. It is known that KL can stimulate the growth of cancer cells (Caceres-Cortes *et al*., 2001), and that addition of KL antibody to cell culture media can inhibit KL bioactivity (Huss *et al*., 1996). Following 48 h of treatment, our results demonstrate that HMGA1a-ON cells are significantly more susceptible than parental MCF-7 cells to growth inhibition by KL neutralization ($P<0.0001$, $n=3$; Figure 4). Interestingly, addition of the KL antibody to the culture medium reduced growth of both HMGA1a-ON and parental cell lines after 72 h. This may be explained, in part, by the fact that parental, nontransgenic MCF-7 cells have been reported to express low levels of KL (Hines *et al*., 1995). In addition, after 48 h, KL neutralization decreased HMGA1a-ON cell growth by more than 50%, implicating KL targeted therapeutics as inhibitors of HMGA1-positive carcinomas. This possibility is also supported by the observation that KL protein is detected in tissue cultured HMGA1a-ON MCF-7 cells by immunocytochemistry but not in HMGA1a-OFF cells (Figure 4b).

Immunohistochemical analysis also demonstrated that human KL is expressed in primary tumors derived from HMGA1-ON MCF-7 cells injected into the mammary fat pad of nude mice (Figure 4c) (Reeves *et al*., 2001). Given these observations, the results of future work to determine the mechanism(s) of inhibition of tumor cell growth by anti-KL antibodies (e.g. by apoptosis, necrosis or other means) should prove to be of considerable interest.

Together, these data indicate that HMGA1 directly regulates the transcription of *KL* in cancer cells. Also supporting this conclusion is the fact that many previous reports characterizing the role of KL in cancer biology are consistent with observations of the influence of HMGA1 on cancer cells. For example, HMGA1 overexpression in MCF-7 breast cancer cells results in increased sensitivity to EGF activation of Ras/ERK signaling (Treff *et al*., 2004). A similar observation (e.g. an increased sensitivity to EGF activation of Ras/ERK signaling) was made when the KL receptor, c-kit, was ectopically expressed in MCF-7 cells (Hines *et al*., 1999). In addition, we have previously demonstrated that increased expression of HMGA1 protein in MCF-7 cells induces their progression to a much more metastatic and malignant phenotype as evidenced by their ability to grow in soft agarose and form tumors in nude mice (Reeves *et al*., 2001). Again, a similar observation was made for KL, in that the addition of KL to the culture medium of DLD-1 colon carcinoma cells leads to their anchorage-independent growth (Bellone *et al*., 2001). This cumulative evidence implicates serum KL as a potential diagnostic marker for HMGA1-specific carcinomas. In addition, the data presented here suggest that HMGA1 may regulate KL expression in other tissues such as the testis.

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