

# A Novel High-Throughput Screening System Identifies a Small Molecule Repressive for Matrix Metalloproteinase-9 Expression

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## ABSTRACT

Aberrant gene expression is one of the driving forces for cancer progression and is considered an ideal target for chemical intervention. Although emerging bioluminescence reporter systems allow high-throughput searches for small molecules regulatory for gene expression, frequent silencing of reporter genes by epigenetic mechanisms hinders wide application of this drug discovery strategy. Here we report a novel system that directs the integration of a promoter-reporter construct to an open chromosomal location by Flp-mediated homologous recombination, thereby overcoming reporter-gene silencing. Using this system, we have screened more than 8000 compounds in the DIVERSet chemical library for repressors of a matrix metalloproteinase-9 (MMP-9) promoter and identified 5-meth-

yl-2-(4-methylphenyl)-1H-benzimidazole (MPBD) inhibitory for MMP-9 gene expression. Consistent with this effect, MPBD inhibits MMP-9-dependent invasion of UMSCC-1 oral cancer cells, preosteoclast migration, and receptor activator of nuclear factor- $\kappa$ B ligand-induced osteoclast activity over concentration ranges that repressed MMP-9 expression. Mechanistic studies indicated that MPBD antagonizes AP-1 function by inhibiting its transactivation activity. We conclude that the Flp-mediated homologous recombination system to direct reporter integration into open chromatin regions represents a novel strategy allowing for the development of high-throughput systems screening for lead compounds targeting aberrant gene expression in cancer.

Aberrant gene expression is a hallmark of cancer. Expression of genes such as matrix metalloproteinase-9 (MMP-9, 92-kDa type IV collagenase/gelatinase B) (Van den Steen et al., 2002) that are essential for normal cellular homeostasis is often altered, resulting in growth and cancer progression. Therefore, targeting the expression of these genes with small molecules has been considered an attractive approach to prevent and/or eliminate cancer (Pandolfi, 2001). Indeed, in this postgenomic era, because many of the genes that are implicated in the genesis and progression of cancer have been identified, large-scale searches for lead compounds that serve as prototypes for therapeutic agents rectifying aberration of gene expression in cancer cells are imperative.

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Because of their simplicity and sensitivity, reporter gene systems are often used for developing high-throughput assays screening for small-molecule gene regulators. In general, a reporter (e.g., firefly luciferase) fused to a natural or synthetic promoter and stably integrated into the genome of a cell line allows for high-throughput, cost-efficient screening amenable to robotic manipulations (Shoemaker et al., 2002). An integrated or transgenic reporter has obvious advantage over a transiently expressed reporter given that the latter has to be introduced into cells for every assay, and transfection efficiency varies. Indeed, several groups have successfully used this technology to identify compounds regulatory for the activities of transcription factors such as CEPB $\alpha$  (Shoemaker et al., 2002), p53 (Wang et al., 2006), or hypoxia-inducible factor-1 (Rapisarda et al., 2002) and their respective downstream targets. Notwithstanding these advances, such systems are nevertheless hindered by transgene position effects, a common phenomenon whereby the activity of

**ABBREVIATIONS:** MMP, matrix metalloproteinase; AP-1, activator protein-1; FRT, Flp recombination target; HTS, high-throughput screening; JNK, Jun N-terminal kinase; MPBD, 5-methyl-2-(4-methylphenyl)-1H-benzimidazole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PBD, 2-(4-methylphenyl)-1H-benzimidazole; PMA, phorbol myristate acetate; RANKL, receptor activator of nuclear factor- $\kappa$ B ligand; kb, kilobase(s); DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; TRAP, telomeric repeat amplification protocol; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one 1,9-pyrazoloanthrone.

reporter genes integrated into nonpermissive chromosomal locations are either repressed or unstable as a result of a closed chromatin structure (Wakimoto, 1998; Yan and Boyd, 2006). Thus, reporters are silenced or variegated in their expression, making them unsuitable for compound screening. Indeed, this is a real obstacle, given that nonpermissive chromosomal locations are widely distributed throughout the human genome (Barski et al., 2007) and that conventional DNA delivery approaches confer random, rather than site-specific, integration of transgenes.

We developed a system that allows reporter genes to integrate at specified chromosomal locations tagged with an FRT sequence through FLP-mediated homologous recombination (Yan et al., 2004). This advance prompted us to ask whether this system could be exploited to introduce reporter genes into a specific chromosomal location where the chromatin structure is open, and whether the derived cells are of utility for high-throughput compound screening. To address this, we introduced a firefly luciferase reporter under the control of a 2.2-kb MMP-9 promoter into an HT1080 clone in which the FRT recombination fragment is located within an open chromatin region (F55 site) (Yan and Boyd, 2006), generating cells bearing an integrated MMP-9 promoter-driven reporter for high-throughput screening (HTS). We chose MMP-9 as a test gene, given its role in promoting multiple steps across the tumor progression spectrum including angiogenesis, invasion/metastasis, and osteolysis (Van den Steen et al., 2002; Hjertner et al., 2005). MMP-9 is overexpressed in many cancers and contributes to cancer progression in part by degrading extracellular matrix and activating proangiogenic growth factors such as transforming growth factor- $\beta$ . Regulation of MMP-9 protein levels has been largely ascribed to transcriptional activation of the gene through binding sites for AP-1 and NF- $\kappa$ B transcription factors that are localized in the 2.2-kb promoter region (Yan and Boyd, 2007), and therefore aberrant MMP-9 expression could be an ideal target for cancer therapy. Indeed, small molecules that inhibit the enzymatic activity of MMP-9 have shown preclinical efficacy in the inhibition of growth and progression of various cancers in experimental models. However, these agents targeted many other metalloproteinases and thus caused unexpected side effects in clinical trials (Coussens et al., 2002), mandating a need to search for small molecules that directly target MMP-9 expression.

Using this novel system, we identified a small molecule that inhibits MMP-9 expression without affecting the expression of two related metalloproteinases. It is noteworthy that this compound counters various MMP-9-dependent biological events, including cancer cell invasion, osteoclast migration, and activity, and thus represents a new class of MMP-9 antagonists that could be of therapeutic benefit. These results collectively demonstrate that this FLP-mediated homologous recombination system represents a useful technology for developing HTS assays to screen compound libraries for regulators of gene expression for cancer therapy.

## Materials and Methods

**Cell Culture and Transfections.** HT1080 and UMSCC-1 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum and antibiotics. RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium/F-12 medium. Transfections were

performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

**Flp-Mediated Integration of the MMP-9 Promoter.** This was performed essentially as described previously (Yan et al., 2004). In brief, 1  $\mu$ g of pCM/MMP-9/Luc2.2k, an FRT-harboring plasmid containing a luciferase gene driven by a 2.2-kb MMP-9 promoter (Yan et al., 2004), was cotransfected with 9  $\mu$ g of Flp-expressing pOG44 plasmid into HT1080/F55 cells (Yan and Boyd, 2006) in a 100-mm plate followed by selection of the transfected cells with 200  $\mu$ g/ml Hygromycin B (Calbiochem, San Diego, CA). The obtained resistant clones were lysed for luciferase activity assays, whereas a random clone designated as HT/MMP9/F55 was expanded for HTS assays.

**High-Throughput Compound Screening.** HT/MMP9/F55 cells were plated in 96-well plates at a density of  $1.5 \times 10^4$ /well. The next day, 5  $\mu$ M compounds (final concentration) from the DIVERSet chemical library (ChemBridge, San Diego, CA) or a corresponding amount of DMSO was added, and cells were treated overnight (18 h). After washes with phosphate-buffered saline, the cells were lysed in 100  $\mu$ l of Passive Lysis buffer (Promega, Madison, WI), and 20  $\mu$ l of cell lysate was assayed for luciferase activity as described previously (Yan et al., 2004).

**Real-Time Reverse Transcription-Polymerase Chain Reaction and Gelatin Zymography.** Total RNA was prepared, reverse-transcribed, and subjected to real-time PCR assays essentially as described previously (Yan and Boyd, 2006). The primer sequences used for amplifying MMP-9, MMP-2, and  $\beta$ -actin cDNA were described in our previous report (Yan et al., 2001). MMP-1 primer sequences were 5'-GGA GAT CAT CGG GAC AAC T-3' and 5'-GGG TAT CCG TGT AGC ACA TTC-3'. PCR specificity was confirmed by the identification of a single amplified product in an agarose gel. Zymography was carried out as described previously (Yan et al., 2001), using aliquots of conditioned medium corrected for any differences in cell number.

**AP-1 Reporter Assays, c-Jun Transactivation Assays, and Western Blotting.** To determine AP-1 activity, HT1080 cells were plated in 96-well plates and transfected with 0.1  $\mu$ g of pAP-1-luc containing seven tandem repeats of AP-1 consensus binding sites (see Fig. 6B) (Stratagene, La Jolla, CA). Twenty-four hours later, 5-methyl-2-(4-methylphenyl)-1H-benzimidazole (MPBD) was added and treated the cells overnight (16 h) before the cells were lysed for luciferase activity assays as described previously (Yan et al., 2004). To determine the transactivation activity of c-Jun, the PathDetect system (Stratagene) was used according to the manufacturer's recommendations (Shin et al., 2002). In brief, HT1080 cells were transfected with pFR-luc (expressing a Gal4-driven luciferase reporter; see Fig. 6C), pFac-Jun (expressing a Gal4-c-Jun fusion protein), or pFCdbd empty vector in 96-well plates. Twenty-four hours after transfections, compound MPBD was added and treated the cells for an additional 16 h. The luciferase activity was determined as described previously (Yan et al., 2004). Western blotting was performed as described previously (Yan et al., 2001). The antibodies against phosphorylated c-Jun (Ser73) was purchased from Cell Signaling Technology (Danvers, MA).

**Invasion and Motility Assays.** For invasion assays, UMSCC-1 cells were detached with 2 mM EDTA, suspended in serum-free medium containing 1% bovine serum albumin, and  $5 \times 10^4$  cells were plated into a Matrigel-coated transwell. Invaded cells were stained and quantitated using a Cell Invasion Assay Kit (Millipore Bioscience Research Reagents, Temecula, CA) 20 h later by following the manufacturer's instructions. For cancer cell-mobilized migration assays, RAW264.7 cells were stimulated with 100 ng/ml RANKL for 72 h, detached, and suspended as above. Cells ( $5 \times 10^4$ ) were plated into a transwell, and the latter was inserted into a 24-well plate containing  $1 \times 10^5$ /well UMSCC-1 cells. Migrated RAW264.7 cells were stained and enumerated 40 h later.

**TRAP Staining and Osteoclastic Activity Assays.** TRAP staining to determine osteoclastogenesis was performed using a Leukocyte Acid Phosphatase Kit (Sigma, St. Louis, IL) according to the

manufacturer's recommendations. In brief, RAW264.7 cells were stimulated with 100 ng/ml RANKL for 4 days and then fixed in citrate/acetone solution for 30 s. After rinsing and drying, the cells were incubated with a mixture containing acetone solution, naphthol AS-BI phosphoric acid, tartrate solution, and Fast Garnet GBC salt at 37°C for 1 h and observed under a microscope. For osteoclastic activity assays, RANKL-stimulated cells were detached and suspended in RANKL-containing medium with or without 20  $\mu$ M MPBD and plated on 16-well coated quartz slides (BioCoat Osteologic Bone Cell Culture System; BD Biosciences, Bedford, MA). After 3 days, the cells were washed with phosphate-buffered saline followed by the addition of bleach for 3 min. After extensive washes, the slides were dried, and images were captured using a light microscope (magnification, 40 $\times$ ). Quantitation of the resorbed area of the bone was with Image J software.

## Results

**Integration of an MMP-9 Promoter into an Open Chromosomal Location through Homologous Recombination.** The first and critical step toward developing a reporter gene-based system for high-throughput compound screening is to establish cells that stably express the reporter gene under the control of a promoter of interest. Because of transgene position effects, reporter genes are often silenced, necessitating that the reporter gene is directed to an open chromosomal location. We have characterized previously the F55 site as a "permissive" chromosomal location that is defined by histone H3 acetylation and H3-Lys4 methylation and not subject to transgene silencing over extended periods (Yan and Boyd, 2006). We thus used the FLP-mediated homologous recombination system as described in our previous report (Yan et al., 2004) to direct the integration of a luciferase cDNA driven by a 2.2-kb MMP-9 promoter at this chromosomal location. Toward this end, we cotransfected the endogenous MMP-9-expressing HT1080 cells harboring the F55 site (Yan and Boyd, 2006) with a FLP-expressing plasmid and the promoter-luciferase construct that also carries an FRT fragment (Fig. 1A). After selection with Hygromycin B for 2 weeks (Yan et al., 2004), we obtained several resistant clones that ubiquitously expressed high levels of luciferase (data not shown), consistent with our previous report that the F55 location is permissive for transgene expression (Yan and Boyd, 2006). Because these isogenic clones expressed comparable levels of luciferase, we randomly expanded one clone (HT/MMP9/F55) and determined whether these cells respond to a known MMP-9 stimulus (PMA) (Yan et al., 2004). The cells were plated into a 96-well plate at different densities ( $1.0 \times 10^4$ ,  $1.5 \times 10^4$ ,  $2.0 \times 10^4$ ,  $2.5 \times 10^4$ , and  $3.0 \times 10^4$ /well) and subjected to treatments with varying amounts of PMA. As expected, PMA increased the cellular luciferase activity in a dose-dependent manner over a wide range of cell density (Fig. 1B), suggesting that the activity of the transgenic MMP-9 promoter mirrors its endogenous counterpart, and thus alterations in the expression level of the reporter gene probably reflect altered transcription of the endogenous MMP-9 gene. We chose a plating density of  $1.5 \times 10^4$ /well for the subsequent HTS studies because the cells plated at this density grew as a nearly confluent monolayer and responded well to the chemical treatment (Fig. 1B).

**High-Throughput Screening for Compounds Inhibiting MMP-9 Promoter Activity.** We then used these cells for high-throughput screening for compounds repressive for

MMP-9 expression. Toward this end, we treated HT/MMP9/F55 cells cultured in 96-well plates with 5  $\mu$ M concentrations of individual compounds from the DIVERSet library (composed of >8000 compounds) and then measured the luciferase activity as an index of promoter activity. To ensure that the assay conditions have been optimized, we first treated two plates of cells with 1  $\mu$ M actinomycin D (positive control), a general transcription inhibitor (Sobell, 1985), which also repressed the transgenic MMP-9 promoter (Fig. 1C), and calculated assay variability within and between plates. The intraplate variability (CV) ranged from 10.9 to 12.1%, whereas the plate-to-plate variation was negligible (Fig. 1D). In addition, the value of Z'-factor (Zhang et al., 1999) equaled 0.64 (Fig. 1D), indicating that the assay system could be used for high-throughput screening for MMP-9-repressing agents. Moreover, to further ensure the reproducibility of the screening data, we performed a small-scale screening with 240 compounds from the library and generated a Q-Q plot (Huang et al., 2004) based on the effects of these compounds on the transgenic promoter in two independent treatments. The quantile distributions derived from the duplicate assays (scatter squares) only slightly deviated from the quantiles of a normal distribution (straight line) (Fig. 1E), indicating a high reproducibility between independent experiments (Huang et al., 2004).

We thus screened the entire library (8471 compounds) for small molecules repressive for transgenic promoter activity. We excluded those compounds that reduced luminescence signal to the background level because they most likely interfered with the luciferase-enzyme reaction or were severely toxic to the cells. Although the majority of compounds (94.8%) had no or modest effects (relative luciferase activity greater than 0.5 but smaller than 2;  $\log_2$  scale), 5.2% either increased (405 compounds or 4.8%) or reduced (35 or 0.4%) the luciferase activity by 1-fold (relative luciferase activity greater than 2 or smaller than 0.5;  $\log_2$  scale) (Fig. 2A). Of the latter group, four compounds (7561157, 7642828, 7661218, and 5691508) yielded a greater than 75% reduction in luminescence signal (Fig. 2A) and were therefore pursued for further investigation. We confirmed the effects of these compounds in an independent experiment showing that luciferase activity was dramatically reduced in a dose-dependent manner (Fig. 2B). Compounds 7561157 and 7642828 resulted in 90% reduction in the luciferase activity at a concentration as low as 2.5  $\mu$ M (Fig. 2B). Cytotoxicity assays (Fig. 2C) ruled out the possibility that repressed reporter activity was merely a consequence of cell death.

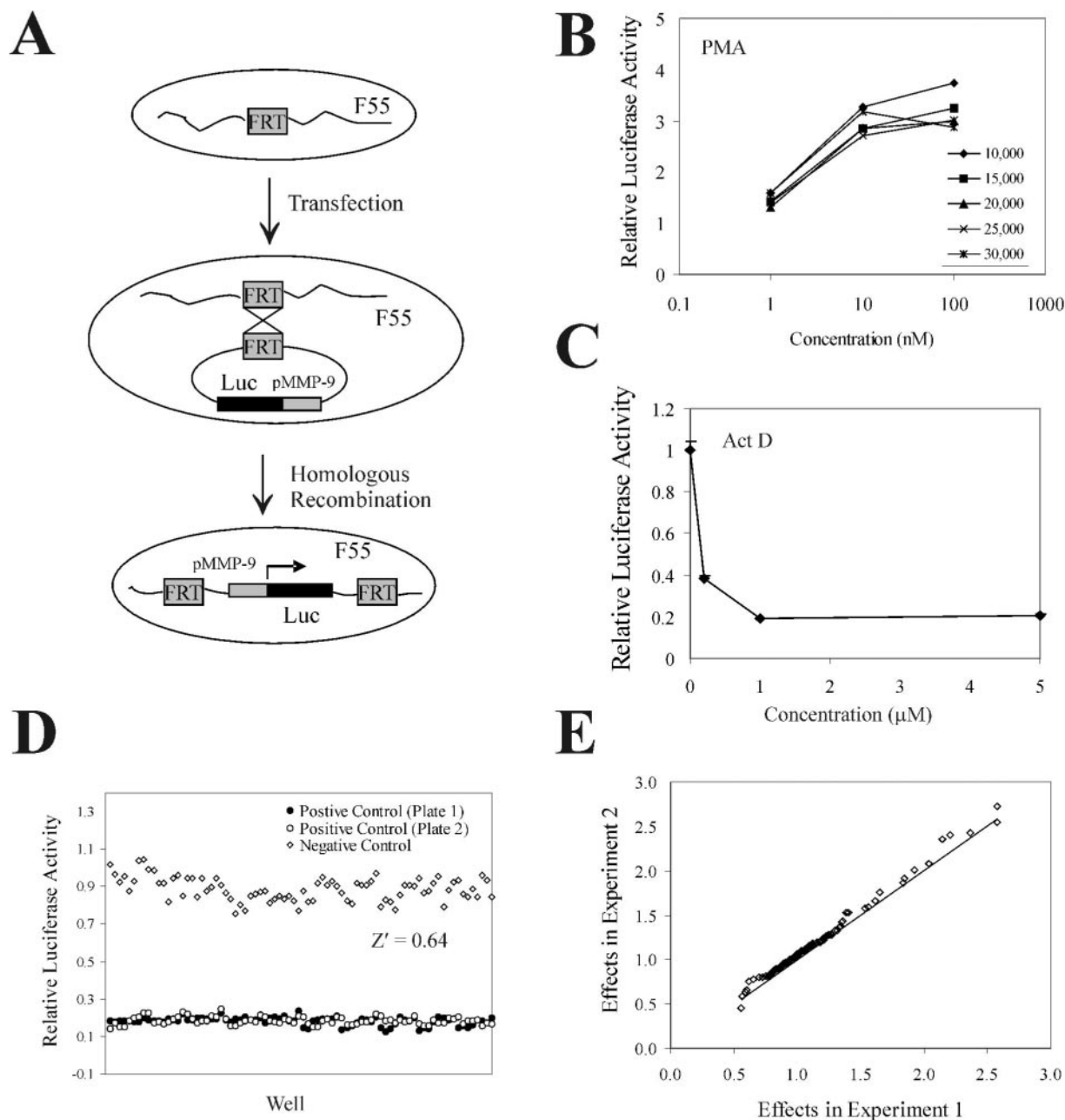
**A Benzimidazole Derivative Specifically Repressed MMP-9 Expression.** Next, we validated the effect of these four small molecules on endogenous MMP-9 expression. We first treated HT1080 cells with these compounds and measured MMP-9 protein levels with zymography. Of the four compounds, 7561157, a benzimidazole derivative, MPBD (see Fig. 7A for the chemical structure), dose-dependently inhibited PMA-induced elevation of MMP-9 levels (Fig. 3A) but had minimal effect on the basal MMP-9 level and the level of MMP-2, a constitutively expressed gelatinase functionally related to MMP-9 but differing in that it is unresponsive to PMA (Sternlicht and Werb, 2001) (Fig. 3A). This effect was most likely attributable to the repression of MMP-9 transcription evident by the reduction of MMP-9 mRNA levels as measured by real-time reverse transcription-polymerase



chain reaction (Fig. 3B) and consistent with the result from a reporter assay showing that MPBD inhibited PMA-induced transactivation of the integrated MMP-9 promoter (data not shown). This observation is notable because PMA stimulates the mitogen-activated protein kinase signaling pathway, a convergence node for divergent growth factors (Van den Steen et al., 2002). Indeed, 20  $\mu$ M MPBD reduced MMP-9 induction by 80% (Fig. 3B). The lack of effect of MPBD on basal MMP-9 mRNA levels may reflect the prolonged half-life

of the MMP-9 transcript ( $\sim 19$  h) (Sehgal and Thompson, 1999) coupled with a low level of transcription under basal conditions. It is interesting that MPBD did not inhibit MMP-2 expression (Fig. 3C), whereas it only slightly repressed PMA-induced MMP-1 expression ( $p > 0.05$ , Fig. 3D), although the promoter of the latter gene bears strong similarity to that of MMP-9 (Yan and Boyd, 2007).

**MPBD Inhibits Cancer Cell Invasion and Blocks Preosteoclast Migration and Osteoclastic Activity.** We



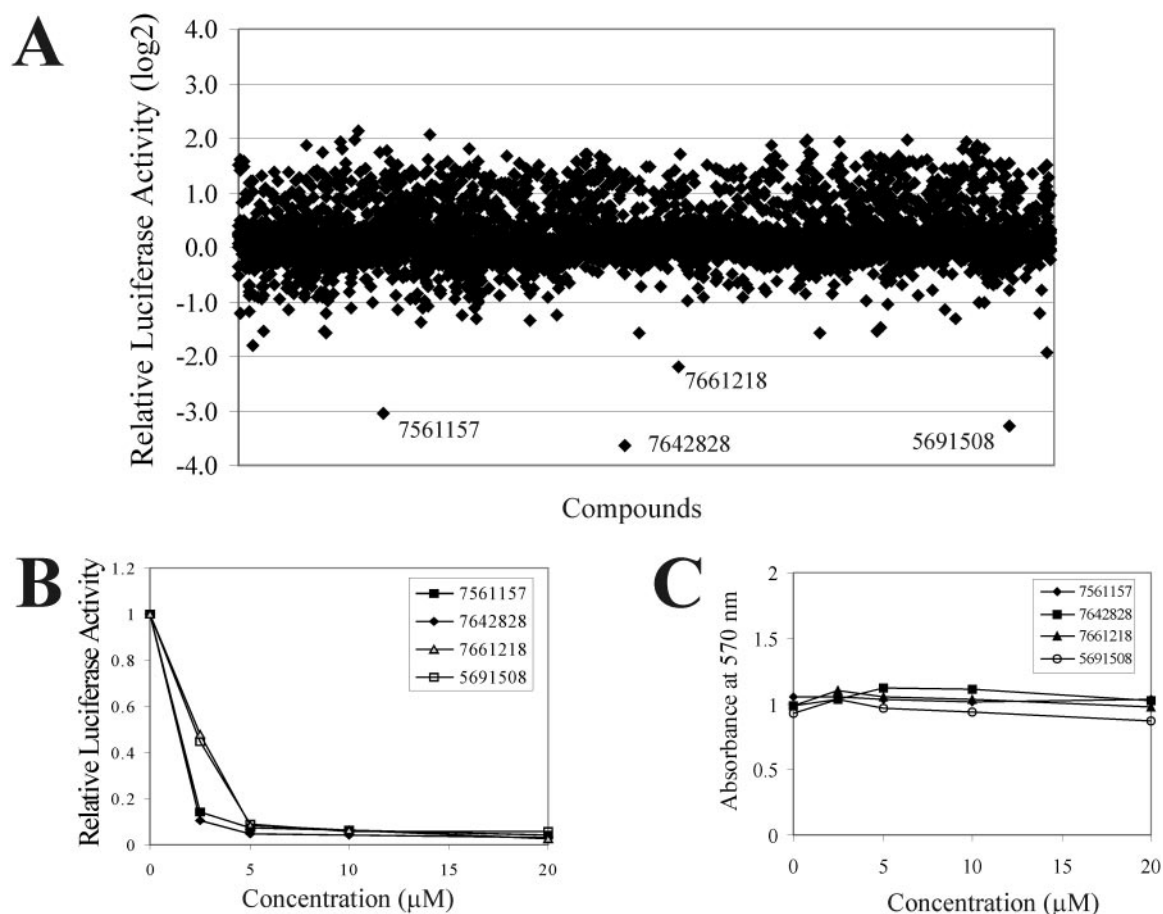
**Fig. 1.** Development of a novel system for high-throughput screening for small MMP-9 transcription regulators. A, schematic showing recombination between FRT fragments allowing for integration of a luciferase reporter gene driven by a MMP-9 promoter into an open chromosomal location (the F55 site). B, HT/MMP9/F55 cells were plated into a 96-well plate at indicated densities, treated with PMA for 5 h, and lysed for luciferase activity assays. C, HT/MMP9/F55 cells ( $1.5 \times 10^4$ /well) were plated into a 96-well plate, treated with indicated amounts of actinomycin D overnight, and lysed for luciferase activity assays. D, the transgenic cells cultured in 96-well plates were treated with 1  $\mu$ M actinomycin D (positive control, two plates) or DMSO (negative control, one plate) overnight and lysed for luciferase activity assays. The assay results from the two positive-control plates were combined and used to calculate  $Z'$  as described previously (Zhang et al., 1999). E, the transgenic cells were treated with 5  $\mu$ M concentrations of 240 randomly selected compounds overnight and lysed for luciferase activity assays. Results from two independent experiments were used to generate a Q-Q plot to determine the reproducibility of the screening assays.

then queried the ability of MPBD to interfere with two known biological functions of MMP-9 in cancer: 1) invasiveness of cancer cells, and 2) cancer-driven osteoclast activity. Regarding the former, we reported previously that the *in vitro* invasiveness of UMSSC-1 oral cancer cells, which do not express MMP-2, is partly dependent on MMP-9 expression (Juarez et al., 1993). Like HT1080 cells, MPBD also inhibited PMA-induced MMP-9 expression in this oral cancer line, as evident with zymography (Fig. 4A). We thus treated UMSSC-1 cells with 20  $\mu$ M MPBD for 24 h, stimulated them with PMA, and determined cell invasion using transwell chambers coated with Matrigel. Consistent with its inhibition of MMP-9 expression (Fig. 4A), MPBD reduced the number of cells invading through the reconstituted basement membrane over an identical concentration range (Fig. 4B). Quantitation with a cell staining method (see *Materials and Methods*) indicated that MPBD inhibited the invasiveness of UMSSC-1 cells by more than 70% (Fig. 4B).

As mentioned above, MMP-9 also contributes to bone resorption with some osteolytic malignancies including breast, multiple myeloma, and oral cancers (Ash et al., 2000; Van Valckenborgh et al., 2004; Guise et al., 2005). For cancers metastatic to the bone, osteoclasts and their precursors (macrophage lineage) rather than cancer cells express MMP-9 in response to cytokines, including receptor activator of nuclear

factor- $\kappa$ B ligand (RANKL), resulting in bone lysis, cancer cell colonization/growth, and further recruitment of preosteoclasts to the remodeling bone (Guise et al., 2005). Because MPBD inhibited MMP-9 expression in cancer cells, we asked whether it could also blunt MMP-9 expression in pre/osteoclasts, their recruitment/migration induced by cancer cells, and subsequent osteoclastic activity. First, we treated RAW264.7 osteoclast precursors with RANKL for 3 days and assayed for MMP-9 levels by zymography. As reported previously (Dong et al., 2005), RAW264.7 cells expressed low MMP-9 levels (no MMP-2 expression), whereas RANKL stimulation yielded a substantial amount of MMP-9 as detected by zymography (Fig. 5A). Treatment with MPBD, however, dramatically inhibited RANKL-induced MMP-9 expression (Fig. 5A) whereas 20  $\mu$ M returned the MMP-9 amount to the basal level. Thus, this small molecule inhibited MMP-9 expression in osteoclast precursors as well. MPBD did not prevent RANKL-induced osteoclastogenesis (Fig. 5B), arguing against the possibility that the inhibition of MMP-9 expression was due to toxicity.

Because preosteoclast migration is MMP-9-dependent (Dong et al., 2005), we stimulated the RAW 264.7 osteoclast precursors with RANKL and determined the effect of MPBD on their migration across a transwell filter induced by conditioned media from UMSSC oral cancer cells. Oral cancers



**Fig. 2.** Identification of small molecules repressive for transgenic MMP-9 promoter. A, HT/MMP9/F55 cells were plated into 96-well plates and treated overnight with 5  $\mu$ M concentrations of compounds from a diversity-set chemical library. Cell lysates were prepared for luciferase activity assays. The relative luciferase activities were converted into logarithm values (binary logarithm, i.e., log<sub>2</sub>) and plotted for each compound. For compounds that inhibited luciferase activity more than 75%, average values from two independent assays were used for analyses. B and C, the cells were treated with the indicated amounts of the four compounds for 5 h and then lysed for luciferase activity (B) or MTT assays as described previously (Mosmann, 1983) (C).

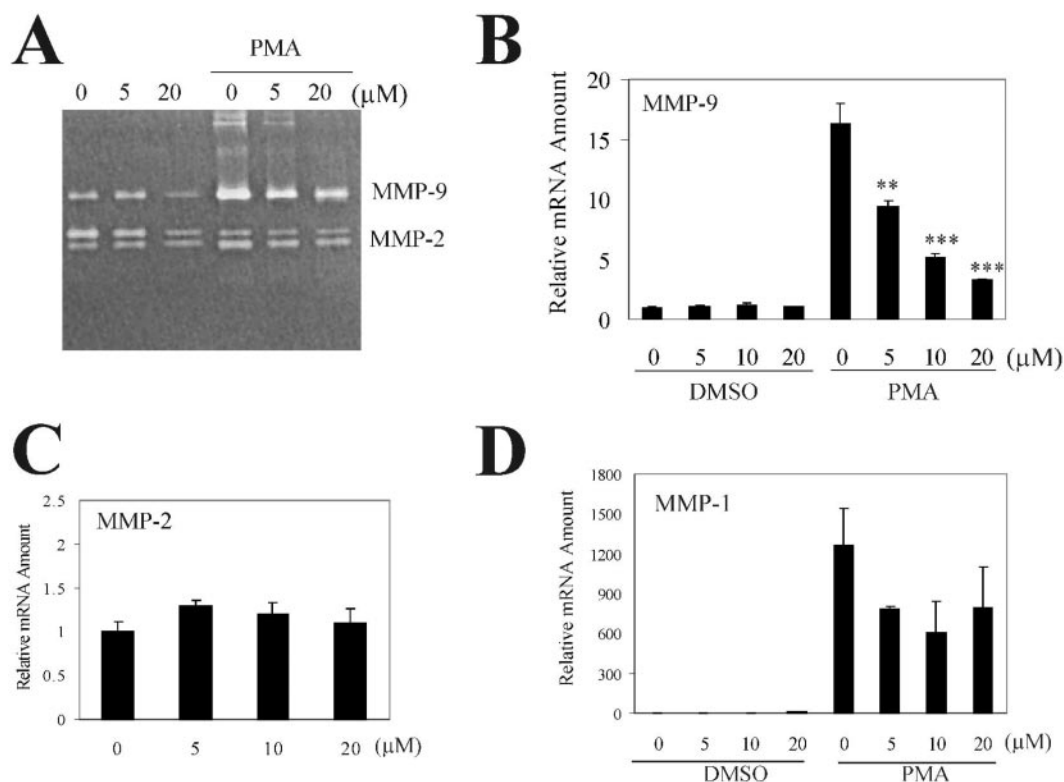
are known for their ability to promote osteolysis (Ash et al., 2000). As expected, RANKL increased cell migration across the membrane by 100% (Fig. 5C). It is noteworthy that MPBD inhibited RANKL-induced migration (Fig. 5C), whereas 2-(4-methylphenyl)-1H-benzimidazole (PBD), an MPBD analog that lacks the 5-methyl group (Fig. 7A) and that failed to inhibit RANKL-induced MMP-9 expression (see Fig. 7D, lane 5), had no effect on migration of stimulated cells (Fig. 5C). These results suggest that MPBD effectively counters MMP-9-dependent preosteoclast migration (Dong et al., 2005).

Because MMP-9 secreted by osteoclasts contributes to bone destruction by osteolytic cancers (Guise et al., 2005), we also asked whether the repression of MMP-9 expression by MPBD counters osteoclastic activity. We thus pretreated RAW264.7 cells with RANKL, plated them on a slide coated with a synthetic bone biomaterial mimicking bone tissue, and measured osteoclastic activity. As expected, RANKL-stimulated RAW264.7 cells exhibited a marked osteoclastic activity as evident with the large "resorbed" areas shown as clear, coating-free surfaces (Fig. 5D). In contrast, upon treatment of the osteoclasts with 20  $\mu$ M MPBD, a concentration which reduces MMP-9 expression to basal level, the activity of these cells in this assay was almost completely abrogated with only small areas of "resorption" now evident (Fig. 5D, arrows). Again, the efficacy of MPBD in strongly antagonizing osteoclast activity was not caused by toxicity as indicated by the presence of TRAP-positive multinucleated cells after MPBD treatment (Fig. 5E, arrows). These biological data provide further support for the view that MPBD is a novel repressor of MMP-9 expression.

**MPBD Reduces MMP-9 Promoter Activity by Antagonizing AP-1 Function.** What is the mechanism underlying the repression of the MMP-9 promoter? The human MMP-9

promoter contains a proximal AP-1 binding site (–73) and an NF- $\kappa$ B motif (–600) (Yan and Boyd, 2007) crucial for regulating MMP-9 expression in response to diverse stimuli, including PMA (Yan et al., 2004) and RANKL (Sundaram et al., 2007). We used a set of HT1080 clones that harbor a single-copy wild-type MMP-9 promoter or mutants in the AP-1 or the NF- $\kappa$ B binding site in an identical chromosomal location (the F8 site) (Yan et al., 2004) and determined whether MPBD affected the activities of these transgenic promoters. The advantage of this system is that the flanking sequence is identical, thus excluding the confounding influence of different neighboring sequences on reporter activity (Yan et al., 2004). As expected, this compound inhibited the wild-type MMP-9 promoter (Fig. 6A). In contrast, point mutation at the proximal AP-1 binding site (mTRE), but not the NF- $\kappa$ B binding site (mkB), compromised (although not completely abrogated) the inhibitory effect of MPBD on the MMP-9 promoter (Fig. 6A), consistent with the notion that the compound represses MMP-9 promoter activity in part via antagonizing AP-1 function at the proximal binding site. Indeed, MPBD dose-dependently repressed the activity of a transiently expressed AP-1 reporter (pAP-1-luc; Fig. 6B) containing seven tandem repeats of the AP-1 consensus binding site (Fig. 6B), confirming that AP-1 is one of the molecular targets of this small molecule. Nevertheless, it is worth emphasizing that because the repressive effect of MPBD was not completely blocked by the AP-1 mutation (Fig. 6A), it is likely that the inhibition of MMP-9 expression by this compound was mediated through multiple *cis* elements, including AP-1.

One interesting question that emerged was how MPBD antagonized the AP-1 function. Such an effect could be achieved by either inhibiting the AP-1 DNA-binding activity or repressing its transactivation activity. It is interesting that although electrophoresis motility shift assays showed no

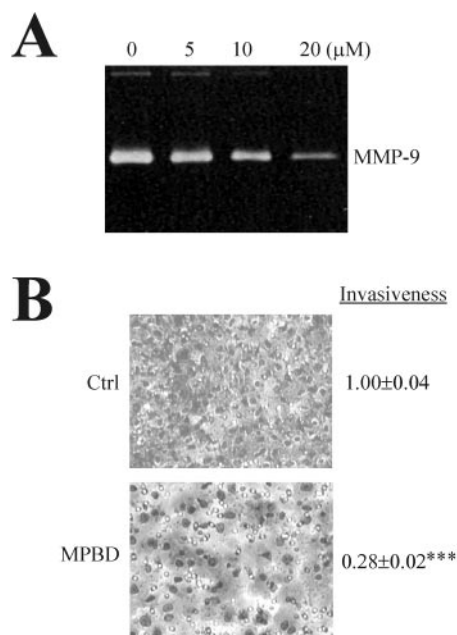


**Fig. 3.** A benzimidazole derivative inhibits MMP-9 expression in cancer cells. A, HT1080 cells were treated with the indicated amounts of MPBD or DMSO for 24 h followed by the addition of 50 nM PMA or DMSO into the medium. Condition media were collected and subjected to zymography. B, C, and D, HT1080 cells were treated as in A. Total RNA was prepared, reverse-transcribed, and cDNA was subjected to real-time PCR assays for MMP-9 (B), MMP-2 (C), MMP-1 (D), and  $\beta$ -actin mRNA amounts. The  $\beta$ -actin mRNA amounts were used to normalize loaded cDNA amounts. Data are depicted as average  $\pm$  S.D. values of three determinations. \*\*,  $p < 0.01$  versus PMA alone (Student's *t* test).



evidence that MPBD altered the DNA-bound AP-1 protein level (data not shown), this small molecule significantly inhibited AP-1 transactivation activity (Fig. 6C). Thus, although a transiently expressed luciferase reporter driven by Gal4-binding *cis*-elements (pFR-luc, Fig. 6C) was strongly transactivated by a c-Jun protein recruited to the promoter via a fused Gal4 DNA-binding domain (pFA c-Jun, Fig. 6C) (Shin et al., 2002), MPBD dramatically repressed the c-Jun transactivation activity in a dose-dependent manner (Fig. 6C). This repressive effect was unlikely due to an inhibition of the general transcription machinery because the small molecule did not modulate expression of the basal reporter activity driven by the empty vector (Fig. 6C, see pFc-dbd). Therefore, MPBD inhibits MMP-9 expression partly through repressing AP-1 transactivation activity.

Because phosphorylation of c-Jun by the Jun N-terminal kinase (JNK) contributes to AP-1 transactivation activity (Bogoyevitch and Kobe, 2006), it might be that MPBD repressed AP-1 activity through inhibiting phosphorylation of the AP-1 family member. However, under the same conditions in which AP-1 activity was repressed (Fig. 6, B and C), MPBD affected neither the phosphorylation level of the endogenous c-Jun (Fig. 6D) nor that of the transfected Gal4-c-Jun fusion protein (Fig. 6E), whereas a JNK inhibitor, SP600125 (Shin et al., 2002), clearly inhibited the c-Jun phosphorylation (Fig. 6E). These results suggest that the c-Jun/JNK pathway might not be the target of this novel AP-1 antagonist. Likewise, MPBD did not affect the activation of the mitogen-activated protein kinase pathway (data not shown), indicating that this compound may not target upstream cell signaling as well.



**Fig. 4.** MPBD inhibits invasion of UMSCC-1 oral cancer cells. **A**, UMSCC-1 cells were treated with indicated concentrations of MPBD or DMSO for 24 h and then cultured in serum-free medium (containing the compound) with 50 nM PMA for 24 h. Conditioned media from an equal number of cells were used for zymography. **B**, cells were treated with 20  $\mu$ M MPBD or DMSO for 24 h followed by the addition of 50 nM PMA into the medium. Twenty-four hours later, cells were plated into transwells coated with Matrigel and allowed to invade through the reconstituted basement membrane for 20 h, after which invaded cells were stained and quantified. Numbers indicate relative invasive capability. \*\*\*,  $p < 0.001$  versus the DMSO control (Student's  $t$  test).

**Analogues to MPBD Inhibit MMP-9 Expression.** Because we have identified MPBD as a novel small molecule inhibiting MMP-9 expression, we asked whether it could serve as a lead compound for drug discovery. We thus queried the ChemBridge compound inventory (available at <http://www.hit2lead.com/>) for MPBD analogs based on structural similarity and compared these compounds for MMP-9 repression. We identified eight compounds similar to MPBD (Fig. 7A), none of which was contained in the DIVERSet chemical library screened previously (Fig. 2). We thus treated the HT/MMP9/F55 cells with these small molecules and measured the luciferase activity. Two compounds (7933942 and 7951841) repressed the MMP-9 promoter (Fig. 7B) with little toxicity, as evident with MTT assays (Fig. 7C). Although the extent of reduction was smaller than that achieved with MPBD, 20  $\mu$ M concentrations of these two compounds inhibited RANKL-induced MMP-9 expression in RAW264.7 cells (Fig. 7D), whereas compound 793942 inhibited PMA-induced MMP-9 expression in UMSCC-1 cells as well (Fig. 7E). These studies point to a number of structural features necessary for repression. First and foremost, the methyl group at the benzimidazole ring is probably important for inhibition of MMP-9 expression, because compound 5129073 (PBD) that lacks this group failed to repress the transgenic promoter and MMP-9 expression (Fig. 7, B, D, and E). However, the proximity of this group to the ring structure seems requisite because its displacement with an ethoxy group (compound 511260) generates a null compound. Nevertheless, the ring position of the methyl group did not seem critical, as evidenced by the efficacy of 7933942 in repressing MMP-9 expression. Likewise, transfer of the methyl group to the phenyl ring combined with the addition of a second methyl group (compound 7951841) yielded a molecule partially effective in suppressing MMP-9 expression, at least in RANKL-stimulated RAW 264.7 cells. We thus conclude that MPBD serves as a lead compound for the development of therapeutic reagents treating MMP-9-dependent diseases (e.g., cancer, arthritis).

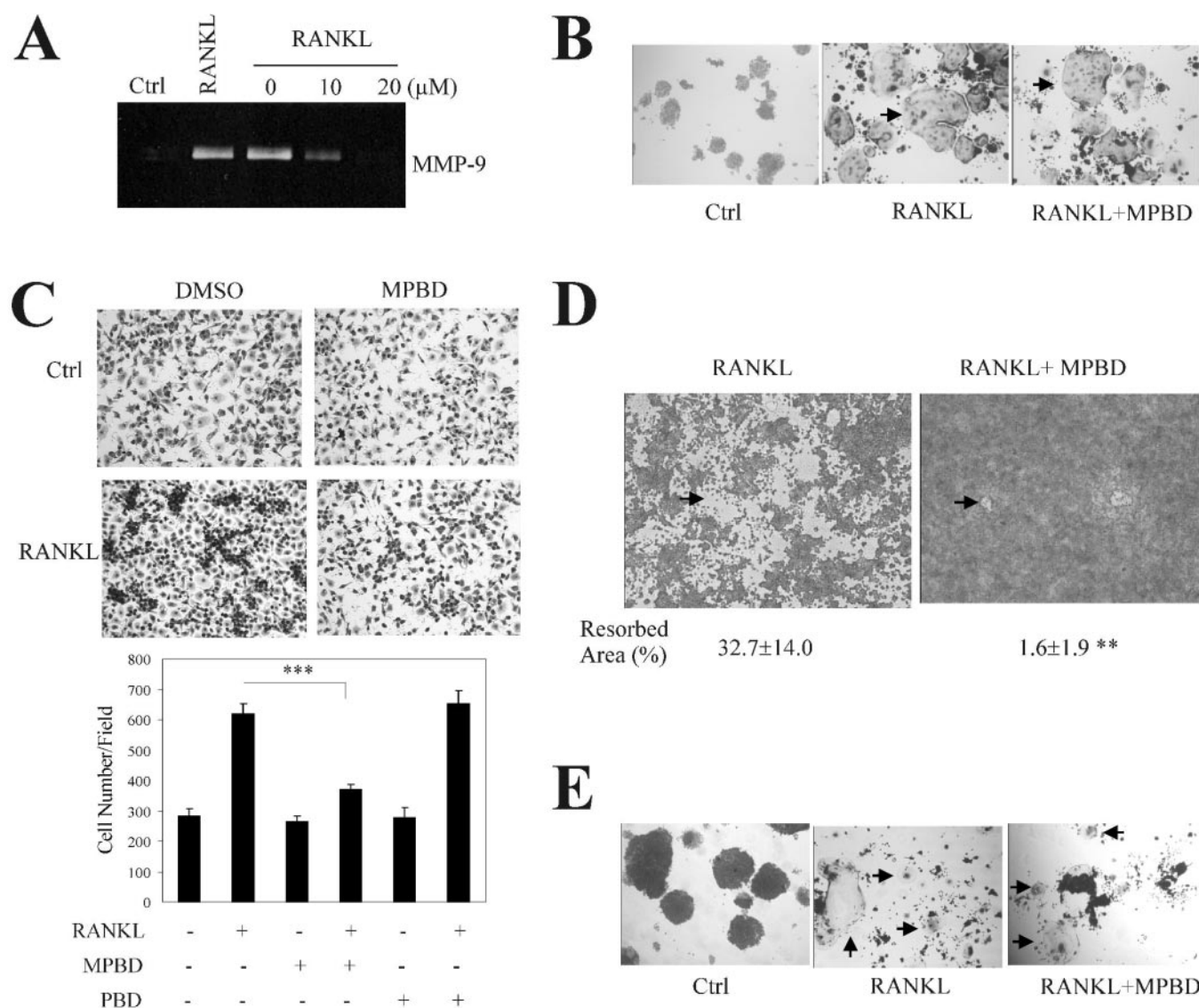
## Discussion

High-throughput screening of chemical libraries using bioluminescence gene reporting technology is an innovative approach to search for small molecules capable of rectifying aberrant gene expression in cancer. However, frequent silencing of reporter genes by epigenetic mechanisms (Wakimoto, 1998; Yan and Boyd, 2006) hinders wide applications of this drug discovery strategy. In this study, a FLP-mediated homologous recombination system (Yan et al., 2004) has been exploited to readily generate cells that carry the reporter in an open chromosomal location, thereby overcoming silencing effects imposed by surrounding chromatin. We have successfully demonstrated the utility of such an HTS system to identify a small molecule antagonist of MMP-9 expression. Thus, the FLP-mediated homologous recombination system serves as a useful technology to develop HTS for lead compounds that restore normal gene expression in cancer cells. Indeed, in addition to the inhibitors of MMP-9 expression, we have also identified compounds capable of repressing expression of an apoptosis suppressor cellular FLICE-inhibitory protein and a p53 antagonist MDM4 (Q. Gao, Z. Wang, C.

Yan, unpublished data), further demonstrating the versatility of this novel system.

MMP-9 has multiple roles in cancer biology, participating in both early and late events in the cancer progression spectrum (Van den Steen et al., 2002; Hjertner et al., 2005), and therefore drugs that interfere with its expression could be of therapeutic benefit. For example, recent studies have implicated this metalloproteinase in the early stages of lung colonization (Acuff et al., 2006), and it can be envisaged that drugs interfering with MMP-9 expression could benefit patients at high risk for tumor dissemination. In this regard, these drugs could be alternatives to MMP-9 enzymatic inhibitors that exhibited severe side effects in clinical trials, whereas these side effects were presumably caused by their

wide spectrum of activity across the metalloproteinase family (Coussens et al., 2002). Given that regulation of transcription is a result of interplay of multiple transcription factors (Yan and Boyd, 2007), drugs repressing MMP-9 expression might be more specific, and thus their side effects could be minimized. Second, there is a substantial body of evidence suggesting that tumor-driven bone destruction with osteolytic cancers is MMP-9-dependent (Brown et al., 2005; Dong et al., 2005). Indeed, our data clearly demonstrate the ability of MPBD to counter RANKL-induced MMP-9 expression, preosteoclast migration, and osteoclast activity, raising the possibility that anti-MMP-9 agents could be of therapeutic utility in managing this phase of the disease. Such agents could be used to complement the use of bisphosphonates, which are



**Fig. 5.** MPBD inhibits MMP-9 expression, preosteoclast migration, and osteoclastic activity. **A**, RAW264.7 cells were stimulated with 100 ng/ml RANKL for 24 h followed by the addition of the indicated amounts of MPBD or DMSO. Twenty-four hours later, cells were placed in serum-free medium and cultured for another 24 h. Conditioned media were collected and subjected to zymography after normalization for any cell number differences. **B**, RAW264.7 cells were stimulated with 100 ng/ml RANKL for 4 days, and MPBD was added into the medium on the third day of the treatments. The stimulated cells and control cells were then stained for TRAP activity. **C**, RAW264.7 cells were stimulated with 100 ng/ml RANKL for 24 h followed by the addition of 20 μM MPBD, PBD, or DMSO into the culture medium. Forty-eight hours later, cells were plated into transwells, and the latter were inserted into a 24-well plate containing UMSCC-1 oral cancer cells. Migratory cells were stained and counted after 40 h of incubation. \*\*\*,  $p < 0.001$ , Student's  $t$  test. **D**, RAW264.7 cells were stimulated with 100 ng/ml RANKL for 3 days and then transferred to 16-well BioCoat Osteologic slides containing 20 μM PBD or DMSO. Cells were removed by bleach 3 days later, and the slides photographed (magnification, 40×). \*\*,  $p < 0.01$  versus RANKL alone (Student's  $t$  test). **E**, RAW264.7 cells were treated as in **D**, and osteoclasts were stained for TRAP.



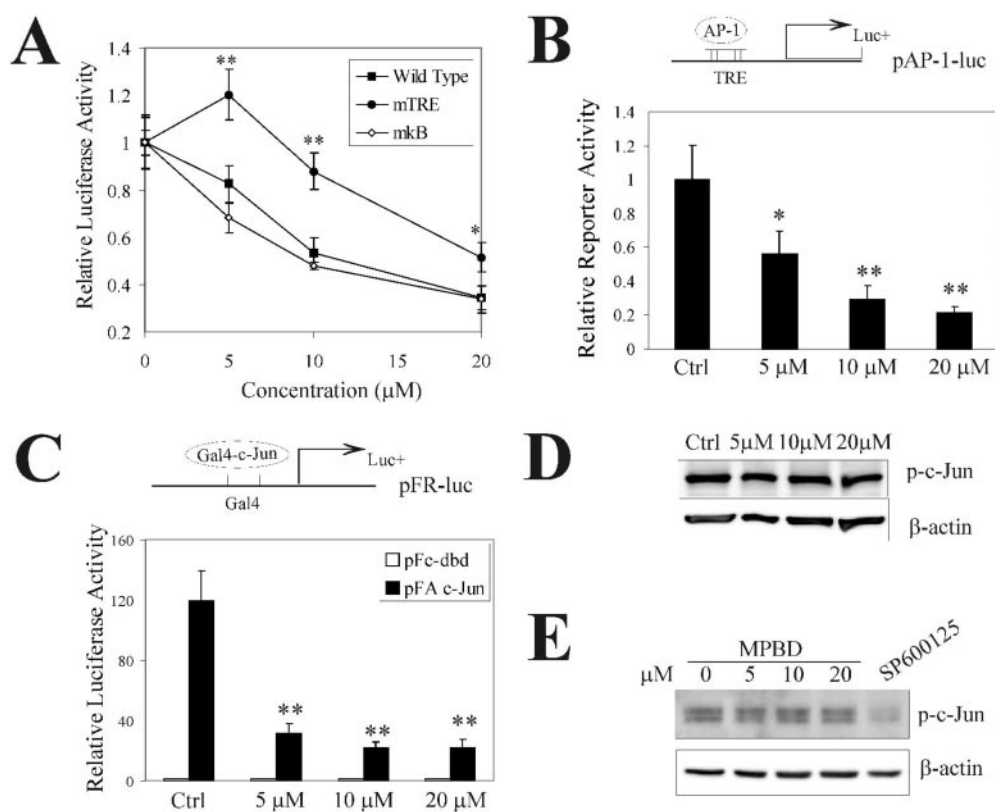
only partially effective in countering bone resorption with osteolytic cancers such as breast and multiple myeloma (Brown et al., 2005) and sometimes have debilitating side effects, including osteonecrosis of the jaw (Migliorati et al., 2005; Dunstan et al., 2007).

How does MPBD, a benzimidazole derivative, repress MMP-9 expression? Most likely, given its potent activity in our reporter assays, this compound targets MMP-9 transcription. Indeed, because of structural similarity to purine bases, it might be that the benzimidazole ring binds the minor groove of DNA to regulate transcription (Briehn et al., 2003; Chenoweth et al., 2007). However, we found no evidence that MPBD affects DNA-protein complex formation at the MMP-9 AP-1 motif, whereas such a mechanism would not explain how the transactivation activity of the Gal4-c-Jun fusion protein is repressed either. Given that MPBD inhibits the ability of the DNA-bound c-Jun to transactivate transcription, it might be that this compound interferes with the interaction of c-Jun with the basal transcription factors, an event essential for promoter transactivation (Papavassiliou, 1998). One possibility is that MPBD blocks cell signaling that leads to phosphorylation of c-Jun, because the latter event enhances the interplay of transcription factors (Bogoyevitch and Kobe, 2006). However, this mechanism is not supported by our observations that the phosphorylation levels of c-Jun remained unchanged upon MPBD treatment. Most likely, MPBD directly binds to the AP-1 protein and affects its interactions with one or more components of the basal transcription machinery (Papavassiliou, 1998).

If MPBD targets AP-1, it raises the question as to how MMP-1, which also harbors such a motif (Yan and Boyd, 2007), was relatively unaffected in its expression. It may be

that chromatin (epigenetic) factors associated with the MMP-1 promoter blunt the response of the promoter to altered AP-1 activity. On the other hand, the MMP-1 promoter may lack other regulatory elements present in the MMP-9 regulatory sequence that are required for repression. Indeed, our observation that substitution of the AP-1 binding site only partially impaired the inhibitory effect is consistent with the notion that MPBD targets multiple *cis* elements. Therefore, our finding that MPBD inhibits MMP-9 expression by antagonizing the AP-1 function adds a new function to the benzimidazole family of compounds that also inhibit the activity of casein kinase II, topoisomerase I, or peroxisome proliferator-activated receptor- $\gamma$  at a concentration range similar to MPBD (Rangarajan et al., 2000; Fujimura et al., 2005; Tapia et al., 2006).

Our finding that MPBD failed to inhibit the basal MMP-9 expression, although it strongly antagonized the AP-1 function and reduced the transgenic MMP-9 promoter activity, deserves some discussion. The most likely possibility is that under basal conditions, transcription is low with protein levels largely reflecting the prolonged half-life of the transcript ( $\sim 19$  h) (Sehgal and Thompson, 1999). Thus, a drug targeting transcription of the MMP-9 gene would have little repressive effect under these conditions. On the other hand, it may be that the endogenous MMP-9 promoter in quiescent cells is assembled into relatively condensed chromatin (Yan and Boyd, 2007) that renders it unresponsive to altered AP-1 activity. In contrast, PMA-induced MMP-9 expression is accompanied by recruitment of chromatin remodeling motors to the promoter (Ma et al., 2004), which may confer responsiveness to changes in AP-1 activity. Indeed, under these latter conditions, MPBD suppressed MMP-9 expression. This latter

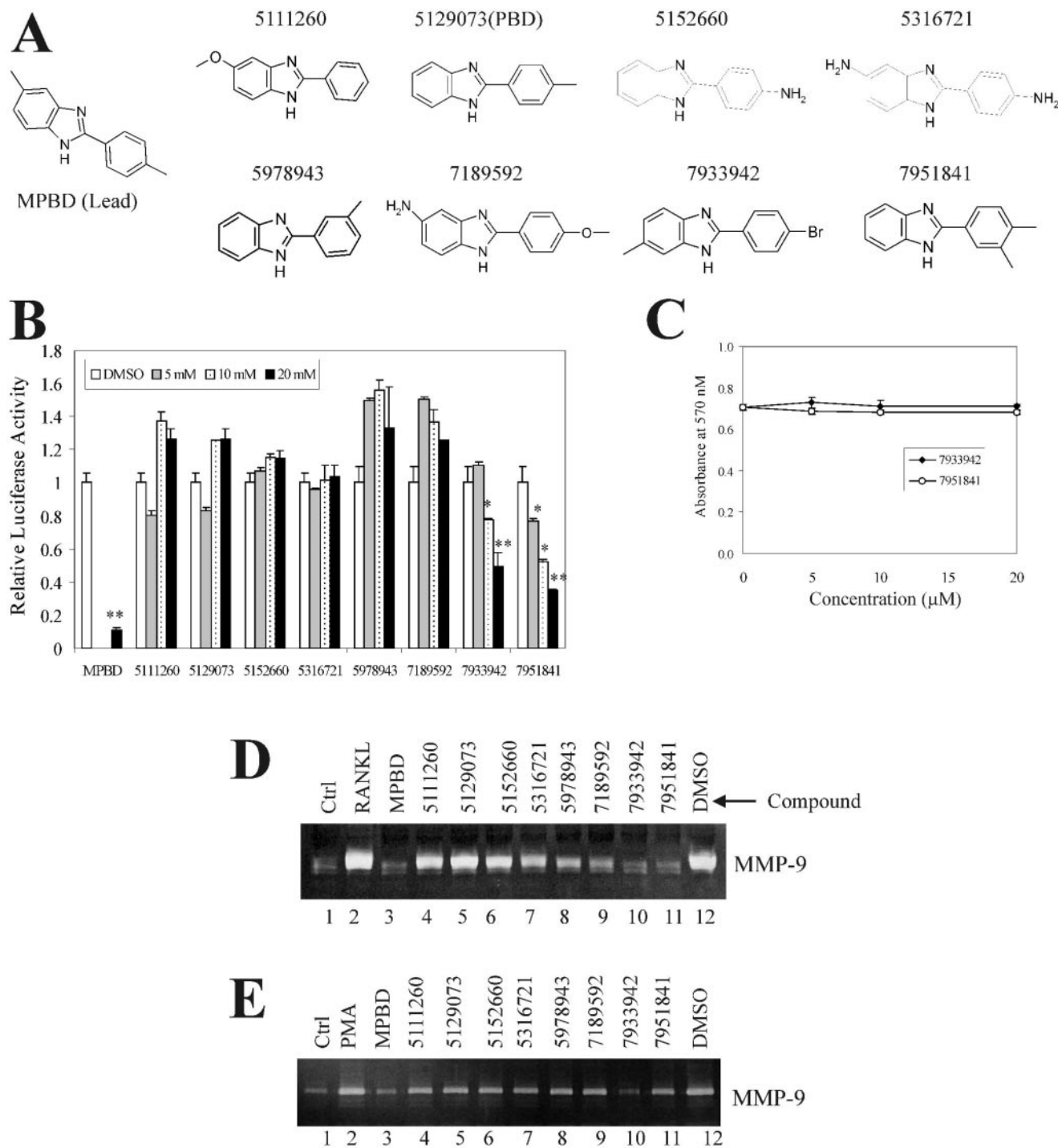


**Fig. 6.** MPBD antagonizes AP-1 function. **A**, HT1080 cells harboring wild-type, the proximal AP-1-site-mutated, or NF- $\kappa$ B-site-mutated MMP-9 promoter were treated with the indicated amounts of MPBD for 5 h and then lysed for luciferase activity assays. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus wild-type promoter (Student's  $t$  test). **B** and **D**, HT1080 cells were plated in 96-well plates, transfected with pAP-1-luc for 24 h, and then treated with indicated amounts of MPBD or DMSO overnight. The cells were lysed for luciferase activity assays (**B**) or Western blotting (**D**). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus DMSO control (Student's  $t$  test). **C** and **E**, luciferase reporter containing Gal4-binding site (pFR-luc) was cotransfected with a construct (pFA c-Jun) expressing a Gal4-c-Jun fusion protein or the empty vector (pFc-dbd) into HT1080 cells. Twenty-four hours later, the cells were treated with the indicated amounts of MPBD for 16 h and then lysed for luciferase activity assays (**C**) or Western blotting (**E**). For the JNK-inhibition control, the cells were treated with 20  $\mu$ M SP600125 for 16 h. \*\*,  $p < 0.01$  versus DMSO control (Student's  $t$  test). Data are depicted as average  $\pm$  S.D. values of three independent experiments.

scenario is given further weight by the observation that the extent to which the transgenic promoter is repressed in the relatively condensed F8 chromosomal location (C. Yan and D. Boyd, unpublished data) is smaller than in the open F55 location (Yan and Boyd, 2006) (Fig. 5).

In summary, we have established a bioluminescence-based high-throughput screening system that overcomes reporter-gene silencing induced by flanking chromatin. Using this

system, we have identified a benzimidazole derivative repressive for MMP-9 expression and consequently inhibiting cell invasion, preosteoclast migration, and osteoclastic activity. This novel compound could serve as a prototype for a new class of anti-MMP-9 therapeutic agents benefiting patients with early disease but who are at high risk for tumor dissemination, or for more effectively managing cancer-driven bone destruction.



**Fig. 7.** Structure-activity studies with MPBD analogs. A, structures of MPBD and its analogs. B and C, HT/MMP9/F55 cells were treated with the indicated amounts of analogs for 5 h and then lysed for luciferase activity assays (B) or MTT assays (C). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  versus DMSO control (Student's  $t$  test). D, UMSCC-1 cells were treated with 20  $\mu$ M MPBD or its analogs as in Fig. 4A, and conditioned media were subjected to zymography after normalization for cell number differences. E, RAW264.7 cells were treated with 20  $\mu$ M MPBD or its analogs as in Fig. 5A for gelatin zymography. For B and C, data are shown as average  $\pm$  S.D. values of 3 separate determinations.

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