

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 $\beta 1$ (TR β) appears to be associated with cancer onset and progression. We found that expression of TR β 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 TR β . These data provide a molecular basis for the anti-cancer 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 TR $\beta 1$, 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 TR $\beta 1$ 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 $\beta 1$ 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 TR β induce regression of carcinogen-induced nodules, reducing the incidence of HCC and lung metastasis in rodents (27, 28), and TR β 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 TR β 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 cyclooxygenase 2 (*COX2*),

DNA-binding protein inhibitor 1 (*ID1*), *C-MET*, matrix metalloproteinase (*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. 1A) and in MDA-MB-468 (MDA) breast cancer cells (Fig. 1B). Because TR β expression significantly increased NCoR mRNA and protein levels in SK and MDA cells (Fig. S1A), and prometastatic genes are repressed by TR β , we reasoned that increased NCoR levels could play a role in this repression. Thus, we also measured prometastatic gene expression in SK-TR β and MDA-TR β cells, finding that the repressive effect of TR β was reversed to a significant extent, or even totally in some cases, in NCoR-depleted cells (Fig. 1). Surprisingly, most of the effect of TR β appears to be ligand independent, because incubation with triiodothyronine (T3) had little effect on transcript levels of *NCoR* and prometastatic genes (Fig. S1B). Similar changes were observed in human HepG2 HCC cells and in nontumoral HH4 human hepatocytes (Fig. S2A and B), showing that TR β 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-TR β cells reversed the effect of the siNCoR in prometastatic gene expression, eliminating the off-target effects of the siRNA pool used (Fig. S1C).

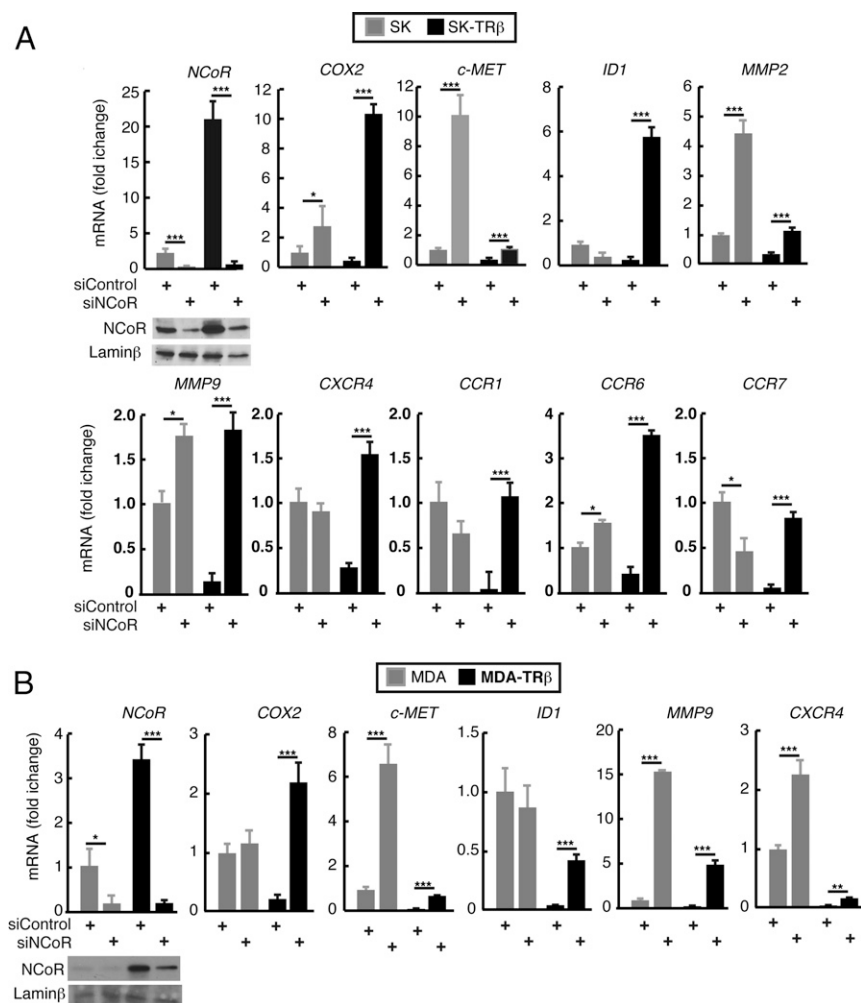


Fig. 1. NCoR depletion increases the expression of prometastatic genes. (A) mRNA levels of prometastatic genes in SK and SK-TR β cells transfected with siControl or siNCoR. Data (means \pm SD) are expressed relative to the values obtained in SK cells transfected with siControl. A Western blot of NCoR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

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 TR β 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-TR β 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 TR β . 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-TR β cells transfected with control or NCoR siRNAs were injected into the tail vein of nude mice.

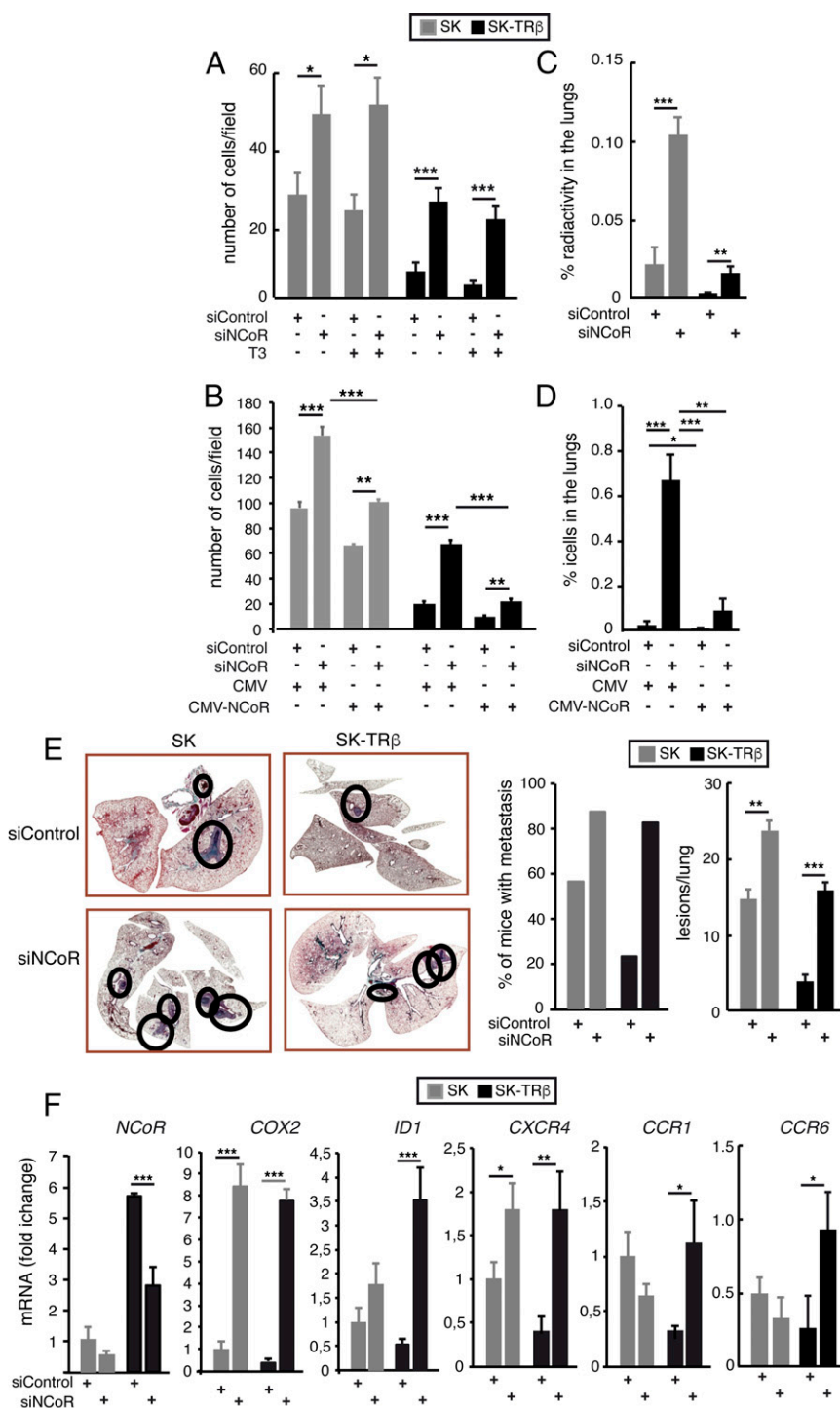


Fig. 3. NCoR inhibits invasion, extravasation, and metastatic growth. (A) Invasion assays in Matrigel of SK and SK-TR β cells that had been transfected with siControl or siNCoR 72 h previously. Invasion lasted for the last 16 h in the presence and absence of T3. The number of cells per field passing the filter (mean \pm 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 \pm SE) are expressed as the percentage of the inoculated cells found in the lung. (D) Extravasation of SK-TR β 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 \pm SE). (F) Relative transcript levels of NCoR and the indicated prometastatic genes (mean \pm 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 TR β -expressing cells to levels similar to those found in the parental cells (Fig. 3C). The augmented extravasation of TR β -expressing cells transfected with siNCoR was again reversed by NCoR expression (Fig. 3D), 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-TR β cells 30 d after i.v. injection. The incidence of metastasis was increased in mice inoculated with SK cells transfected with siNCoR. Furthermore, SK-TR β 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. 3E). 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-TR β cells that expressed higher levels of the corepressor. Furthermore, prometastatic genes were derepressed in the metastasis after NCoR depletion (Fig. 3F). 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-TR β cells transfected with siControl or siNCoR. NCoR depletion increased tumor volume in both parental and TR β -expressing cells. Differences were observed as early as 2 wk after inoculation and were maintained during the whole experimental period (6 wk) (Fig. 4A). In explants from tumors obtained at 2 wk, NCoR depletion was maintained in both SK and SK-TR β cells. Furthermore, there was a significant positive correlation between the levels of *NCoR* and *TR β* 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. 4B). Tumors that originated from parental SK cells were more proliferative than those formed by SK-TR β 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-TR β 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. 4C). NCoR depletion also enhanced the invasive capacity of SK-TR β tumors (Fig. 4D). Tumors formed by SK-TR β 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 TR β , and this protector effect was lost upon NCoR depletion (Fig. 4D). NCoR knockdown also increased the growth rate of tumors formed by inoculation of MDA and MDA-TR β cells into nude mice (Fig. S5A), 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. S5B). In addition, the noninvasive TR β -expressing breast tumors became highly infiltrative in the absence of the corepressor (Fig. S5B). Therefore, NCoR acts not only as a suppressor of tumor growth but also as an inhibitor of tumor invasion in vivo.

NCoR and TR β 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. 5A). 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. TR β transcripts also were reduced in the HCCs, and *Dio1*, a bona fide TR target gene in liver cells (23), showed a similar reduction (Fig. 5B). Statistical analysis found a significant positive correlation ($P < 0.001$) when *TR β* mRNA was plotted against *NCoR* or *Dio1* mRNAs (Fig. 5C). Strongly reduced levels of NCoR and TR β 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 *TR β* transcripts ($P < 0.001$) that occurred independently of ER status (Fig. 5D). 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 TR β 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. 6A, 17 d after siNCoR transfection, when several cell doublings had already occurred, *NCoR* mRNA was still depleted in SK and SK-TR β cells. Furthermore, derepression of *ID1* and *COX2* mRNAs was also maintained. In fact, in SK-TR β cells the degree of change was similar at 3, 10, and 17 d after siRNA transfection (Fig. 6B), and at these time points NCoR protein levels were reduced also (Fig. 6C). NCoR autoregulation is not an exclusive characteristic of SK cells, because *NCoR* mRNA levels also were depleted in MDA and MDA-TR β cells 17 d after siNCoR transfection (Fig. 6D).

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. S7A). 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-TR β cells 17 d after transfection with siControl or siNCoR (Fig. S7B). These results were confirmed by pyrosequencing (Fig. S7C), 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-TR β cells (Fig. 7A). Because SMRT also silences gene expression by HDAC3 recruitment (34), there was a strong increase in HDAC3 association with the *NCoR* promoter (Fig. 7A). These changes occurred without alterations in the expression of HDAC3 SMRT (Fig. 7B). 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

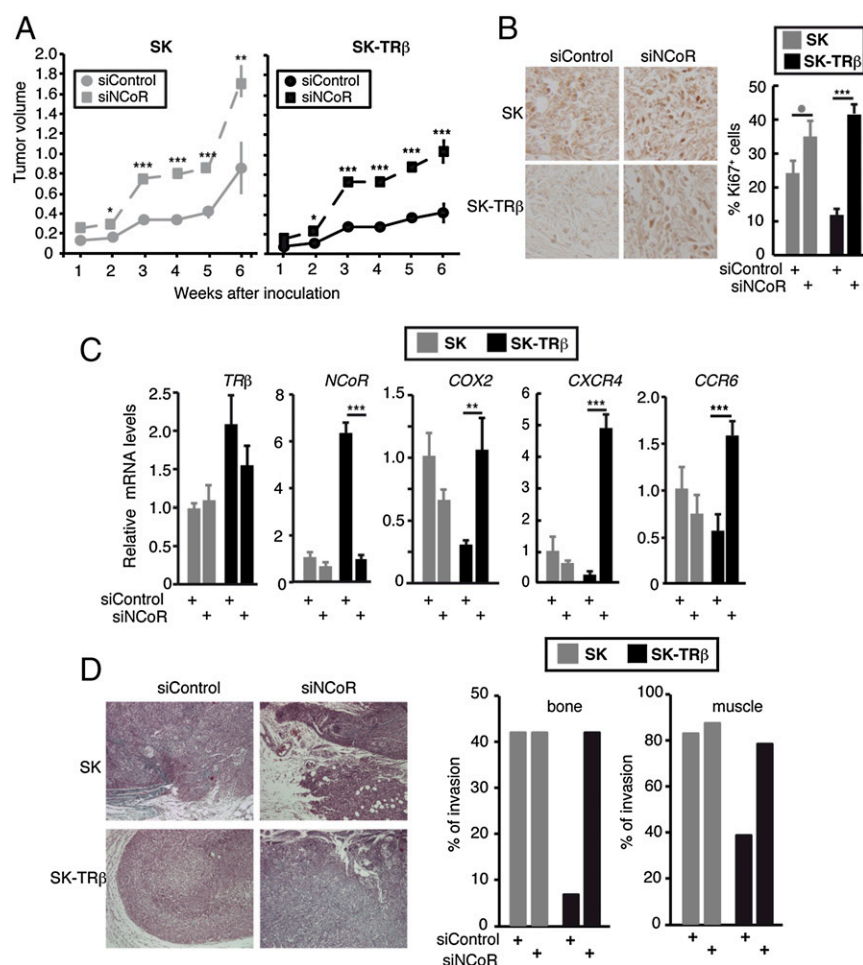


Fig. 4. NCoR inhibits tumor growth and invasion. (A) Volume of the tumors (mean \pm SE) after inoculation of cells transfected with siControl or siNCoR into nude mice. (B) Immunohistochemistry of Ki67 and the percentage of Ki67⁺ cells (mean \pm SE) in tumors excised 6 wk after inoculation. (C) Relative transcript levels of *TRβ*, *NCoR*, and the indicated prometastatic genes (mean \pm 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. 7C). 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. 7C). 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. 7D and E). This effect again was specific, because it was not observed when *SMRT* mRNA levels were determined (Fig. 7D).

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. 7F). 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 HP1 γ was recruited also (Fig. 7G), 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. 7H). 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. 7I). 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-TR β 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. S8A). 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. S8B).

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. 7J).

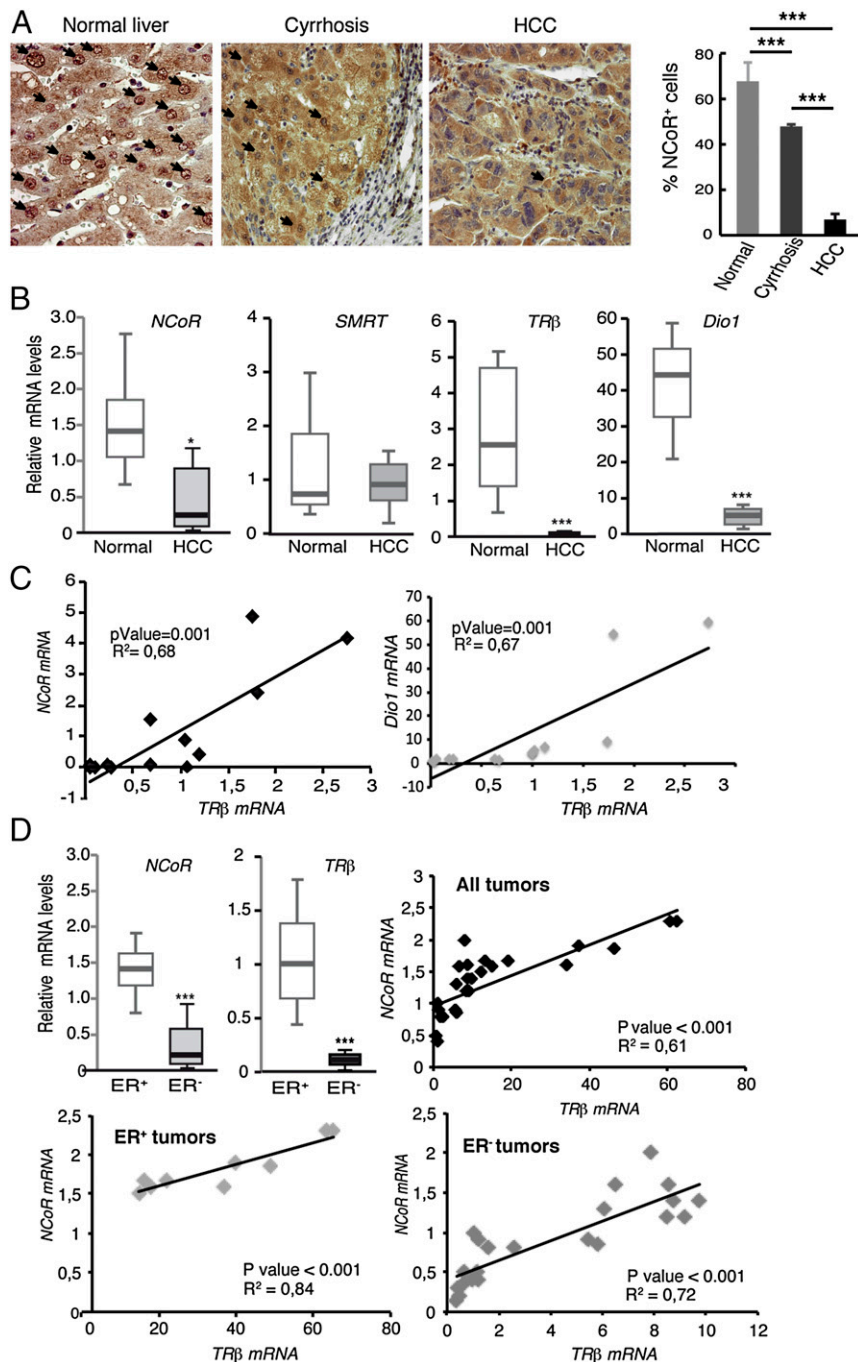


Fig. 5. NCoR and TR β expression in human HCC and breast cancer tumors. (*A, Left*) NCoR immunohistochemistry of normal liver, the surrounding cirrhotic tissue, and HCC NCoR-positive cell nuclei are indicated by arrows. (*Right*) The percent of NCoR-positive cells in the different groups (mean \pm SD). (*B*) Whisker plot of *NCoR*, *SMRT*, *TRβ*, and *Dio1* mRNAs (mean \pm 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 \pm 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

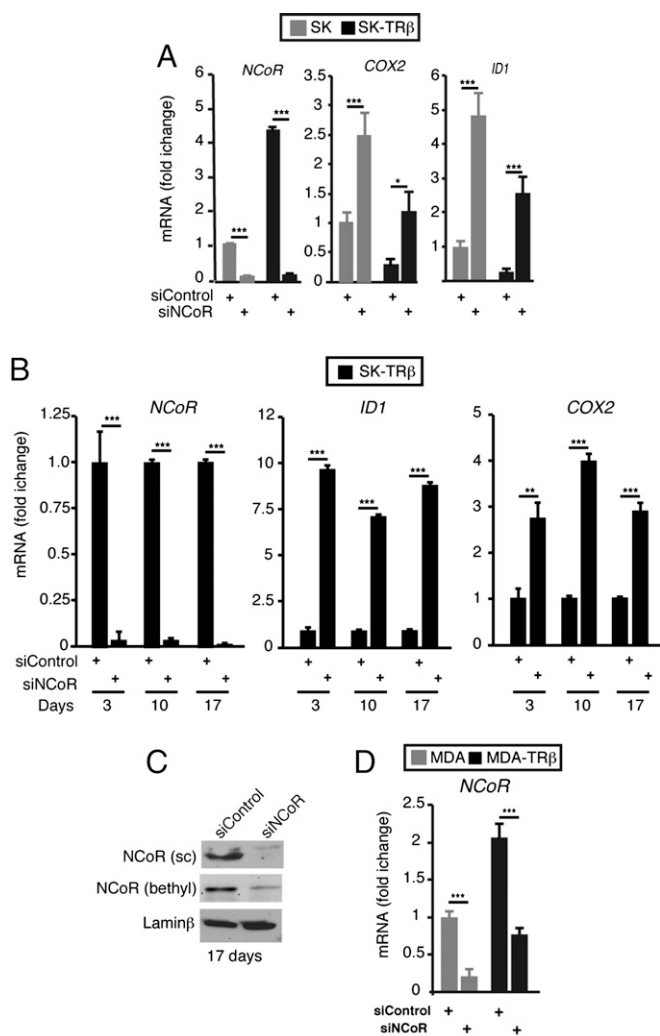


Fig. 6. NCoR depletion with siRNA leads to long-term reduction of *NCoR* gene expression. (A) *NCoR*, *COX2*, and *ID1* transcripts (means \pm SD) in SK and SK-TR β cells that had been transfected with siControl or siNCoR 17 d previously. (B) The same mRNAs quantitated at 3, 10, and 17 d posttransfection in SK-TR β cells. Data are means \pm SD. (C) Western blot analysis of NCoR expression with Santa Cruz (SC) and Bethyl antibodies 17 d after SK-TR β cells were transfected with siControl or siNCoR. (D) *NCoR* transcripts (means \pm SD) in MDA and MDA-TR β 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 HP1 γ 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 post-translational 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 TR β 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, TR β 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 TR β . TR β -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 TR β 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 TR β in prometastatic gene expression. This action would be particularly important for transcription factors preferentially interacting with NCoR rather than SMRT, because TR β 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

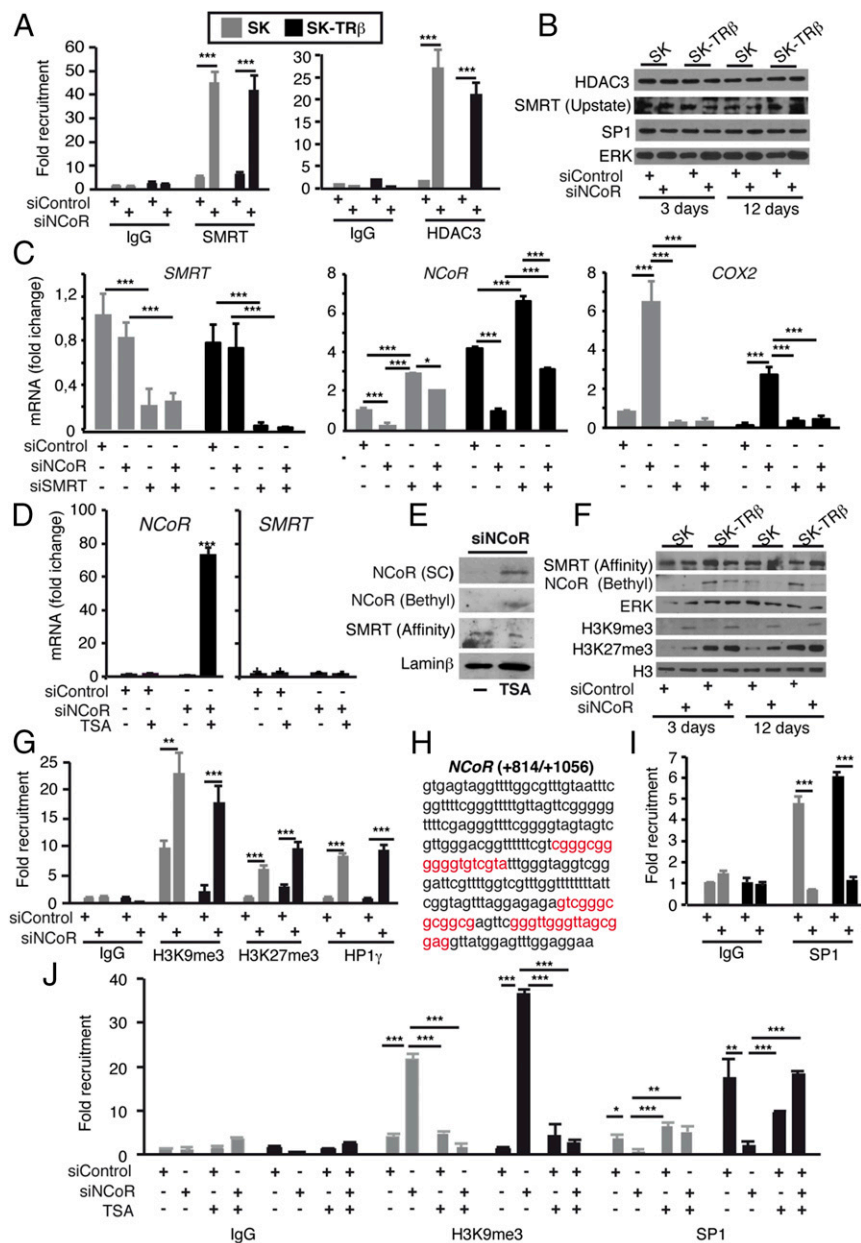


Fig. 7. Heterochromatinization of the *NCoR* gene after NCoR depletion with siRNA. (A) ChIP assays of SMRT and HDAC3 with the +814/+1056 region of the *NCoR* gene with respect to the first exon in SK and SK-TR β cells that had been transfected with siControl or siNCoR 24 d previously. (B) Western blot analysis of HDAC3, SMRT (with Upstate antibodies), SP1, and ERK in SK and SK-TR β cells that had been transfected with siControl or siNCoR 3 and 12 d previously. (C) *SMRT*, *NCoR*, and *COX2* transcripts (means \pm 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-TR β 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-TR β 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 HP1 γ to the +814/+1056 region of the *NCoR* gene. ChIP assays were performed in SK and SK-TR β 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.

TR β down-regulation has been reported recently to be an early event in human and rat HCC development (29). Interestingly, we found that TR β 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 TR β 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 TR β 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 TR β -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 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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- Perissi V, Jepsen K, Glass CK, Rosenfeld MG (2010) Deconstructing repression: Evolving models of co-repressor action. *Nat Rev Genet* 11(2):109–123.
- Chen JD, Evans RM (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* 377(6548):454–457.
- Hörlein AJ, et al. (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* 377(6548):397–404.
- Perissi V, Aggarwal A, Glass CK, Rose DW, Rosenfeld MG (2004) A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. *Cell* 116(4):511–526.
- Codina A, et al. (2005) Structural insights into the interaction and activation of histone deacetylase 3 by nuclear receptor corepressors. *Proc Natl Acad Sci USA* 102(17):6009–6014.
- Yoon HG, et al. (2003) Purification and functional characterization of the human N-CoR complex: The roles of HDAC3, TBL1 and TBLR1. *EMBO J* 22(6):1336–1346.
- Privalsky ML (2004) The role of corepressors in transcriptional regulation by nuclear hormone receptors. *Annu Rev Physiol* 66:315–360.
- Hartman HB, Yu J, Alenghat T, Ishizuka T, Lazar MA (2005) The histone-binding code of nuclear receptor co-repressors matches the substrate specificity of histone deacetylase 3. *EMBO Rep* 6(5):445–451.
- Wen YD, et al. (2000) The histone deacetylase-3 complex contains nuclear receptor corepressors. *Proc Natl Acad Sci USA* 97(13):7202–7207.
- Astapova I, et al. (2008) The nuclear corepressor, NCoR, regulates thyroid hormone action in vivo. *Proc Natl Acad Sci USA* 105(49):19544–19549.
- Ishizuka T, Lazar MA (2003) The N-CoR/histone deacetylase 3 complex is required for repression by thyroid hormone receptor. *Mol Cell Biol* 23(15):5122–5131.
- Mottis A, Mouchiroud L, Auwerx J (2013) Emerging roles of the corepressors NCoR1 and SMRT in homeostasis. *Genes Dev* 27(8):819–835.
- Kurebayashi J, et al. (2000) Expression levels of estrogen receptor- α , estrogen receptor- β , coactivators, and corepressors in breast cancer. *Clin Cancer Res* 6(2):512–518.
- Ciriello G, et al. (2013) The molecular diversity of Luminal A breast tumors. *Breast Cancer Res Treat* 141(3):409–420.
- Green AR, et al. (2008) The prognostic significance of steroid receptor co-regulators in breast cancer: Co-repressor NCoR2/SMRT is an independent indicator of poor outcome. *Breast Cancer Res Treat* 110(3):427–437.
- Girault I, et al. (2003) Expression analysis of estrogen receptor α coregulators in breast carcinoma: Evidence that NCoR1 expression is predictive of the response to tamoxifen. *Clin Cancer Res* 9(4):1259–1266.
- Cancer Genome Atlas Network (2012) Comprehensive molecular portraits of human breast tumours. *Nature* 490(7418):61–70.
- Banerji S, et al. (2012) Sequence analysis of mutations and translocations across breast cancer subtypes. *Nature* 486(7403):405–409.
- Stephens PJ, et al.; Oslo Breast Cancer Consortium (OSBREAC) (2012) The landscape of cancer genes and mutational processes in breast cancer. *Nature* 486(7403):400–404.
- Xu XR, et al. (2001) Insight into hepatocellular carcinogenesis at transcriptome level by comparing gene expression profiles of hepatocellular carcinoma with those of corresponding noncancerous liver. *Proc Natl Acad Sci USA* 98(26):15089–15094.
- Guichard C, et al. (2012) Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. *Nat Genet* 44(6):694–698.
- Bhaskara S, et al. (2010) Hdac3 is essential for the maintenance of chromatin structure and genome stability. *Cancer Cell* 18(5):436–447.
- Aranda A, Martínez-Iglesias O, Ruiz-Llorente L, García-Carpizo V, Zambrano A (2009) Thyroid receptor: Roles in cancer. *Trends Endocrinol Metab* 20(7):318–324.
- Martínez-Iglesias O, et al. (2009) Thyroid hormone receptor β 1 acts as a potent suppressor of tumor invasiveness and metastasis. *Cancer Res* 69(2):501–509.
- Ruiz-Llorente L, Ardila-González S, Fanjul LF, Martínez-Iglesias O, Aranda A (2014) microRNAs 424 and 503 are mediators of the anti-proliferative and anti-invasive action of the thyroid hormone receptor β . *Oncotarget* 5(10):2918–2933.
- García-Silva S, Martínez-Iglesias O, Ruiz-Llorente L, Aranda A (2011) Thyroid hormone receptor β 1 domains responsible for the antagonism with the ras oncogene: Role of corepressors. *Oncogene* 30(7):854–864.
- Ledda-Columbano GM, Perra A, Loi R, Shinozuka H, Columbano A (2000) Cell proliferation induced by triiodothyronine in rat liver is associated with nodule regression and reduction of hepatocellular carcinomas. *Cancer Res* 60(3):603–609.
- Perra A, Kowalik MA, Pibiri M, Ledda-Columbano GM, Columbano A (2009) Thyroid hormone receptor ligands induce regression of rat preneoplastic liver lesions causing their reversion to a differentiated phenotype. *Hepatology* 49(4):1287–1296.
- Frau C, et al. (2015) Local hypothyroidism favors the progression of preneoplastic lesions to hepatocellular carcinoma in rats. *Hepatology* 61(1):249–259.
- Chan IH, Privalsky ML (2009) Thyroid hormone receptor mutants implicated in human hepatocellular carcinoma display an altered target gene repertoire. *Oncogene* 28(47):4162–4174.
- Lin KH, Wu YH, Chen SL (2001) Impaired interaction of mutant thyroid hormone receptors associated with human hepatocellular carcinoma with transcriptional coregulators. *Endocrinology* 142(2):653–662.
- Eun JR, et al. (2014) Hepatoma SK Hep-1 cells exhibit characteristics of oncogenic mesenchymal stem cells with highly metastatic capacity. *PLoS One* 9(10):e110744.
- Jordà M, et al. (2007) Id-1 is induced in MDCK epithelial cells by activated Erk/MAPK pathway in response to expression of the Snail and E47 transcription factors. *Exp Cell Res* 313(11):2389–2403.
- Feng XH, Lin X, Derynck R (2000) Smad2, Smad3 and Smad4 cooperate with Sp1 to induce p15(Ink4B) transcription in response to TGF- β . *EMBO J* 19(19):5178–5193.
- Kouzarides T (2007) Chromatin modifications and their function. *Cell* 128(4):693–705.
- Schwartz YB, Pirrotta V (2013) A new world of Polycombs: Unexpected partnerships and emerging functions. *Nat Rev Genet* 14(12):853–864.
- Cheng SY, Leonard JL, Davis PJ (2010) Molecular aspects of thyroid hormone actions. *Endocr Rev* 31(2):139–170.
- Greer EL, Shi Y (2012) Histone methylation: A dynamic mark in health, disease and inheritance. *Nat Rev Genet* 13(5):343–357.
- Huang F, Geng XP (2010) Chemokines and hepatocellular carcinoma. *World J Gastroenterol* 16(15):1832–1836.
- Lengyel E, et al. (2005) C-Met overexpression in node-positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu. *Int J Cancer* 113(4):678–682.
- Costamagna E, García B, Santisteban P (2004) The functional interaction between the paired domain transcription factor Pax8 and Smad3 is involved in transforming growth factor- β repression of the sodium/iodide symporter gene. *J Biol Chem* 279(5):3439–3446.
- Minn AJ, et al. (2005) Genes that mediate breast cancer metastasis to lung. *Nature* 436(7050):518–524.
- Schimanski CC, et al. (2006) Chemokine receptor CCR7 enhances intrahepatic and lymphatic dissemination of human hepatocellular cancer. *Oncol Rep* 16(1):109–113.
- Yang X, et al. (2006) Essential contribution of a chemokine, CCL3, and its receptor, CCR1, to hepatocellular carcinoma progression. *Int J Cancer* 118(8):1869–1876.
- You SH, et al. (2013) Nuclear receptor co-repressors are required for the histone-deacetylase activity of HDAC3 in vivo. *Nat Struct Mol Biol* 20(2):182–187.
- Vastenhouw NL, et al. (2006) Gene expression: Long-term gene silencing by RNAi. *Nature* 442(7105):882.
- Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* 410(6824):116–120.
- Bannister AJ, et al. (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* 410(6824):120–124.
- Groner AC, et al. (2010) KRAB-zinc finger proteins and KAP1 can mediate long-range transcriptional repression through heterochromatin spreading. *PLoS Genet* 6(3):e1000869.
- Strunnikova M, et al. (2005) Chromatin inactivation precedes de novo DNA methylation during the progressive epigenetic silencing of the RASSF1A promoter. *Mol Cell Biol* 25(10):3923–3933.
- Hassan MM, et al. (2009) Association between hypothyroidism and hepatocellular carcinoma: A case-control study in the United States. *Hepatology* 49(5):1563–1570.
- Reddy A, et al. (2007) Hypothyroidism: A possible risk factor for liver cancer in patients with no known underlying cause of liver disease. *Clin Gastroenterol Hepatol* 5(1):118–123.
- Corral RS, Iñiguez MA, Duque J, López-Pérez R, Fresno M (2007) Bombesin induces cyclooxygenase-2 expression through the activation of the nuclear factor of activated T cells and enhances cell migration in Caco-2 colon carcinoma cells. *Oncogene* 26(7):958–969.
- Chandrasekar B, et al. (2006) Interleukin-18-induced human coronary artery smooth muscle cell migration is dependent on NF- κ B- and AP-1-mediated matrix metalloproteinase-9 expression and is inhibited by atorvastatin. *J Biol Chem* 281(22):15099–15109.
- Mengeling BJ, Phan TQ, Goodson ML, Privalsky ML (2011) Aberrant corepressor interactions implicated in PML-RAR(α) and PLZF-RAR(α) leukemogenesis reflect an altered recruitment and release of specific NCoR and SMRT splice variants. *J Biol Chem* 286(6):4236–4247.
- Vella KR, et al. (2014) Thyroid hormone signaling in vivo requires a balance between coactivators and corepressors. *Mol Cell Biol* 34(9):1564–1575.