Loss of Hypermethylated in Cancer 1 (HIC1) in Breast Cancer Cells Contributes to Stress-induced Migration and Invasion through β-2 Adrenergic Receptor (ADRB2) Misregulation*

The transcriptional repressor HIC1 (Hypermethylated in Cancer 1) is a tumor suppressor gene inactivated in many human cancers including breast carcinomas. In this study, we show that HIC1 is a direct transcriptional repressor of β-2 adrenergic receptor (ADRB2). Through promoter luciferase activity, chromatin immunoprecipitation (ChIP) and sequential ChIP experiments, we demonstrate that ADRB2 is a direct target gene of HIC1, endogenously in WI-38 cells and following HIC1 re-expression in breast cancer cells. Agonist-mediated stimulation of ADRB2 increases the migration and invasion of highly malignant MDA-MB-231 breast cancer cells but these effects are abolished following HIC1 re-expression or specific down-regulation of ADRB2 by siRNA treatment. Our results suggest that early inactivation of HIC1 in breast carcinomas could predispose to stress-induced metastasis through up-regulation of the β-2 adrenergic receptor.  

HIC1 (hypermethylated in cancer 1) is a tumor suppressor gene located at 17p13.3 on the short arm of human chromosome 17, in a region including the tumor suppressor gene p53 (17p13.1). This region is silenced in many human cancers by hypermethylation or deletions (1). HIC1 is hemi-methylated in normal breast tissue and is found to be epigenetically or deleterionally (loss of heterozygosity) inactivated in many cases of breast carcinomas (2, 3). Expression of HIC1 is associated with an improved prognosis in human breast cancer (4).  

HIC1 encodes a transcriptional repressor composed of two autonomous repression domains, an N-terminal BTB/POZ (Broad complex Tramtrack and Bric à brac/POxviruses and Zinc finger) domain and a central region, followed by five Krüppel like C2H2 zinc fingers able to bind a specific motif consisting of a (C/G)NG(C/G)GGCA motif named HiRE (HIC1 Responsive Element) (5, 6).

HIC1 can recruit different co-repressor complexes to its target genes, using short motifs in its central region notably CtBP through a GLDLSKK motif (7), and MTA1, a component of the NuRD complex, through a SUMOylation-dependent ΨKXEP motif (8). HIC1 also recruits BRG1-ARID1A containing SWI/SNF complexes (9).

Although an increasing number have been described in the last several years, bona fide target genes of HIC1 are still few. To date, ten target genes play supporting roles in developmental and cell cycle control: histone deacetylase SIRT1 (10), the transcription factors ATOH1 (11), Sox9 (12), and ΔNp73 (13), the G-protein coupled receptor CXCR7 (14), Cyclin D1 and P57KIP2 (CDKN1C) (8) and EFN1A, a cell surface ligand for Eph tyrosine kinase receptors (15).

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6 The abbreviations used are: HIC1, hypermethylated in cancer 1; ADRB2, β-2 adrenergic receptor; GPCR, G-protein-coupled receptor; HiRE, HIC1-responsive elements.
ADRB2 Is a Direct Target Gene of HIC1

Depending on the cell type, re-expression of HIC1 leads to proliferation arrest, differentiation, and apoptosis (1, 14). Our recent results also demonstrate a role for HIC1 in the regulation of cell migration and invasion. These biological effects are partially mediated through transcriptional repression of the ligand/receptor couple EFNA1 and EphA2 in different cells (15, 34).

In this study, we demonstrate that ADRB2 is a new direct target gene of HIC1. ADRB2 encodes a G-protein-coupled receptor (GPCR) activated by adrenaline/noradrenaline, which are released in vivo under stress conditions (16). Ex vivo, ADRB2 stimulation by agonists induces migration and invasion (17, 18, 19). In vivo, ADRB2 activation promotes tumor growth and metastasis (16, 20, 21, 22 or 16, 20–22). A recent clinical study in breast cancer patients using β-blockers demonstrates a strong diminution of metastasis and heightened survival supporting the impact of stress in breast cancer progression (23).

Through molecular and biological approaches, we demonstrate that ADRB2 is a new bona fide HIC1 target gene. Firstly, in WI-38 normal lung embryonic fibroblasts, endogenous HIC1 directly regulates ADRB2 as demonstrated by chromatin immunoprecipitation (ChIP and sequential ChIP), siRNA targeting HIC1 and retroviral overexpression of HIC1. In MDA-MB-231, a metastatic breast cancer cell line expressing high levels of ADRB2 and no HIC1, HIC1 re-expression strongly represses ADRB2 expression and prevents its activation of migration and invasion. Furthermore, in these HIC1 re-expressing cells, concomitant expression of ADRB2 partially rescues these phenotypes. Our results suggest that HIC1 silencing, which occurs in the early stages of breast tumorigenesis, could also contribute to later stages of tumor progression such as metastasis.

EXPERIMENTAL PROCEDURES

Cell Culture—WI-38 cells (ATCC, passage 14) were grown in MEM (Invitrogen, Carlsbad, CA) supplemented with sodium pyruvate, NEAA, 10% fetal calf serum (FCS, Invitrogen) and gentamicin (Invitrogen). U2OS, the packaging cell line HEK293 GP and human mammary adenocarcinoma cells MDA-MB-231 were cultured in Dulbecco’s modified Eagle’s medium (DME, Invitrogen) supplemented with 10% FCS and gentamicin (Invitrogen). U2OS, the packaging cell line HEK293 GP and human mammary adenocarcinoma cells MDA-MB-231 were cultured in Dulbecco’s modified Eagle’s medium (DME, Invitrogen) supplemented with 10% FCS and gentamicin (Invitrogen). Cells were cultured at 37 °C in water-saturated 5% CO2 buffer saline (PBS) 48 h after transfection and lysed with the Luc reporter to generate the pGL3 basic control vector. The value obtained for each construct was divided by the repressive effect elicited by HIC1 on the empty pGL3 basic vector to obtain the final fold of activation. Results represented are the mean values and S.D. from a representative experiment performed in duplicate.

Quantitative RT-PCR—Total RNA was reverse transcribed using random primers and MultiScribe™ reverse transcriptase (Applied Biosystems). Real-time PCR analysis was performed with Power SYBR Green (Applied Biosystems) in a MX3005P fluorescence temperature cycler (Stratagene) according to the manufacturer’s instructions. Results were normalized with respect to 18 S RNAs used as internal control. Primers were used at a concentration of 0.5 μM. According to a melting point analysis, only one PCR product was amplified under these conditions. RNAs extracted from pBABE infected cells were used to generate a standard curve for each gene. Results were normalized with respect to the internal controls and are expressed relative to the levels found in pBABE-infected cells.

Relevant primers used are: HIC1 (for-CGACGACTACAAGACGACGACG, rev-CAGGTTGTCACCAGAAGCTTCTC); ADRB1 (for-TGCACAGCGAGATGAAAGACCTTG, rev-GAGAGGCTTCGCCGCTG); ADRB2 (for-GCAAGGAGGAGGTGTGG, rev-AGACGCTCAACTTGCAAT).
ADRB3 (for-GGAGTGAGCTTGGGCTTTTGATA, rev-GCGTGGCTTTTGCTACTCAATG); EphA2 (for-TGTGC-CAGCGAGGCTACG, rev-CTCCAAGCAGGGGCTCTCA); CyclinD1 (for-CTCTCAACCGCAGACTCTTCC, rev-TG-TCCAATGAAATCGTGCCG); 18 S (for-GGCGCCCCCTC-GATGCTITTAG, rev-GCTCGGGCCTGCTTGAACACT-TCT).

Small Interfering RNA—WI-38 cells were reverse-transfected with RNAiMax according to manufacturer’s instructions using 10 nM small interfering RNA targeting HIC1 (HIC1 siGENOME Smart Pool M-006532-01, Dharmacon) or a scrambled sequence, as previously described (34). 72 h later, cells were lysed for RNA or protein extraction. MDA-MB-231 cells were forward-transfected with Lipofectamine 2000 according to manufacturer’s instructions using 10 nM small interfering RNA targeting ADRB2 (ADRB2 siGENOME Smart Pool M-005426-02, Dharmacon) or a scrambled sequence. 48 h later, cells were harvested for RNA/protein extraction or seeded for bioassays.

Chromatin Immunoprecipitation—ChIP was performed as previously described (8). Alternatively, we used the protocol previously described by Dahl and Collas (25). The purified DNAs were used for PCR analyses with Fast Start TaqDNA Polymerase (Roche) using the relevant primers for ADRB2 (for-TCGGTATAAGTCTGAGCATGTCTG; rev-ACATT-GGAAGGAAACGAGA), and GAPDH (for-TCCCTCGTT-TCATCCAGGC; rev-TAGTACGGCGCCCTACTTCTT).

Type I Collagen and Fibronectin Coatings—Six-well plates were incubated with a solution of rat-tail Type I collagen at a density of 0.22 cm² per well (24-well format). For each treatment condition, cells were seeded onto the BD BioCat™ gelatin or type I collagen and incubated at 37 °C for 2 h or with human fibronectin (both from BD Biosciences, Bedford, MA) at 20 μg/ml for 1 h at room temperature. Then, plates were washed twice with PBS(−/−) containing neither Ca²⁺ nor Mg²⁺ (Invitrogen) and stored at 4 °C in PBS(−/−) before use.

Adhesion Assay—WI-38 cells were serum-starved for 20 h, trypsinized, centrifuged, and resuspended in serum free medium containing PBS or 100 nM isoproterenol (Sigma-Aldrich). Then, 20,000 cells were seeded on 6-well plates precoated with fibronectin or type I collagen and incubated at 37 °C for 1 h 30 min on type I collagen or 2 h 30 min on fibronectin. Finally, cells were washed twice with PBS, fixed 10 min at −20 °C with ice-cold methanol, and conserved in PBS for later observation on a phase contrast microscope. Similarly, MDA-MB-231 cells were serum starved for 1 h and treated with 10 μM isoproterenol. Cells were then seeded in medium containing 0.5% FCS and incubated for 4 h on collagen or 5 h on fibronectin. Experiments were performed at least twice in triplicate.

Migration Assay (Wound Healing Assay)—Wound healing assays were performed using culture-insert μ-Dish (ibidi, Martinsried, Germany) composed of two chambers (growth area per well 0.22 cm²) separated by a wall (width of 500 μm). Culture inserts were put in six-well plates precoated with fibronectin or type I collagen. MDA-MB-231 were starved in serum-free DMEM medium for 10 min, and then 30,000 cells were seeded into the chambers. After cell attachment overnight at 37 °C, culture-inserts were gently removed to form the cell-free gap. For each condition, pictures were taken at a precise localization every 3 h to monitor the healing of the cell-free gap. To calculate the percentage of closure of the wound, 8-bit image analysis was performed with the Fiji software, an image-processing package based on ImageJ (NIH). First, cells were localized by edge detection. Then the mask of empty areas was created using background subtraction. Then, particle analysis enabled the detection and measurement of the uncolonized areas. Finally, the percentage of closure was obtained by subtracting the area at a given time by the original area at T0 in each condition.

Invasion Assay—MDA-MB-231 cells were starved in serum-free DMEM medium for 4 h, split and counted. When applicable, cells were pretreated with PBS or isoproterenol (10 μM) in serum-free DMEM medium for 10 min. Cells in serum-free DMEM medium were then seeded onto the BD BioCat™ growth factor reduced matrigel™ invasion chamber with 8-μm pore size (BD Biosciences), at a density of 50,000 cells per well (24-well format). For each treatment condition, cells were seeded in triplicate. Next, the cells were tested for their ability to invade the matrigel and migrate across the filters in response to chemoattraction of 10% FCS-DMEM medium placed in the lower chambers. After 24 h of incubation at 37 °C, non-migrating cells were scraped from the top face of the inserts, while cells that had migrated across the filter pores to the lower face were fixed in methanol and stained with Hoechst 33258. The number of cells that had migrated was analyzed on each filter using an Axioplan 2 (Zeiss, Germany) microscope. Ten images of randomly chosen optical fields were captured on each migration filter using AxioVision® Software for microscopy image analysis (Zeiss), and migrating cells were counted with the Colony1.0 software.

Statistics—Experiments were performed at least twice independently in duplicates or triplicates. Statistical analyses were performed by Student’s t test. * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, and NS indicates a non-significant variation.

RESULTS

ADRB2 Is a Direct Target Gene of HIC1—We recently described the role of HIC1 in cell migration and invasion as partially relying on direct transcriptional repression of the tyrosine kinase receptor EphA2 gene. Nevertheless, we hypothesized that other target genes could also be implicated in these important biological processes. In a previous report of gene expression profiling, we published a list of genes repressed in U2OS osteosarcoma cancer cells following adenoviral infection and reexpression of HIC1 (14). Among them, we already validated the receptors coding genes CXCR7 and EphA2 as new HIC1 target genes (14, 34). Another gene present in our list, ADBR2, coding for a G-protein-coupled receptor, was repressed 5-fold in Ad-HIC1-infected cells as compared with control infected cells as early than 8 h postinfection (Fig. 1A). In this current work, we first confirmed ADBR2 down-regulation in U2OS cells infected with a retrovirus expressing FLAG-tagged HIC1. After puromycin selection, HIC1-expressing cells were harvested and then mRNAs and proteins were extracted. qRT-PCR and immunoblot with an antibody specific for ADBR2 confirmed the repression induced by HIC1 re-expres-
ADRB2 is a Direct Target Gene of HIC1

Human lung embryonic fibroblasts (WI-38) are normal diploid cells expressing endogenous HIC1 (8, 10) and ADRB2 (Fig. 1E). Overexpression of HIC1 in these cells by retroviral infection induced a marked decrease of ADRB2 mRNA (Fig. 1D) and a slight decrease of protein levels (Fig. 1E). In these conditions, Cyclin D1 and EphA2 were also repressed (Fig. 1, D and E) confirming previous (8) and ongoing results (34). Conversely, inhibition of endogenous HIC1 expression in WI-38 cells by
siRNA resulted in a concomitant increase in ADRB2 transcripts (Fig. 1F) and proteins (Fig. 1G).

To determine whether ADRB2 is a direct target gene of HIC1, we first scanned its promoter for the presence of HIC1-responsive elements (HiRE) (5, 26). These analyses identified many putative HiRE, particularly 600 bp upstream of the translation start site (Fig. 1H, left panel). We cloned a length of genomic DNA corresponding to the region 750 bp upstream of the ATG codon into the pGL3 basic reporter vector and performed luciferase promoter-reporter assays in U2OS cells in the presence or absence of transiently transfected pcDNA3-FLAG-HIC1. Under former conditions, the promoter activity was repressed 2-fold (Fig. 1H, right panel) in accordance with our results showing similar repression of endogenous ADRB2 following HIC1 re-expression in these cells (Fig. 1A). Furthermore, HIC1 was no longer able to repress a mutant with deletion of the region 600 bp upstream of the ATG and lacking a cluster of binding sites (Fig. 1H, pGL3-ADRB2 −440/−6).

Finally, in WI-38 cells, chromatin immunoprecipitation (ChIP) experiments demonstrated the specific binding of HIC1 on the ADRB2 promoter with primers flanking these HIC1 binding sites located 600 bp upstream of the translation start site (Fig. 1, H and I, left panels). Moreover, sequential ChIP showed concomitant fixation of HIC1 and at least two of its transcriptional co-repressors, MTA1, and CtBP on the ADRB2 promoter (Fig. 1I, right panel) (7, 8). Taken together, these results demonstrate that ADRB2 is a direct target gene of HIC1.

**ADRB2-activated Primary Fibroblasts WI-38 Exhibit a Specific Adhesion Phenotype Suppressed by HIC1 Overexpression**

We next attempted to decipher the functional link between HIC1 and this newly characterized target gene. Many studies performed on different tumor cell types have highlighted various important roles for epinephrine and norepinephrine, the natural ligands of adrenergic receptors, in biological processes (27). We focused on ADRB2 function during cell adhesion on substrates like type I collagen and fibronectin as the natural microenvironment of normal fibroblasts. Indeed, a previous study showed that the activation of ADRB2 with isoproterenol, a synthetic catecholamine targeting and activating β subtype adrenergic receptors, could accelerate the Ovcar3 ovarian cancer cell’s adhesion on fibronectin through a pathway involving integrins (28).

**FIGURE 1. ADRB2 is a direct target gene of HIC1.** A, effects of HIC1 overexpression on expression of ADRB2 in infected cells. Total RNAs from U2OS cells (HIC1 null) infected with Ad-FLAG-HIC1 and Ad-GFP were prepared at the indicated times (from 8 to 24 h) and Affymetrix HG U133A chips were used to measure the gene expression. Expression values were normalized to Ad-GFP-infected control cells at the same time points. % of control corresponds to the ratio between the expression levels of ADRB2 measured in Ad-GFP and Ad-FLAG-HIC1-infected cells at each time point. B, quantitative Real Time PCR analyses of ADRB2 and (C) immunoblot assays of HIC1 and of ADRB2 in U2OS cells infected with pBABE-FLAG-HIC1 (abbreviated as pBABE-HIC1 in all figures) or by pBABE-FLAG (pBABE) as control. Actin protein levels were used as a loading control. D, quantitative real time PCR analyses and (E) immunoblot assays of HIC1 and of relevant target genes in WI-38 cells infected by pBABE-FLAG-HIC1 or by pBABE as control. $^*$, detection of SUMOylated forms of HIC1 (33). F and G, inactivation of endogenous HIC1 in WI-38 cells up-regulates ADRB2. F, qRT-PCR and G, Western blot analyses of HIC1 and of ADRB2 in WI-38 cells following RNA interference of HIC1 or with non-targeting control siRNA. Quantification of ADRB2 protein levels was realized using ImageJ by dividing the value obtained for ADRB2 by the value of actin. ** indicates p < 0.01 and *** indicates p < 0.001. H, schematic drawing of the 5’ promoter region and the unique coding exon of ADRB2 are shown on the left. The transcription start site (bent arrow) as well as a portion of the coding exon are described in GenBank under accession number NG_016421 (26). Numbering is relative to the ADRB2 translational start site (ATG, nt +1) as in Ref. 26. The potential HiREs are shown as white ovals and as black ovals for those which are highly homologous to the consensus (5). The two small arrows indicate the position of the primers used to amplify the relevant region of ADRB2 in the ChIP and sequential ChIP experiments. Below are shown schematic drawings of the two human ADRB2 promoter constructs subcloned in the luciferase reporter plasmid pGL3 basic. Luciferase promoter reporter assay in U2OS cells (right). Results are expressed relative to a value of 1.0 for cells transfected with the pGL3 empty vector. Each condition was performed at least twice in duplicate and a representative experiment is shown. J, ChIP analyses of HIC1 on ADRB2 promoter in WI-38 cells (left panels) and sequential ChIP experiments demonstrate that HIC1/MTA1 and HIC1/CtBP might form a stable complex on the ADRB2 promoter (right panels). Normal WI-38 lung fibroblasts chromatin was immunoprecipitated with anti-HIC1 antibody (32S) (7). The bound material was eluted, divided in two and subjected to a second round of immunoprecipitation with anti-MTA1 antibodies, anti-CtBP, or with normal rabbit IgG (8). PCR amplifications were performed with primers flanking HIC1 binding sites in ADRB2. GAPDH was used as an internal non-binding control.
In normal WI-38 cells, isoproterenol did not affect adherent cell number on either type of coating (Fig. 2A), in contrast to previous results obtained in transformed cell lines (28). However, ADRB2 activation in WI-38 cells induced a particular adhesion phenotype illustrated by the establishment of focal adhesion sites usually associated with integrin recruitment (Fig. 2B, right panels). Following HIC1 retroviral over-expression in WI-38 cells, concomitant with a strong decrease of ADRB2 levels (Fig. 1F), the isoproterenol-induced phenotype was completely abolished (Fig. 2, C and D, bottom right panels). Specific activation of ADRB2 could therefore induce localization of integrins on focal sites that are essential for cell migration. Based on these phenotypic results affecting adhesion, we hypothesized that other physiologic events could rely on ADRB2 activation and therefore could be regulated by transcriptional repression induced by HIC1.

Re-expression of HIC1 Decreases High ADRB2 Levels in MDA-MB-231 Breast Cancer Cells—Given our previous results and the link between norepinephrine and migration, we decided to focus on HIC1’s impact on migration. To that end, we switched to a cellular model widely used to study migration properties, the metastatic breast cancer cell line MDA-MB-231. As expected for MDA-MB-231 breast cancer cells, HIC1 was not detected by immunoblot (Fig. 3A) (2). Conversely, ADRB2, but not the related ADRB1 and ADRB3 receptors, is highly expressed in these cells (Fig. 3B).

We first established that ADRB2 activation had similar effects upon MDA-MB-231 cell adhesion and induced focal adhesion sites on type I collagen and fibronectin (supplemental Fig. S1A). In these cells, isoproterenol induced a slight increase in adherent cells (supplemental Fig. S1B). Again, re-expression of HIC1 induced a loss of the isoproterenol-induced phenotype (Fig. 3, C and D) correlated with a robust decrease of ADRB2 transcripts (Fig. 3E) and protein (Fig. 3F) following HIC1 binding on the ADRB2 promoter (Fig. 3G). In conclusion, ADRB2 is the only member of the β-Adrenergic receptor family highly expressed in MDA-MB-231 breast cancer cells and re-expression of HIC1 in these cells is able to extinguish its expression.

HIC1 Blocks an ADRB2-mediated Boost of Migration and Invasion—We next verified that ADRB2 activation could stimulate migration of MDA-MB-231 as previously described (17). In a wound healing assay, pretreatment of cells with isoproterenol during adhesion could indeed accelerate migration on type I collagen and fibronectin (supplemental Fig. S1, C and D and
cells re-expressing HIC1 showed a diminution of migration compared with the empty vector-infected cells (Fig. 4, A and B, bottom right panels). The lack of isoproterenol effect in HIC1 re-expressing cells could therefore be caused by the loss of ADRB2 expression.

MDA-MB-231 cells are metastatic and highly invasive. In invasion assays, cells were seeded on the top of the invasion chamber (coated with a matrigel layer) and were stimulated with isoproterenol during the adhesion process. In agreement with the migration assays, we observed a significant increase of invasion (supplemental Fig. S1). Again, HIC1 re-expression dramatically abolished these invasive properties upon isoproterenol stimulation and notably also in basal conditions (Fig. 4C). These results demonstrate that activation of β-subtype adrenoreceptor ADRB2 enhances both the migration and invasion of MDA-MB-231 breast cancer cells, and that both phenotypes are abolished by HIC1 re-expression.

Inhibition of ADRB2 in MDA-MB-231 Mimics the Effects of HIC1 Re-expression on Adhesion, Migration, and Invasion—To ensure that phenotypes observed following isoproterenol treatment and abolished in the presence of HIC1 are specific to ADRB2-mediated pathways, we inhibited ADRB2 by RNA interference in MDA-MB-231. Focal adhesion sites were severely impaired despite isoproterenol treatment but phenotypes were not fully abolished (Fig. 5A, bottom right panels). This could be explained by the presence of residual receptors on cells. Indeed, in our experimental conditions, ADRB2 levels were less decreased by siRNA ADRB2 treatment than following HIC1 re-expression (compare Fig. 3E and inset in Fig. 5C). Nevertheless, cellular migration was...
also severely impaired (Fig. 5B, compare bottom panels). Finally, invasion was also strongly inhibited in the presence and absence of isoproterenol, recapitulating the HIC1-induced phenotype (Fig. 5C).

**ADRB2 Partially Rescues HIC1-induced Phenotypes—**To more directly demonstrate that ADRB2 is a key target gene involved in phenotypes caused by HIC1 re-expression, we repeated invasion assays after reintroducing ADRB2 expression by retroviral infection. ADRB2 expression alone induced a 2-fold increase of invasion comparable to the results obtained with empty vector-infected cells treated with isoproterenol (Fig. 6, compare lanes 2 and 3). The lack of increased invasion in ADRB2 infected cells treated with isoproterenol (lane 4) could reflect saturation of the membrane due to the large number of cells. However, and in accordance with all of our previous results, co-expression of ADRB2 in HIC1-infected MDA-MB-231 cells partially rescued HIC1-induced abolition of invasion by a significant 3-fold increase in invading cells in the absence of isoproterenol (compare lanes 5 and 7). Furthermore, HIC1 and ADRB2 co-infected cells were statistically more invasive in the presence of isoproterenol (compare lanes 7 and 8). Altogether these results demonstrate that phenotypes induced by re-expression of HIC1 in MDA-MB-231 breast cancer cells are in part due to transcriptional repression of one target gene, **ADRB2**, coding for a cell membrane receptor whose activation promotes migration and invasion.

**DISCUSSION**

In this study, we demonstrate that **ADRB2** is a new direct target gene of HIC1, a tumor suppressor inactivated in many cancers, particularly in breast and prostate. In these tissues, **HIC1** is already hemi-methylated in normal conditions and its extinction is correlated with the aggressiveness of tumors (2, 4). The identification of HIC1 target genes is thus a crucial step in understanding how its inactivation could contribute to tumorigenesis.

**ADRB2** is a cell membrane GPCR, overexpressed in breast cancers whose activation by the stress-released hormones adrenaline/noradrenaline stimulates tumor growth, migration, and invasion. Recent studies and our results demonstrate that HIC1 re-expression strongly impairs these phenotypes in breast cancer cells (15, 34). **ADRB2** repression consistently sup-
ports these observations although re-expression of a transcription factor involves a network of multiple target genes, which in the case of HIC1, seem to be involved in cell cycle regulation (8, 15) and cell motility (14, 34).

We did not accumulate evidence for a role of ADRB2 in cell cycle progression but these effects seem to be highly cell type specific (29, 30). It is nevertheless conceivable that HIC1 could inhibit cell cycle progression by two means: directly by repressing \textit{Cyclin D1} (8) but also indirectly by decreasing ADRB2 levels. In our experiments with both WI-38 and MDA-MB-231 cells, expression of HIC1 induced a decrease of \textit{Cyclin D1} transcripts and proteins levels in accordance with a retardation of proliferation (1, 14, 34).

In our experiments, we used isoproterenol, an adrenaline/noradrenaline mimetic, to activate ADRB2 as previously described (16, 20, 28, 29). Isoproterenol is also able to target

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![Figure 5](image_url)

**FIGURE 5.** Inhibition of ADRB2 in MDA-MB-231 mimics the effects of HIC1 re-expression on adhesion, migration, and invasion. MDA-MB-231 cells were transfected with non-targeting control siRNA or with ADRB2 siRNA. After 48 h, cells were trypsinized, treated with PBS or isoproterenol (10 μM) and seeded for (A) adhesion assay on type I collagen or fibronectin (Bar, 50 μm), (B) wound test assay on type I collagen for 8 h and fibronectin for 11 h (Bar, 200 μm) and (C) matrigel invasion assay as previously described in Figs. 3 and 4. Bar, 500 μm. ADRB2 immunoblot and mRNA levels measured by qRT-PCR in cells treated by siADRB2 or control siRNA are shown as right inset. ** indicates p < 0.01, and *** indicates p < 0.001. In B, at the bottom left, the percentage of wound closure was established by image analysis as described under “Experimental Procedures.”
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A matrigel invasion assays of 50,000 MDA-MB-231 cells infected by a combination of viruses coding for HIC1 and ADRB2 as indicated on the bottom panel and as previously described. v means “empty vector” used to clone coding sequences of HIC1 or ADRB2. * indicates p < 0.05, ** indicates p < 0.01, *** indicates p < 0.001, and NS indicates non-significant. In the absence of bars, asterisks, or NS compare PBS and isoproterenol-treated conditions. B, immunoblot of MDA-MB-231-infected cells used for Matrigel invasion assays. Overexpression of ADRB2 proteins is detected as a ladder of multiple bands.

ADRB1 and ADRB3. Both were undetectable at the mRNA levels in WI-38 cells (Fig. 3B). In MDA-MB-231 breast cancer cells, ADRB3 was undetectable and ADRB1 was only slightly expressed and its transcription was not affected by re-expression of HIC1 (Fig. 3B and data not shown).

Surprisingly, ADRB2 stimulation during adhesion induced arborized shapes on matrix substrates, a phenotype abolished following HIC1 expression or specific inhibition of ADRB2. This phenotype has been previously described on fibronectin following dibutyryl cAMP treatment of transformed fibroblasts (BHK21 and NIH-3T3) (31) and is consistent with an increase in total cAMP in HEK-293 cells following ADRB2 activation (32).

To the best of our knowledge, our siRNA results are the first direct evidence that specific stimulation of ADRB2 is able to promote both migration and invasion of breast cancer cells. It is noteworthy that in our experiments, preactivation of ADRB2 by isoproterenol occurred during cell seeding and adhesion and then had an impact on migration and invasion. These biological effects would thus be due to activation of pathways downstream of ADRB2. Previous studies have shown that stress results in higher levels of tissue catecholamine followed by increased levels of proangiogenic factors such as IL-8 (interleukine-8), VEGF (vascular endothelial growth factor), and MMP (matrix metalloproteinase) resulting in enhanced tumor vascularization (16, 21) and invasion (20).

In our experiments, the migration and invasion of MDA-MB-231 cells are affected after HIC1 re-expression in the presence but also in the absence of isoproterenol (Fig. 4C, right panels). Although other target genes must be involved in these phenotypes, it is essential to note that the same results are obtained after ADRB2 inhibition by siRNA even in the absence of isoproterenol (Fig. 5C), strongly suggesting that basal cell culture conditions are able to activate ADRB2 and that ADRB2 is a major player involved in migration and invasion of breast cancer cells. In agreement with this idea, ADRB2 overexpression increases invasion even in the absence of specific activation by isoproterenol treatment.

Our results are particularly significant in Matrigel invasion assays (Figs. 4 and 5). Wound-healing assays were more difficult to quantify because treatments effects are more visual and depend on the behavior of cells on the substrates. In particular, the HIC1-infected cells migrate differently on fibronectin (Fig. 4B). Nevertheless, on the whole, our results are consistent with the link established between HIC1 and ADRB2 on cell migration.

Strikingly, in immortalized normal mammary epithelial HMEC-hTERT cells, despite the presence of ADRB2, isoproterenol treatment did not induce any consistent phenotype during the adhesion process (data not shown). Furthermore, isoproterenol treatment delayed migration in wound healing assays. Thus, although siRNA mediated inhibition of HIC1 led to increased migration in wound healing assays as previously described in transwell migration assays (34), they were still delayed after isoproterenol treatment (supplemental Fig. S2). We speculate that some ADRB2 downstream effectors are absent in these normal cells, which in contrast with MDA-MB-231, have not undergone an epithelial-mesenchymal transition. Consistent with a multi step process of breast cancer progression, ADRB2 up-regulation could not favor cell motility in these normal cells. In conclusion, our data suggest that, in breast epithelial cells, loss of HIC1 in tumorigenesis could favor metastasis through up-regulation of β2 adrenergic receptor.

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