Autoregulatory loop of nuclear corepressor 1 expression controls invasion, tumor growth, and metastasis

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Nuclear corepressor 1 (NCoR) associates with nuclear receptors and other transcription factors leading to transcriptional repression. We show here that NCoR depletion enhances cancer cell invasion and increases tumor growth and metastatic potential in nude mice. These changes are related to repressed transcription of genes associated with increased metastasis and poor prognosis in patients. Strikingly, transient NCoR silencing leads to heterochromatinization and stable silencing of the *NCoR* gene, suggesting that NCoR loss can be propagated, contributing to tumor progression even in the absence of *NCoR* gene mutations. Down-regulation of the thyroid hormone receptor β1 (TRβ) appears to be associated with cancer onset and progression. We found that expression of TRβ increases NCoR levels and that this induction is essential in mediating inhibition of tumor growth and metastasis by this receptor. Moreover, NCoR is down-regulated in human hepatocarcinomas and in the more aggressive breast cancer tumors, and its expression correlates positively with that of TRβ. These data provide a molecular basis for the anticancer actions of this corepressor and identify NCoR as a potential molecular target for development of novel cancer therapies.

nuclear corepressor 1 | thyroid hormone receptor | tumor growth | metastasis | transcription

Corepressors play a central role in bridging chromatin-modifying enzymes and transcription factors (1). NCoR (nuclear corepressor 1) and the homologous protein SMRT (silencing mediator or retinoic and thyroid hormone receptors or NCoR2) were identified by their interaction with unliganded thyroid hormone receptors (TRs) and retinoic acid receptors (2, 3), although later studies demonstrated that they also could bind to other transcription factors (4). NCoR and SMRT belong to large complexes that contain histone deacetylases (HDACs), thereby inducing chromatin compaction and gene silencing (4–7). Although these corepressors interact with multiple HDACs, HDAC3 plays a key role in mediating their actions (8, 9) and is essential for repression by TRs (10, 11).

As expected from their prevalent role in integrating the action of many transcription factors, NCoR and HDAC3 affect numerous developmental and homeostatic processes (12). In addition, there is increasing evidence that NCoR could play a significant role in cancer. Alterations in NCoR expression or subcellular localization have been linked to various solid tumors. Thus, reduced NCoR expression has been associated with invasive breast tumors (13, 14), shorter relapse-free survival (15), and resistance to antiestrogen treatment (16). Unbiased pathway analysis recently has revealed mutations of NCoR (17, 18) among the driver mutations in breast tumors (19). The human *NCoR* gene is located on a region of chromosome 17p frequently deleted in hepatocarcinoma (HCC) (20, 21), suggesting that loss of this corepressor could drive liver cancer also. In agreement with this idea, liver-specific deletion of HDAC3 caused spontaneous development of HCC in mice, showing its essential role in the maintenance of chromatin structure and genome stability (22). Furthermore, the expression of HDAC3 and NCoR was down-regulated in a subset of human HCCs (22). All these findings suggest that NCoR could be an important suppressor of cancer initiation or progression, but the mechanisms by which the corepressor exerts its tumor-suppressing role have not yet been examined.

TRs, and in particular TRβ1, can act as tumor suppressors (23). We have shown that this receptor retards tumor growth and suppresses invasion, extravasation, and metastasis formation in nude mice (23–26). These tumor-suppressing effects are associated with a decreased expression of prometastatic genes (23). The role of TRβ1 appears to be particularly relevant in liver cancer. Thus, thyroid

Significance

Inactivating nuclear corepressor 1 (*NCoR*) mutations have been found in human tumors. Here, we report that NCoR has a tumor-suppressive role, inhibiting extravasation, metastasis formation, and tumor growth in mice. These changes are related to repressed transcription of genes associated with increased metastasis and poor prognosis in cancer patients. An autoregulatory loop maintains *NCoR* gene expression, suggesting that NCoR loss can be propagated, contributing to tumor progression in the absence of *NCoR* gene mutations. The nuclear receptor thyroid hormone receptor β1 increases NCoR expression, and this induction is essential in mediating its tumor-suppressive actions. Both are reduced in human hepatocarcinoma and aggressive breast cancer tumors, identifying NCoR as a potential molecular target for the development of novel therapies.

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hormones binding to TRβ1 induce regression of carcinogen-induced nodules, reducing the incidence of HCC and lung metastasis in rodents (27, 28), and TRβ1 down-regulation appears to be associated with HCC onset and progression (29). In addition, aberrant TRs that act as dominant-negative inhibitors of wild-type TR activity and that have altered association with corepressors have been found frequently in human HCCs (30, 31).

Here, we show that NCoR depletion enhances cellular invasion in vitro and increases tumor growth and the metastatic potential in nude mice. These actions are related to the regulation of genes associated with metastatic growth and poor outcome in cancer patients. Furthermore, we demonstrate the existence of a positive autoregulatory loop that maintains *NCoR* gene expression. NCoR depletion results in heterochromatinization and long-term silencing of *NCoR* transcription. Silencing could represent an important oncogenic mechanism in tumors in which inactivating mutations in the *NCoR* gene are not present. Finally, we show that induction of NCoR is an essential mediator of the tumor-suppressing actions of TRβ1 and that both are down-regulated in human HCC and in estrogen receptor-negative (ER^-) breast tumors, demonstrating a positive correlation between the expression of the receptor and the corepressor. Taken together, our results define NCoR as a potent tumor suppressor and as a potential target for cancer therapy.

Results

NCoR Represses Expression of Prometastatic Genes. mRNAs of selected prometastatic genes, including cyclooxigenase 2 (*COX2*), DNA-binding protein inhibitor 1 (*ID1*), *C-MET*, matrix metallopeptidase (*MMP*)*2*, *MMP9*, *CXCR4*, *CCR1*, *CCR6*, and *CCR7*, were measured in SK-hep1 (SK) cells transfected with a control siRNA or with an NCoR-specific siRNA. These cells were derived from a patient with liver adenocarcinoma and recently have been shown to have an oncogenic mesenchymal stem cell phenotype (32). Most of these genes were significantly increased upon NCoR depletion in SK cells (Fig. 1*A*) and in MDA-MB-468 (MDA) breast cancer cells (Fig. 1*B*). Because TRβ expression significantly increased NCoR mRNA and protein levels in SK and MDA cells (Fig. S1*A*), and prometastatic genes are repressed by TRβ, we reasoned that increased NCoR levels could play a role in this repression. Thus, we also measured prometastatic gene expression in SK-TRβ and MDA-TRβ cells, finding that the repressive effect of TRβ was reversed to a significant extent, or even totally in some cases, in NCoR-depleted cells (Fig. 1). Surprisingly, most of the effect of TRβ appears to be ligand independent, because incubation with triiodothyronine (T3) had little effect on transcript levels of *NCoR* and prometastatic genes (Fig. S1*B*). Similar changes were observed in human HepG2 HCC cells and in nontumoral HH4 human hepatocytes (Fig. S2 *A* and *B*), showing that TRβ also has a role as a direct regulator of NCoR in hepatocytes and the importance of the corepressor as an inhibitor of prometastatic gene expression. NCoR overexpression in SK and SK-TRβ cells reversed the effect of the siNCoR in prometastatic gene expression, eliminating the off-target effects of the siRNA pool used (Fig. S1*C*).

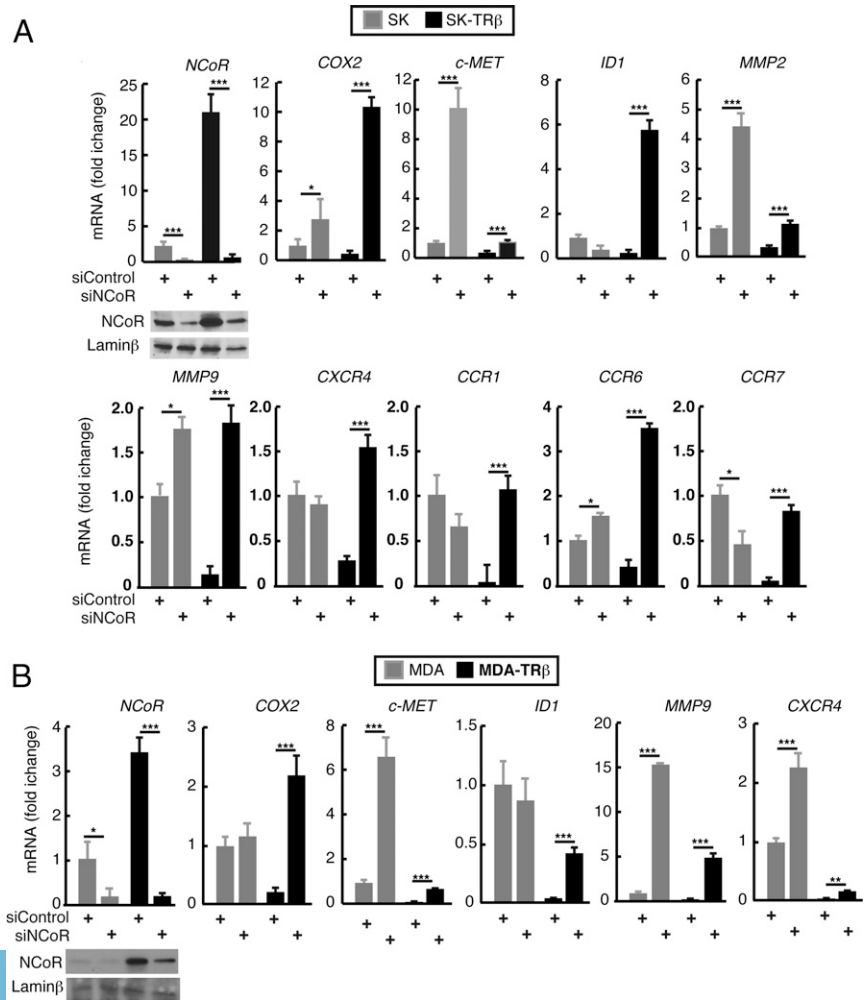

Fig. 1. NCoR depletion increases the expression of prometastatic genes. (*A*) mRNA levels of prometastatic genes in SK and SK-TRβ cells transfected with siControl or siNCoR. Data (means ± SD) are expressed relative to the values obtained in SK cells transfected with siControl. A Western blot of NCoR protein levels is shown. (*B*) An experiment in MDA and MDA-TRβ cells similar to that shown in *A*.

In contrast with the crucial role of NCoR, efficient knockdown of the SMRT corepressor did not increase the expression of these genes, indicating that it does not participate in their regulation (Fig. S2 *C* and *D*).

We next conducted transient transfection studies with luciferase promoter constructs of prometastatic genes in SK cells. Proximal promoter sequences of the *COX2*, *ID1*, and *MMP9* genes containing binding sites for various transcription factors appear to mediate both basal promoter activity and TRβ inhibition (Fig. 2*A*). Furthermore, in SK-TRβ cells the receptor was constitutively bound to these sequences in ChIP assays, and NCoR and HDAC3 recruitment to the promoters was enhanced significantly in comparison with the parental cells. In parallel with this increase, histone H3 acetylation was reduced and the repressive histone marker H3K9me3 was increased in SK-TRβ cells (Fig. 2*B*). In the *ID1* gene additional control regions have been identified (33), and although an irrelevant upstream region and the −1006/−746 control region were unaffected, the −1817/−1609 fragment also bound NCoR and TRβ, and the epigenetic changes observed were similar to those found with the more proximal promoter sequences (Fig. 2*B*). To analyze the functional role of NCoR in promoter regulation, the impact of NCoR gain of function and loss of function was evaluated in transfection assays. Overexpression of NCoR reduced promoter activity in SK cells to levels similar to those found in SK-TRβ cells. Conversely, NCoR depletion normalized promoter activity in the cells expressing the receptor (Fig. 2*C*), demonstrating the essential role of this corepressor in the inhibitory effect of TRβ on

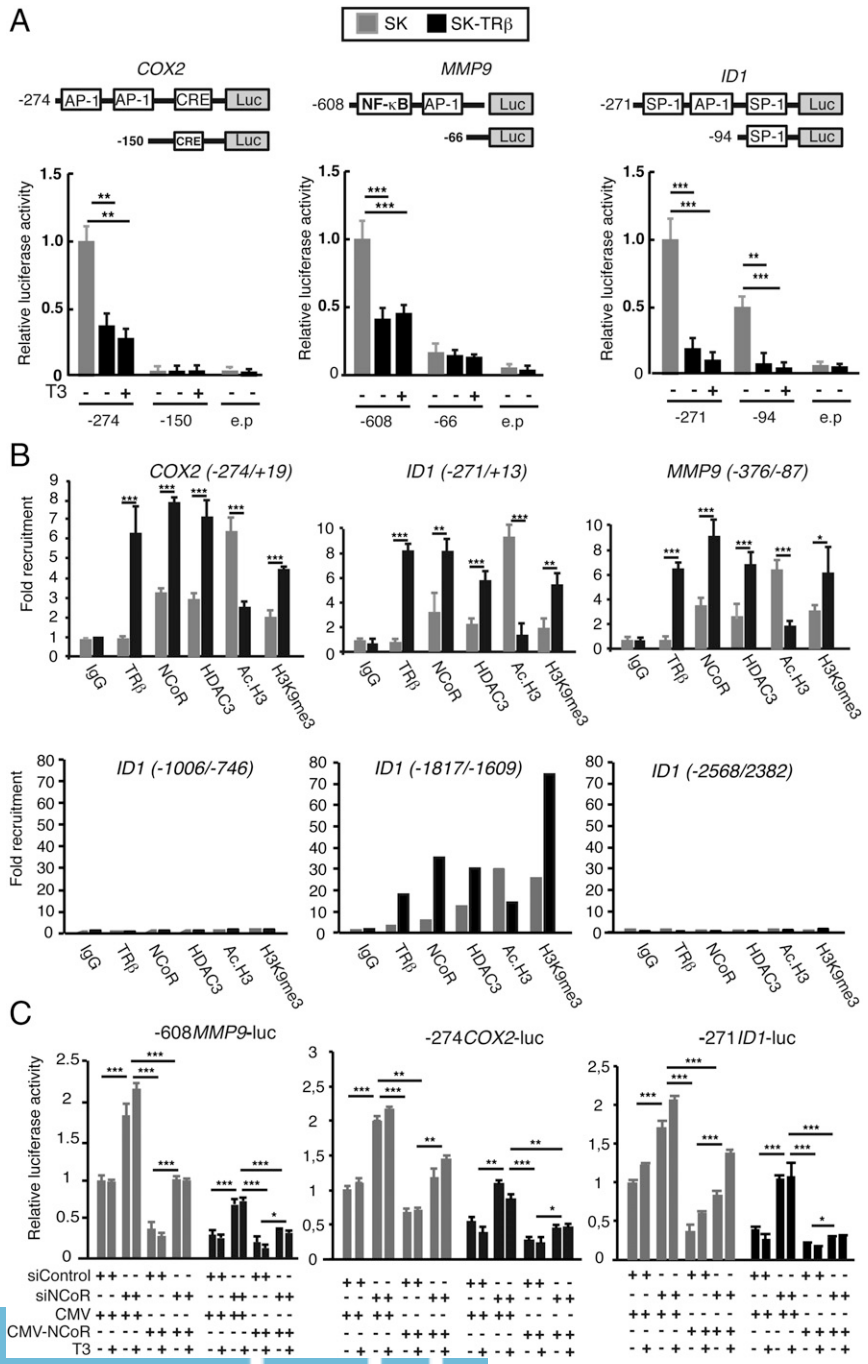

Fig. 2. NCoR represses promoter activity of prometastatic genes. (*A*) Transient transfection assays in SK and SK-TRβ cells with luciferase reporters of the *COX2*, *MMP9*, and *ID1* promoters or an empty plasmid (e.p). Schematics of the plasmids used showing the putative binding motifs for different transcription factors are illustrated. Cells were treated with and without 5 nM T3 for 36 h as indicated. Data are means ± SD and are expressed relative to the luciferase activity obtained in untreated SK cells. (*B*) ChIP assays in SK and SK-TRβ cells with the antibodies and the promoter regions indicated. (*C*) Luciferase assays (mean ± SD) with the indicated reporter plasmids in SK and SK-TRβ cells cotransfected with siControl or siNCoR in the presence of an expression vector for NCoR (*CMV-NCoR*) or the empty vector (*CMV*). Luciferase activity (means ± SD) was determined in cells treated with and without T3.

metastatic gene transcription. In addition, NCoR overexpression inhibited the stimulatory effect of siNCoR, again eliminating off-target effects of the siRNA.

NCoR Inhibits Invasion and Metastasis. The identification of NCoR as an important regulator of genes that are well documented as playing a role in cancer progression suggested that this protein might play a role in invasion, tumor growth, or metastasis. Therefore we first investigated the role of NCoR in cell invasion in Transwell Matrigel assays. NCoR depletion increased the invasive capacity of SK, HepG2, and MDA cells and to a significant extent reversed the reduced invasion of TRβ in both the absence and presence of T3 (Fig. 3*A* and Fig. S3*A*). NCoR overexpression reduced invasion and antagonized stimulation by siNCoR in SK and SK-TRβ cells (Fig. 3*B*). Therefore, the corepressor has a functional role as an inhibitor of cellular invasion and plays a critical function in mediating the repressive effect of TRβ. In contrast, depletion of SMRT had no effect (Fig. S3*B*), indicating that SMRT is not a regulator of invasion in these cells.

To analyze whether NCoR also could play a role in cell invasion in vivo, SK and SK-TRβ cells transfected with control or NCoR siRNAs were injected into the tail vein of nude mice.

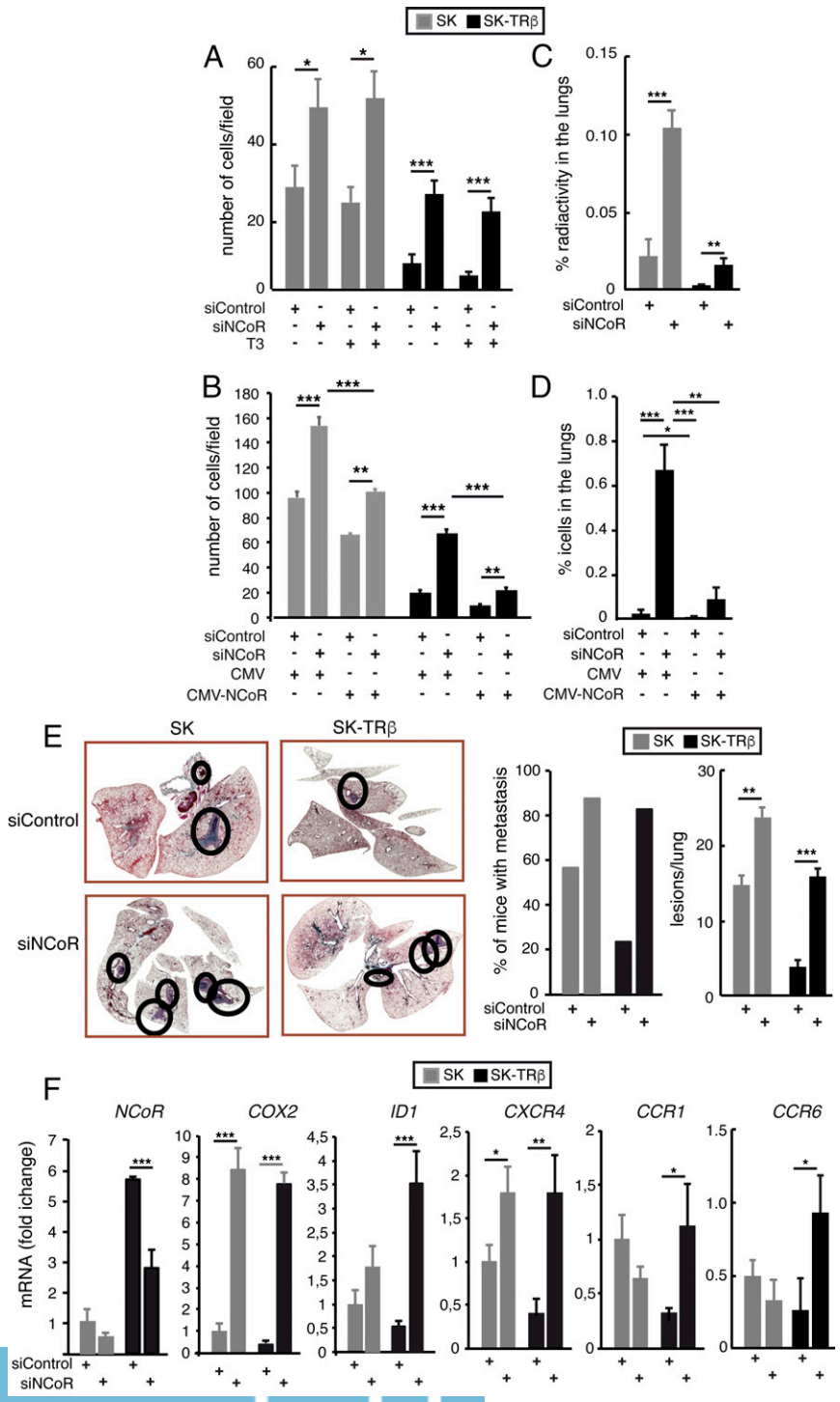

Fig. 3. NCoR inhibits invasion, extravasation, and metastatic growth. (*A*) Invasion assays in Matrigel of SK and SK-TRβ cells that had been transfected with siControl or siNCoR 72 h previously. Invasion lasted for the last 16 h in the presence and absence of T3. The number of cells per field passing the filter (mean ± SD) was scored under the microscope and is shown on the *y* axis. (*B*) Similar assays in cells transfected as indicated with siNCoR, CMV-NCoR, or the corresponding controls. (*C*) Extravasation assays in nude mice of cells transfected with siControl or siNCoR. Data (mean ± SE) are expressed as the percentage of the inoculated cells found in the lung. (*D*) Extravasation of SK-TRβ cells transfected with siNCoR or CMV-NCoR or with the corresponding controls alone and in combination was analyzed by determination of human Alu sequences in the lungs. (*E*, *Left*) Metastatic lesions (surrounded by circles) in lungs from nude mice that had been inoculated 30 d previously via the tail vein with cells transfected with siControl or siNCoR. (*Right*) The percent of animals with metastases and number of lesions per lung (mean ± SE). (*F*) Relative transcript levels of *NCoR* and the indicated prometastatic genes (mean ± SD) in metastases excised from the lungs by laser-capture microdissection.

NCoR depletion increased the amount of SK cells present in the lungs very significantly and increased the extravasation of TRβ-expressing cells to levels similar to those found in the parental cells (Fig. 3*C*). The augmented extravasation of TRβ-expressing cells transfected with siNCoR was again reversed by NCoR expression (Fig. 3*D*), demonstrating the specificity of the effects of the corepressor on the invasive properties of the cancer cells in vivo. Because NCoR limits cancer cell extravasation, it should act as an inhibitor of metastatic growth. Thus we next analyzed the effect of NCoR depletion in the formation of lung metastasis by SK and SK-TRβ cells 30 d after i.v. injection. The incidence of metastasis was increased in mice inoculated with SK cells transfected with siNCoR. Furthermore, SK-TRβ cells showed a reduced metastatic capacity (24), but this capacity increased significantly in the absence of NCoR. Both the incidence of lung metastasis and the number of metastatic lesions were enhanced significantly in the absence of the corepressor (Fig. 3*E*). Therefore, NCoR suppresses the metastatic potential in vivo. We next isolated the metastatic lesions by laser-capture microdissection to analyze the expression of *NCoR* and prometastatic genes. Strikingly, even at 30 d post-injection *NCoR* transcripts were significantly reduced in the metastases formed by cells originally transfected with siNCoR. Reduction was particularly apparent in metastases from SK-TRβ cells that expressed higher levels of the corepressor. Furthermore, prometastatic genes were derepressed in the metastasis after NCoR depletion (Fig. 3*F*). The finding that NCoR knockdown with siRNA lasted for a very long time suggests the existence of a positive autoregulatory loop that maintains NCoR gene expression.

Tumors Formed by NCoR-Depleted Cells Are Bigger and More Invasive. To examine the tumor-suppressive effect of NCoR in vivo further, we conducted xenograft studies with SK and SK-TRβ cells transfected with siControl or siNCoR. NCoR depletion increased tumor volume in both parental and TRβ-expressing cells. Differences were observed as early as 2 wk after inoculation and were maintained during the whole experimental period (6 wk) (Fig. 4*A*). In explants from tumors obtained at 2 wk, NCoR depletion was maintained in both SK and SK-TRβ cells. Furthermore, there was a significant positive correlation between the levels of *NCoR* and *TRβ* mRNAs and an inverse relationship between expression of the corepressor and the mRNA levels of several prometastatic genes, again indicating the crucial role of NCoR in prometastatic gene expression (Fig. S4).

The increased size of tumors formed by cells transfected with siNCoR correlated with a higher proliferation rate (Fig. 4*B*). Tumors that originated from parental SK cells were more proliferative than those formed by SK-TRβ cells, and NCoR depletion markedly increased the number of $Ki67^+$ cells. Even 6 wk after inoculation, strongly reduced *NCoR* mRNA levels were observed in the tumors formed by SK-TRβ cells originally transfected with siNCoR, and transcript levels of prometastatic genes were still significantly higher than in tumors that originated from cells transfected with the control siRNA (Fig. 4*C*). NCoR depletion also enhanced the invasive capacity of SK-TRβ tumors (Fig. 4*D*). Tumors formed by SK-TRβ cells were very compact and were surrounded by a pseudocapsule, which was lost in NCoR-deficient tumors that were highly infiltrative. Furthermore, the percentage of tumors invading bone and muscle was reduced by TRβ, and this protector effect was lost upon NCoR depletion (Fig. 4*D*). NCoR knockdown also increased the growth rate of tumors formed by inoculation of MDA and MDA-TRβ cells into nude mice (Fig. S5*A*), and significantly reduced *NCoR* transcripts also were found in the breast tumors formed by the cells that had been transfected with siNCoR 33 d previously (Fig. S5*B*). In addition, the noninvasive TRβ-expressing breast tumors became highly infiltrative in the absence of the corepressor (Fig. S5*B*). Therefore, NCoR acts not only as a suppressor of tumor growth but also as an inhibitor of tumor invasion in vivo.

NCoR and TRβ Expression Are Reduced in Human HCC and Breast Tumors. To support the role of NCoR in human cancer further, we collected human HCC and breast tumors samples. The expression of *NCoR* was analyzed by immunohistochemistry in the HCC, in the surrounding cirrhotic tissue, and in normal liver samples (Fig. 5*A*). In the normal tissue most nuclei were positive for NCoR, but this number was reduced in the cirrhotic liver and was markedly inhibited in the tumors, where less than 10% of the nuclei had detectable NCoR. These findings were confirmed by immunohistochemistry in other NCoR-specific antibodies (Fig. S6) and by mRNA analysis. The results revealed a significant decrease in NCoR mRNA in tumors compared with normal liver, whereas no differences in SMRT mRNA levels were found. TRβ transcripts also were reduced in the HCCs, and *Dio1*, a bona fide TR target gene in liver cells (23), showed a similar reduction (Fig. 5*B*). Statistical analysis found a significant positive correlation ($P < 0.001$) when *TRβ* mRNA was plotted against *NCoR* or *Dio1* mRNAs (Fig. 5*C*). Strongly reduced levels of NCoR and TRβ transcripts, without changes in SMRT, also were found in RNA samples from the more aggressive ER^- breast tumors as compared with ER^+ tumors. Again there was a strong correlation between NCoR and *TRβ* transcripts ($P < 0.001$) that occurred independently of ER status (Fig. 5*D*). Taken together these data are consistent with a tumor-suppressive role for the corepressor in HCC and breast cancer and support the notion that NCoR is a downstream effector of TRβ in these tumors.

Epigenetic Changes Responsible for Stable NCoR Depletion. Using cultured cells, we next investigated the mechanism by which transient NCoR knockdown could lead to long-term deficiency. As shown in Fig. 6*A*, 17 d after siNCoR transfection, when several cell doublings had already occurred, *NCoR* mRNA was still depleted in SK and SK-TRβ cells. Furthermore, derepression of *ID1* and *COX2* mRNAs was also maintained. In fact, in SK-TRβ cells the degree of change was similar at 3, 10, and 17 d after siRNA transfection (Fig. 6*B*), and at these time points NCoR protein levels were reduced also (Fig. 6*C*). NCoR autoregulation is not an exclusive characteristic of SK cells, because *NCoR* mRNA levels also were depleted in MDA and MDA-TRβ cells 17 d after siNCoR transfection (Fig. 6*D*).

The long-term NCoR silencing obtained with the siRNA suggested the existence of an epigenetic mechanism that could maintain NCoR repression through cell generations. The most typical inheritable mechanism that could explain these results would be silencing of the *NCoR* gene by DNA methylation. Analysis of the *NCoR* gene revealed the presence of a regulatory CpG island spanning −687 to +1557 with respect to the first exon, with the ATG located in the second exon (Fig. S7*A*). However, bisulfite analysis of this fragment showed that the island was unmethylated to a large extent and that the same nucleotides were modified in SK and SK-TRβ cells 17 d after transfection with siControl or siNCoR (Fig. S7*B*). These results were confirmed by pyrosequencing (Fig. S7*C*), thus suggesting that increased methylation of this region was not responsible for the long-lasting repression of the *NCoR* gene upon siRNA transfection.

We then investigated whether recruitment of repressor proteins or increased repressive histone markers could be related to stable *NCoR* gene silencing in NCoR-depleted cells. Indeed, recruitment of SMRT to the +814/+1056 regulatory region of the *NCoR* gene increased strongly 24 d after transfection with siNCoR in SK and SK-TRβ cells (Fig. 7*A*). Because SMRT also silences gene expression by HDAC3 recruitment (34), there was a strong increase in HDAC3 association with the *NCoR* promoter (Fig. 7*A*). These changes occurred without alterations in the expression of HDAC3 SMRT (Fig. 7*B*). To test the functional significance of this pathway, we next analyzed the effect of SMRT knockdown in cells previously transfected with siNCoR. NCoR depletion did not alter *SMRT* mRNA levels, but the low levels of *NCoR* mRNA in cells transfected with siNCoR increased very

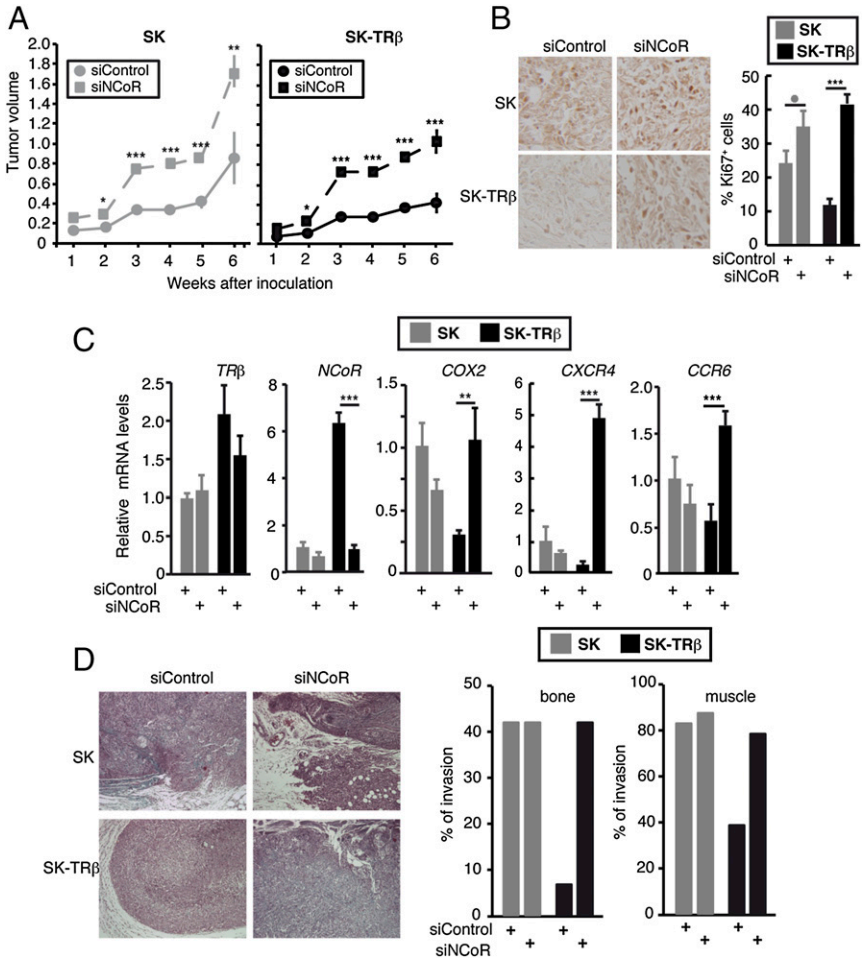

Fig. 4. NCoR inhibits tumor growth and invasion. (*A*) Volume of the tumors (mean ± SE) after inoculation of cells transfected with siControl or siNCoR into nude mice. (*B*) Immunohistochemistry of Ki67 and the percentage of Ki67$^+$ cells (mean ± SE) in tumors excised 6 wk after inoculation. (*C*) Relative transcript levels of *TRβ*, *NCoR*, and the indicated prometastatic genes (mean ± SE) in the tumors. (*D*, *Left*) Representative Masson´s trichrome staining of tumors, showing that tumors formed by cells transfected with siNCoR were more invasive. (*Right*) The percentage of tumors that infiltrated bone and muscle.

significantly upon SMRT depletion, demonstrating the key role of SMRT in long-term silencing of the *NCoR* gene (Fig. 7*C*). Moreover, the enhanced expression of the NCoR target gene *COX2* observed in NCoR-depleted cells was reversed, in parallel with the increased NCoR expression, when SMRT was depleted also (Fig. 7*C*). The importance of histone acetylation in the positive autoregulatory mechanism that maintains NCoR expression was demonstrated further by the finding that the HDAC inhibitor trichostatin A (TSA) was a very strong inducer of NCoR mRNA and protein expression only in cells transfected with siNCoR (Fig. 7 *D* and *E*). This effect again was specific, because it was not observed when *SMRT* mRNA levels were determined (Fig. 7*D*).

Other posttranslational histone modifications such as methylation of H3K9 have a crucial influence on chromatin structure and transcriptional repression. H3K9 trimethylation enables binding of the heterochromatin protein 1 (HP1), which facilitates local heterochromatinization (35). Furthermore, H3K27 methylation, another repressive marker, can synergize with HDAC complexes and H3K9 methylation to silence chromatin (36). Interestingly, we found that NCoR depletion induced a global cellular increase in the levels of H3K9me3 and that H3K27me3 levels also were induced, whereas the total levels of H3 were unaltered in the absence of the corepressor (Fig. 7*F*). ChIP assays also showed a strong increase in the abundance of H3K9me3 and H3K27me3 at the *NCoR* promoter associated with NCoR knockdown, and under these conditions HP1γ was recruited also (Fig. 7*G*), thus creating a stably silenced chromatin state. Examination of the *NCoR* sequences used in the ChIP assays showed the existence of three putative SP1-binding motifs (Fig. 7*H*). One of the mechanisms by which silencing was sustained could involve the inhibition of recruitment of key transcription factors in this repressive chromatin structure. In agreement with this hypothesis, SP1 bound the *NCoR* promoter in cells transfected with siControl, but it was excluded in NCoR-depleted cells (Fig. 7*I*). Therefore, transcriptional repression of the *NCoR* gene in cells transfected with siNCoR appears to be associated with heterochromatinization and SP1 removal from the promoter. This action was specific for the *NCoR* gene, because HDAC3 recruitment to the *ID1* promoter did not increase in cells depleted of NCoR and was even clearly reduced in SK-TRβ cells, as expected for an *NCoR* target gene. The same results were obtained with H3K9me3 levels, which were reduced in the cells transfected with siNCoR despite the higher total levels of this histone modification (Fig. S8*A*). In addition, SP1 did not bind to the *ID1* promoter, but its association to the *p21* promoter, a well-known target gene of this transcription factor, was not affected by NCoR knockdown (Fig. S8*B*).

To clarify why TSA invokes a strong response in NCoR expression when other repressive epigenetic marks are also altered, we tested the possibility that the HDAC inhibitor also could influence H3K9me3 abundance at the *NCoR* regulatory region. ChIP assays showed the expected increase in H3K9 methylation after NCoR silencing, but this increase was totally inhibited in TSA-treated cells, coinciding with NCoR derepression. As expected, heterochromatinization was reversed under these conditions because SP1 was able to associate with the *NCoR* gene (Fig. 7*J*).

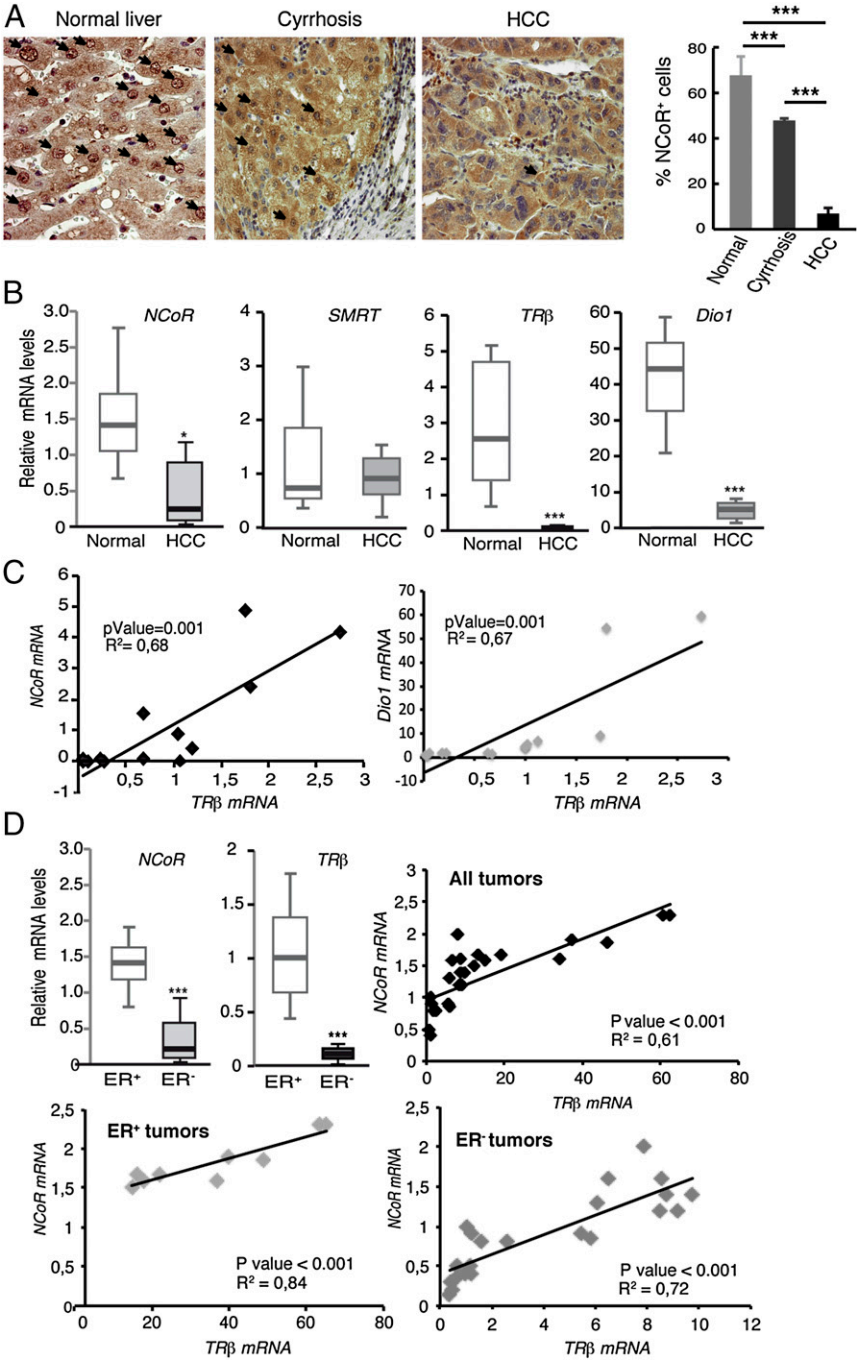

Fig. 5. NCoR and TRβ expression in human HCC and breast cancer tumors. (*A*, *Left*) NCoR immunohistochemistry of normal liver, the surrounding cirrhotic tissue, and HCC NCoR-positive cell nuclei are indicated by arrows. (*Right*) The percent of NCoR-positive cells in the different groups (mean ± SD). (*B*) Whisker plot of *NCoR, SMRT, TRβ*, and *Dio1* mRNAs (mean ± SD) in normal liver and HCC. (*C*) *TRβ* mRNA was plotted against the corresponding *NCoR* or *Dio1* transcripts in each sample. The *P* value and linear regression coefficient obtained are shown. (*D*) *NCoR, SMRT*, and *TRβ* mRNAs (mean ± SD) in ER^+ ($n = 18$) and ER^- ($n = 18$) breast tumors. Correlation between *NCoR* and *TRβ* in all tumors as well as independently in ER^+ and ER^- tumors is illustrated also.

Discussion

This work shows the important tumor-suppressive role of the corepressor NCoR. NCoR inhibits the invasive ability of malignant cells, and knockdown of the corepressor enhances their ability to grow as invasive tumors, to pass into the circulation, and to form metastases, agreeing with the circumstantial evidence of reduced NCoR expression in human tumors, in some cases associated with truncating mutations and homozygous deletions of the *NCoR* gene (19, 21, 22), providing an explanation for these findings and defining NCoR as a potential target for cancer therapy.

Increasing evidence indicates that epigenetic alterations involving an altered pattern of histone modifications lead to the misregulation of gene expression and can contribute to cancer development (37, 38). Our results show that the tumor-suppressive effects of NCoR are linked to the silencing of genes involved in metastatic spreading, whose expression has been associated with poor prognosis in cancer patients (39–44). NCoR, together with HDAC3, the main enzyme responsible for the repressive activity of NCoR (45), binds to the promoters of several prometastatic genes, thereby inhibiting histone acetylation and repressing their transcription.

An unexpected observation was that transient NCoR silencing with siRNA led to very long-lasting inhibition of NCoR expression. This inhibition was observed both in cultured cells and in the tumors and metastatic lesions and was accompanied by derepression of prometastatic genes. Similar to these results, a single episode of RNAi in *Caenorhabditis elegans* can induce

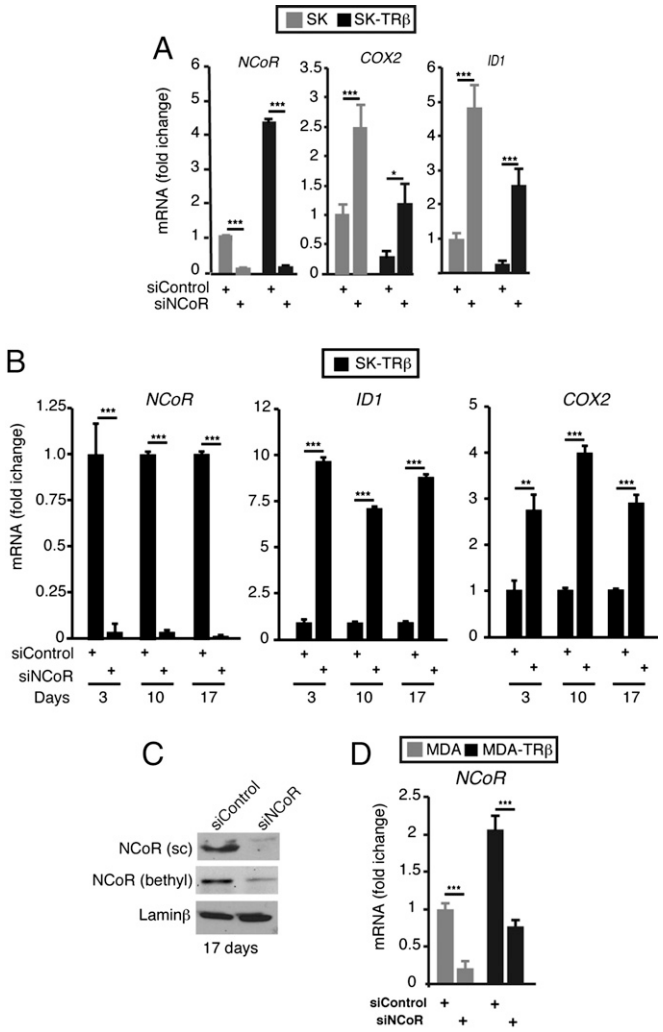

Fig. 6. NCoR depletion with siRNA leads to long-term reduction of *NCoR* gene expression. (*A*) *NCOR*, *COX2*, and *ID1* transcripts (means ± SD) in SK and SK-TRβ cells that had been transfected with siControl or siNCoR 17 d previously. (*B*) The same mRNAs quantitated at 3, 10, and 17 d posttransfection in SK-TRβ cells. Data are means ± SD. (*C*) Western blot analysis of NCoR expression with Santa Cruz (SC) and Bethyl antibodies 17 d after SK-TRβ cells were transfected with siControl or siNCoR. (*D*) *NCoR* transcripts (means ± SD) in MDA and MDA-TRβ cells 17 d after transfection with siControl or siNCoR.

inheritable reduced transcription of some genes, which can be relieved in the presence of TSA (46). Furthermore, some cases of transgenerational epigenetic inheritance seem to involve inherited histone methylation patterns (38), and in the nematode long-term silencing is associated with increased H3K9me3 levels that can be passed through generations without being lost (18).

Stable NCoR silencing after siRNA occurs via a transcriptional mechanism dependent on changes in chromatin structure. Despite the presence of a regulatory CpG island within the *NCoR* gene, DNA methylation of this region was not involved in silencing, suggesting that this is not the main mechanism responsible for NCoR autoregulation. Next, we investigated histone methylation and found that increased abundance of H3K9me3, one of the best markers of heterochromatin, and to a lesser extent of H3K27me3, another repressive marker, were linked to repression. Remarkably, siRNA-mediated suppression of NCoR increased the global levels of H3K9me3, suggesting that chromatin structure is globally altered in the absence of the corepressor. However, we did not find an increase in H3K9me3 association with other genes. It presently is unclear how the gene-target specificity is achieved, but it could involve the assembly of different sets of cofactors and histone-modifying enzymes (1). H3K9 methylation facilitates the recruitment of HP1 (47, 48), allowing local heterochromatinization (49), and accordingly HP1γ also was recruited to the silenced *NCoR* gene. In addition to the increased abundance of transcriptional repressive methylation markers, siRNA depletion of NCoR strongly increased HDAC3 association with the *NCoR* gene. This paradoxical recruitment in the absence of NCoR was caused by SMRT recruitment. The existence of cross-talk between posttranslational histone modifications is well known (37), and we found that inhibiting HDAC activity with TSA also inhibits H3K9me3 abundance at the *NCoR* gene. Thus, both histone deacetylation and methylation appear to play important roles in the establishment of a local repressive state that leads to long-lasting inhibition of *NCoR* gene transcription, presumably by providing a heterochromatic environment that could prevent sequence-specific transcription factor binding. Indeed, under these conditions SP1 could not bind to the promoter, in agreement with findings indicating that occlusion of SP1 binding is associated with increased H3K9me3 (50). Interestingly, NCoR depletion could represent an important mechanism for tumor progression when no *NCoR* mutations are present. A cellular change resulting in a reduction in NCoR levels at a given point in time could be propagated through many cell generations, allowing the cancer cell to proliferate and to become invasive.

Our results show that NCoR levels are increased in cancer cell lines, as well as in nontumoral hepatocytes, after TRβ expression. Surprisingly, this increase occurred in a ligand-independent manner. It is possible that the unliganded receptor could have constitutive effects on NCoR expression when expressed at high concentrations or that residual amounts of thyroid hormones in the serum could be sufficient for maximal stimulation under these conditions. NCoR induction appears to play a crucial role in the antitumorigenic and antimetastatic actions of the receptor (24), because these actions were reversed almost completely upon NCoR silencing. We also have shown that, in contrast with the native receptor, TRβ mutants unable to bind the corepressor cannot antagonize Ras-mediated transformation and tumorigenesis (26), reinforcing the idea that NCoR is an essential mediator of the tumor-suppressive actions of TRβ. TRβ-mediated up-regulation of NCoR could extend the scope of genes that are influenced by the receptor, because the corepressor has been shown to repress transcription through other nuclear receptors and numerous transcription factors with a role in cancer progression (1, 12). Therefore, repression of prometastatic gene expression could be a consequence of NCoR interaction with this receptor or, more likely, with other transcription factors that associate with their promoters. The findings that the effects of TRβ are largely ligand independent and that NCoR binds to the promoters of prometastatic genes in parental SK cells that express very low levels of TR support the hypothesis that NCoR could be recruited to these promoters by other DNA-binding proteins. The increased levels of NCoR would be sufficient to account for the T3-independent action of TRβ in prometastatic gene expression. This action would be particularly important for transcription factors preferentially interacting with NCoR rather than SMRT, because TRβ did not significantly regulate SMRT. Furthermore, SMRT depletion affected neither metastatic gene expression nor the invasive capacity of SK or MDA cancer cells, suggesting the specific role of NCoR in these processes.

Our data confirm that *NCoR* mRNA levels are reduced in human HCC, in agreement with the finding that *Hdac3* deletion in mice causes the appearance of these tumors (22). Furthermore, *NCoR* expression was decreased in the cirrhotic peritumoral tissue, which is considered to be precancerous, further supporting a tumor-suppressive role for NCoR in human HCC. On the other hand, hypothyroidism has been considered to be a risk factor for the development of HCC in humans (51, 52), and

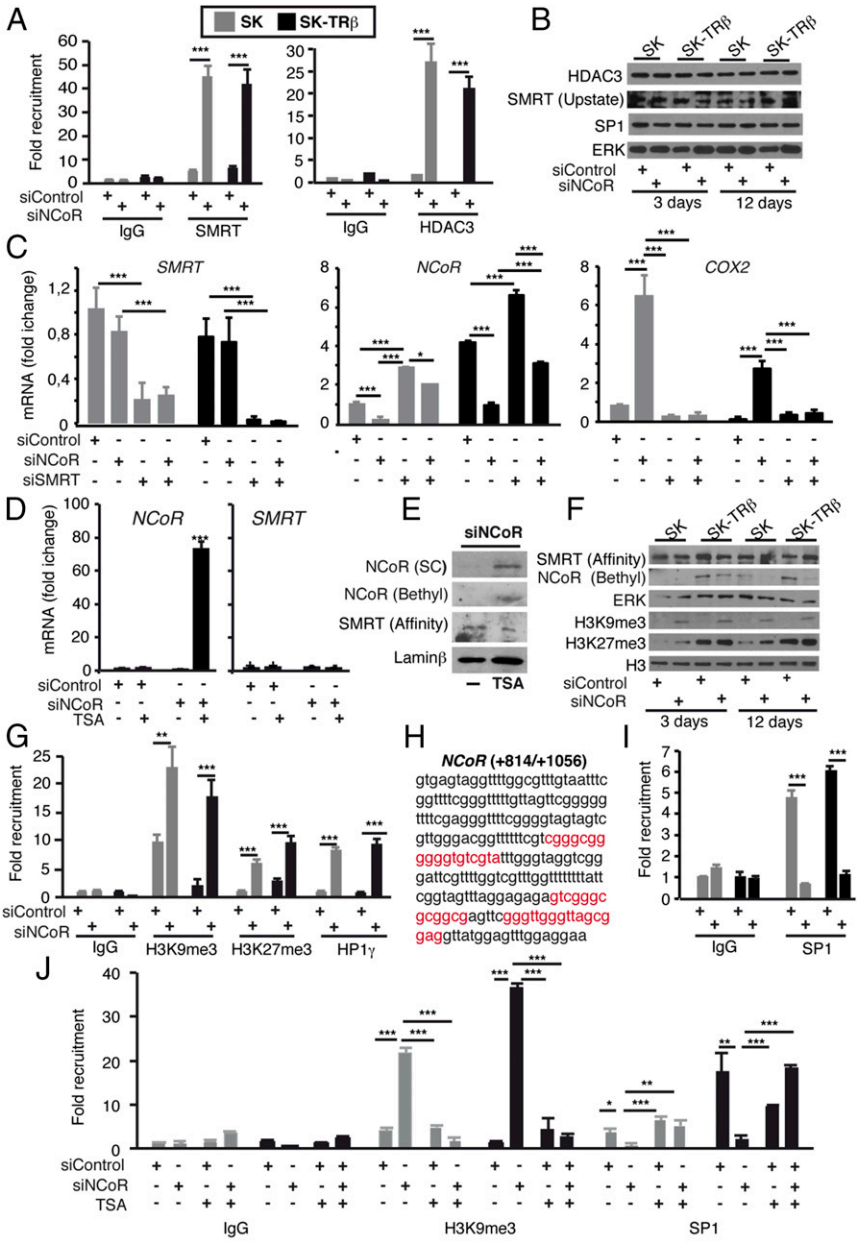

Fig. 7. Heterochromatinization of the *NCoR* gene after NCoR depletion with siRNA. (*A*) ChIP assays of SMRT and HDAC3 with the +814/+1056 region of the *NCoR* gene with respect to the first exon in SK and SK-TRβ cells that had been transfected with siControl or siNCoR 24 d previously. (*B*) Western blot analysis of HDAC3, SMRT (with Upstate antibodies), SP1, and ERK in SK and SK-TRβ cells that had been transfected with siControl or siNCoR 3 and 12 d previously. (*C*) *SMRT, NCoR*, and *COX2* transcripts (means ± SD) in cells transfected with siControl or siNCoR for 17 d and with siSMRT for the final 3 d. (*D*) *NCoR* and *SMRT* mRNA levels in SK-TRβ cells that had been transfected with the control or NCoR siRNAs 17 d previously and treated with 300 nM TSA for the last 24 h. (*E*) Western blot of NCoR and SMRT with the indicated antibodies in SK-TRβ cells that had been transfected with siControl or siNCoR 17 d previously and treated with TSA during the last 24 h. (*F*) Western blot of NCoR, ERK, SMRT, H3K9me3, H3K27me3, and total H3 in the groups shown in *B*. (*G*) Recruitment of H3K9me3, H3K27me3, and HP1γ to the +814/+1056 region of the *NCoR* gene. ChIP assays were performed in SK and SK-TRβ cells that had been transfected with siControl or siNCoR 24 d previously. (*H*) Sequence of the +814/+1056 fragment of the *NCoR* gene showing putative binding sites for SP1 in red. (*I*) SP1 recruitment to the *NCoR* gene in the conditions shown in *G*. (*J*) ChIP assays with IgG, H3K9me3, and SP1 antibodies in cells that had been transfected with siControl or siNCoR for 15 d and with TSA during the last 24 h.

TRβ down-regulation has been reported recently to be an early event in human and rat HCC development (29). Interestingly, we found that *TRβ* transcripts not only were significantly reduced in the tumors compared with normal tissues but also correlated positively with the levels of the corepressor. A strong correlation between *TRβ* and *NCoR* expression also was found in human breast tumors and, interestingly, both genes were markedly down-regulated in ER^- tumors as compared with ER^+ tumors that have a better prognosis. Thus, our results support the possibility that the receptor is an upstream stimulator of *NCoR* gene expression and suggest that NCoR and TRβ could be considered potential biomarkers for some types of human cancers.

Although the mechanisms underlying the role of chromatin regulation in oncogenesis are complex and remain to be defined in a cellular context-dependent manner, our data hold the potential of defining the epigenetic signatures associated with NCoR expression in tumors for potential use as diagnostic or prognostic markers. Elucidating the link between epigenetic mechanisms connecting NCoR loss and tumor progression should prompt the development of more efficient therapeutic strategies as well as facilitate a better understanding of tumor biology.

Materials and Methods

Extended materials and methods are provided in *SI Materials and Methods*. Animal and human studies were approved by the Ethics Committees of the Ramón and Cajal Hospital and the Consejo Superior de Investigaciones Científicas. Informed consent was obtained for the study. NCoR and SMRT were knocked down in parental and TRβ-expressing cells with specific siRNA SMART pools from Dharmacon. Experimental procedures for transfections, luciferase reporter assays, Western blot, mRNA determination by real-time PCR, and ChIP assays have been published previously and are described together with the antibodies and primers used in *SI Materials and Methods*. Clinical and pathological characteristics of HCC and breast cancer samples are given in Tables S1 and S2. Invasion assays in vitro in Transwell plates containing Matrigel and extravasation assays to the lung in vivo were performed as previously described (24, 25). Metastasis formation in the lungs was determined 30 d after inoculation of tumor cells into the tail vein of nude mice, and tumor formation was followed for 6 wk after inoculation into the flanks or the mammary pad. Lung areas affected by metastasis were dissected

by laser-capture microdissection, and RNA was extracted. Histology and immunohistochemistry were performed by standard procedures. Significance of ANOVA posttest or the Student *t* test among the experimental groups indicated in the figures is shown as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

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