NCOA1 Directly Targets M-CSF1 Expression to Promote Breast Cancer Metastasis

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Abstract

In breast cancer, overexpression of the nuclear coactivator NCOA1 (SRC-1) is associated with disease recurrence and resistance to endocrine therapy. To examine the impact of NCOA1 overexpression on morphogenesis and carcinogenesis in the mammary gland (MG), we generated MMTV-hNCOA1 transgenic [Tg(NCOA1)] mice. In the context of two distinct transgenic models of breast cancer, NCOA1 overexpression did not affect the morphology or tumor-forming capability of MG epithelial cells. However, NCOA1 overexpression increased the number of circulating breast cancer cells and the efficiency of lung metastasis. Mechanistic investigations showed that NCOA1 and c-Fos were recruited to a functional AP-1 site in the macrophage attractant CSF1 promoter, directly upregulating colony-simulating factor 1 (CSF1) expression to enhance macrophage recruitment and metastasis. Conversely, silencing NCOA1 reduced CSF1 expression and decreased macrophage recruitment and breast cancer cell metastasis. In a cohort of 453 human breast tumors, NCOA1 and CSF1 levels correlated positively with disease recurrence, higher tumor grade, and poor prognosis. Together, our results define an NCOA1/AP-1/CSF1 regulatory axis that promotes breast cancer metastasis, offering a novel therapeutic target for impeding this process. Cancer Res; 74(13); 1–12. ©2014 AACR.

Introduction

NCOA1 is a member of the p160 SRC family that also contains NCOA2 (GRIP1/TIF2/SRC-2) and NCOA3 (AIB1/ACTR/SRC-3; ref. 1). These NCOAs interact with nuclear hormone receptors and other transcription factors (TF) to facilitate the assembly of transcriptional protein complexes for chromatin remodeling and activation of gene expression (1). Because these coactivators are strong boosters of gene expression, these proteins are usually unstable and present at low concentrations in normal cells (2) and changes in either their concentration or activity significantly affect their target gene expression (3). Accordingly, overexpression of these coactivators is often linked with human diseases such as cancer. Specifically, NCOA3 is amplified and overexpressed in subsets of breast, prostate, ovarian, hepatocellular, and pancreatic cancers (4–8). Forced overexpression of NCOA3 in the mouse mammary gland (MG) epithelium induces tumorogenesis, whereas knockout of NCOA3 suppresses oncogene- or chemical carcinogen–induced MG and prostate tumorogenesis (9–13). Furthermore, NCOA2 is a commonly amplified oncogene that is associated with an enhanced androgen receptor function in prostate cancer (14). Moreover, NCOA1 is also overexpressed in a subset of breast tumors that express HER2 and give poor prognosis (15). However, the in vivo role of NCOA1 overexpression in breast cancer remains to be defined.

Recent studies have suggested that NCOA1 is required for breast cancer metastasis. Knockout of Ncoa1 significantly inhibits mammary tumor metastasis to the lung in transgenic MMTV (mouse mammary tumor virus)-polyoma middle T [Tg (PyMT)] or Tg(Neu) breast cancer mouse models (16, 17). Knockdown of NCOAI in human breast cancer cells also suppresses their invasion and metastasis (18–20). At the molecular level, NCOAI serves as a coactivator for different TFs to upregulate the expression of several genes that promote the epithelial–mesenchymal transition (EMT), migration, invasion, and metastasis of breast cancer cells. The known NCOAI-regulated genes include Twist1, integrin α2, SDF1, HER2, and c-Myc (16, 18, 19, 21–23). Because NCOA1 is a critical coactivator that may control breast cancer metastasis through interaction with multiple TFs important for the metastatic process, further characterization of the TF partners of NCOA1 and their target genes will aid in elucidating the regulatory gene networks of...
cancer metastasis and identifying potential targets for inhibiting cancer metastasis.

CSF1 is expressed in multiple cell types such as osteoblasts, uterine epithelial cells, and different types of cancer cells, and it plays important roles in organ development and physiologic functions such as MG and placental development (24–27). Colony-simulating factor 1 (CSF1) regulates the proliferation, differentiation, and survival of mononuclear phagocytic cells and their bone marrow progenitors (26). CSF1 secreted from breast cancer cells recruits cancer-associated macrophages (CAM) to promote metastasis (28). CSF1 is overexpressed in 70% of breast tumors and its overexpression is associated with macrophage infiltration, tumor cell invasion, advanced tumor grades, and poor prognosis (28, 29). Knockout of CSF1 inhibits lung metastasis from MG tumors, whereas transgenic expression of CSF1 in both CSF1 knockout and wild-type (WT) mammary epithelium restores or enhances macrophage recruitment and lung metastasis in the Tg(PyMT) mouse model (30). A paracrine loop between tumor cells and macrophages has been shown to be required for breast cancer cell migration (31). In this regulatory loop, cancer cells secrete CSF1 to recruit and stimulate macrophages. In turn, macrophages secrete epidermal growth factor (EGF) to stimulate tumor cells to migrate and metastasize. However, the TFs and coactivators that regulate CSF1 expression in breast cancer cells are still unknown.

In this study, we generated both breast cancer mouse models and cell lines with overexpression or knockout/knockdown of NCOA1 or CSF1 to investigate whether NCOA1 directly regulates CSF1 expression to promote breast cancer metastasis.

Materials and Methods

Transgenic mice

The MMTV-hNCOA1 transgene was constructed (Fig. 1A). Tg(NCOA1) mice were generated as described in Supplementary Methods. Tg(NCOA1) mice were crossed with Tg(Neu) mice (32) and Tg(TVA) mice (33), respectively, to generate female Tg(Neu), Tg(NCOA1)×Tg(Neu), Tg(TVA), and Tg(NCOA1)×Tg(TVA) mice for experiments. Mouse genotypes were determined by PCR using transgene-specific primers listed in Supplementary Table S1. All mice have an FVB strain background. Animal protocols were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Cell culture

MDA-MB-231 and MCF-7 cell lines were obtained from the Tissue Culture Core in Baylor College of Medicine (Houston, TX). The two Ncoa1 knockout PygMT (PygMT×Ncoa1-K1/K2) and the two Ncoa1 WT Tg(PygMT) (PygMT×Ncoa1-W1/W2) cell lines were developed from mouse MG tumors as previously described (19). The MDA-231-LM3 breast cancer cell line was developed from a lung metastatic focus derived from a xenograft tumor of MDA-MB-231-LM2 cells (34) in the MG of an SCID mouse. These cells were authenticated by short tandem repeat DNA fingerprinting in The University of Texas MD Anderson Cancer Center Characterized Cell Line Core. Cells were cultured as described previously (19).

Promoter–reporter constructs, cell transfection, and luciferase assay

Two DNA fragments spanning base pairs (bp) −1956 to 284 (F1) or −790 to 284 (F2) of the human CSF1 promoter region were amplified by high-fidelity PCR and subcloned into pGL3 plasmid with a luciferase reporter. The primer sequences used for PCR are listed in Supplementary Table S1. Another set of the mutant CSF1 promoter–reporter plasmids were constructed on the basis of the pGL3-F2 plasmid that contains the F2 DNA fragment. In this set, one, two, or three AP-1–binding sites at bp −614, −300, or −106 positions were deleted individually or in combination by PCR-assisted mutagenesis. Hela cells in 24-well plates were cotransfected with one of the pGL3-based promoter–reporter plasmids, pCR3.1-NCOA1 plasmid, and one of the plasmids for PEAK3, TCF-4, NF-kB, and AP-1 (c-Jun/c-Fos) expression as described previously (18, 19). Mock plasmids were used to compensate for total DNA in each transfection. After 48 hours, cells were lysed for measuring luciferase activity, which were then normalized to total protein used in each assay as described previously (19, 35).

Macrophage recruitment and macrophage-induced tumor cell invasion assays in a Transwell coculture system

Tg(Neu) and Tg(NCOA1)×Tg(Neu) tumor cells isolated from individual mouse MG tumors were cultured in 24-well plate and transfected with nontargeting siRNAs (control) or siRNAs targeting NCOA1 or CSF1. The transfected cells were cultured in DMEM medium with 10% serum for 48 hours and then changed to the serum-free DMEM medium. Matrigel-coated invasion chambers were mounted to the top of the 24-well plate and 5 × 10^4 of RAW-264.7 mouse macrophages suspended in the same serum-free medium were loaded to each upper chamber. After culturing for 20 hours, the non-intruding macrophages above the Transwell membrane were removed, and the macrophages adhered to the bottom surface of this membrane were fixed, stained, and counted as described previously (18, 19, 35). The number of recruited macrophages was normalized to the number of total tumor cells in the lower chamber.

Detection of NCOA1 and CSF1 proteins by IHC in human breast tumors

The tissue microarrays were prepared from archived human breast tumor specimens (n = 560) of a patient cohort as described previously (Supplementary Methods: refs. 15, 35). IHC was performed using NCOA1 and CSF1 antibodies as described in Supplementary Methods. The immunostaining intensities for NCOA1 and CSF1 were independently scored by a pathologist (Z. Yang) and an investigator (L. Qin) according to the Allred scoring system (36), and the average score was used for each sample. The Pearson χ^2 test was used for categorical variables to compare two proportions. Kaplan–Meier estimates of recurrence-free functions were computed and the log-rank test was applied to compare the difference of
the recurrence-free curves among different groups. A P value of less than 0.05 was considered to be statistically significant.

Other methods

Examinations of MG morphology, epithelial proliferation, MG tumor growth, and lung metastasis were performed as described previously (10, 11, 16, 33, 37, 38). IHC, Western blotting, and ELISA were performed as described previously (16, 18, 19). Quantitative real-time RT-PCR (qPCR), knockdown and expression of NCOA1, and chromatin immunoprecipitation (ChIP) assay were also performed as described previously (18, 19). Oligonucleotide primers used in qPCR and ChIP assays are listed in Supplementary Table S1. Please refer to Supplementary Information for detailed description of these methods.

Results

Generation of Tg(NCOA1) mice for NCOA1 overexpression in MECs

To overexpress hNCOA1 in the mouse mammary epithelial cells (MEC), we constructed the MMTV-hNCOA1 transgene (Fig. 1A). Microinjection of the transgene DNA into the fertilized oocytes generated 41 pups, 13 of which harbored the transgene. From these founders with the transgene, we developed 6 Tg(NCOA1) transgenic lines that expressed different levels of the transgene as measured by hNCOA1-specific qPCR. Two of the lines expressed higher hNCOA1, which was similar to the level of endogenous mNCOA1 mRNA (Fig. 1A). The different levels of mNCOA1 expression in different mice might be due to the different phases of estrous cycle. Because total colony number/0.2 mL blood

Figure 1. Generation of Tg(NCOA1) mice and mammary tumor metastasis in Tg(Neu)/C2 Tg(NCOA1)/C2 Tg(Neu) mice. A, the MMTV-hNCOA1 transgene and qPCR analysis of hNCOA1 mRNA expression. RNA was extracted from MGs of WT and Tg(NCOA1) mice and MDA-MB-435 cells. qPCR was performed using hNCOA1 cDNA-specific P1/P2 primers. Human hNCOA1 and mouse mNCOA1 are indicated. bGHpA, bovine growth hormone polyadenylation sequence. B, IHC was performed using an antibody that detects both mNcoa1 and hNCOA1. Arrows, NCOA1-overexpressing MECs. MG sections were prepared from female WT and Tg(NCOA1) mice with indicated weeks of ages. C, tumor growth curves were obtained from 27 Tg(Neu) and 26 Tg(NCOA1)/C2 Tg(Neu) mice by measuring tumor volumes (cm³). Three tumors from each genotype group were assayed. D, the number of tumor cell colonies derived from circulating tumor cells in the blood samples of Tg(Neu) and Tg(NCOA1)/C2 Tg(Neu) mice that had developed mammary tumors for 9 weeks. The Student t test indicated a significant difference (P = 0.001) between the two groups. E, metastatic tumor foci on the lung surfaces and hematoxylin and eosin–stained lung (L) sections with metastatic (M) tumors in Tg(Neu) and Tg(NCOA1)/C2 Tg(Neu) mice. F, lung metastasis indexes are presented as the ratio of tumor area to the total tumor and lung area in Tg(Neu) (n = 15) and Tg(NCOA1)/C2 Tg(Neu) (n = 10) mice.
MG RNA was used in the assay, hNCOA1 was only expressed in MECs, and mNcoa1 was expressed in both MECs and other MG cells, the hNCOA1 mRNA should be much higher than mNcoa1 mRNA in MECs of these Tg(NCOA1) mice. Indeed, IHC revealed a significant increase of NCOA1 protein in MECs of Tg(NCOA1) mice as examined at ages of 6 and 8 weeks compared with age-matched WT mice (Fig. 1B). On the basis of these results, we kept two transgenic lines that displayed higher levels of hNCOA1 expression for further experiments, and these two lines showed similar features in all experiments described below.

WT and Tg(NCOA1) mice showed no significant changes in MG ductal morphogenesis, the MEC proliferation index, and the number of macrophages around MG ducts when examined at 3.5, 6, and 8 weeks of ages (Supplementary Fig. S1A–S1E). Tg (NCOA1) mice also exhibited normal lactation function and developed no MG tumors during the examining period from newborn to 14-month-old. These results demonstrate that NCOA1 is successfully overexpressed in MECs of Tg(NCOA1) mice, and this overexpression does not cause any obvious abnormal phenotypes.

**NCOA1 overexpression promotes breast cancer metastasis in Tg(Neu) and Tg(TVA) + RCAS-PyMT mouse models**

Palpable solid MG tumors comparably developed in Tg(Neu) and Tg(NCOA1) × Tg(Neu) mice during 6 to 12 months of ages and showed similar growth speeds. These tumors exhibited histopathologic morphologies of poorly differentiated adenocarcinomas (data not shown). Total NCOA1 mRNA and protein were increased in Tg(NCOA1) × Tg(Neu) tumors versus Tg (Neu) tumors as measured by qPCR and Western blot analysis (Fig. 1C). Interestingly, after examining the circulating tumor cells by culturing blood samples collected from mice borne mammary tumors for 9 weeks, we found that both the frequency and number of tumor cell colonies formed from the blood samples of Tg(NCOA1) × Tg(Neu) mice were much higher than those from Tg(Neu) mice (Fig. 1D). The metastatic foci in the lung were also more common and greater in area in Tg(NCOA1) × Tg(Neu) mice when compared with Tg (Neu) mice. Statistical analysis of tumor area in lungs revealed a significant increase of the metastatic index in Tg(NCOA1) × Tg(Neu) mice compared with that of Tg(Neu) mice (Fig. 1E and F).

We also induced MG tumorigenesis in Tg(TVA) and Tg (NCOA1) × Tg(TVA) mice by intraductal injection of RCAS-PyMT avian virus as described previously (33). In these mice, the TVA receptor for RCAS virus is expressed in MECs, so that the injected RCAS-PyMT virus specifically infects some of these TVA-expressing cells to express PyMT for tumorigenic transformation (33). Again, no significant differences in MG tumorigenesis and tumor growth were observed in Tg(TVA) + RCAS-PyMT and Tg(NCOA1) × Tg(TVA) + RCAS-PyMT mice (Supplementary Fig. S2A and data not shown). However, the number of circulating tumor cells, the tumor foci in the lung, and the metastatic index were significantly increased in Tg (NCOA1) × Tg(TVA) + RCAS-PyMT mice versus Tg(TVA) + RCAS-PyMT mice (Supplementary Fig. S2B–S2D). Together, the results from both Tg(NCOA1) × Tg(Neu) and Tg(NCOA1) × Tg(TVA) + RCAS-PyMT mouse models indicate that NCOA1 overexpression significantly enhances spontaneous breast cancer metastasis.

**NCOA1 overexpression promotes CSF1 expression in MG tumors**

Macrophage recruitment to the primary tumor site is important for tumor progression to advanced malignant stages (28–31). To examine the effect of NCOA1 overexpression on the recruitment of macrophages, immunostaining against F4/80, a specific macrophage marker, was performed on MG tumor sections from Tg(Neu) and Tg(NCOA1) × Tg(Neu) mice at 2 to 3 weeks (early stage) and 9 weeks (late stage) after tumors were first detected. More macrophages were observed on the sections of Tg(NCOA1) × Tg(Neu) tumors versus the sections of Tg (Neu) tumors at both stages. Quantitative analysis confirmed that the average number of macrophages was significantly increased in Tg(NCOA1) × Tg(Neu) tumors versus Tg(Neu) tumors (Fig. 2A and B). Consistently, qPCR analysis revealed that the expression levels of CSF1 mRNA were higher in all three examined NCOA1-overexpressing tumors compared with NCOA1 WT tumors (Fig. 2C). Furthermore, adenovirus-mediated overexpression of hNCOA1 in MCF-7 and MDA-MB-231 human breast cancer cells significantly upregulated CSF1 expression (Fig. 2D). These results demonstrate that NCOA1 overexpression in MG tumor cells results in upregulation of CSF1 expression.

**NCOA1 regulates CSF1 expression in breast cancer cells**

To determine whether NCOA1 regulates CSF1 expression, we measured Csfi mRNA levels in Ncoa1-negative PyMT × Ncoa1-K1/K2 and Ncoa1-positive PyMT × Ncoa1-W1/W2 mouse MG tumor cells (19). We found that Csfi mRNA levels decreased 3- to 5-fold in PyMT × Ncoa1-K1/K2 versus PyMT × Ncoa1-W1/W2 cells (Fig. 3A). Knockdown of NCOA1 by siRNA significantly reduced Csfi mRNA levels and secreted Csfl protein in both PyMT × Ncoa1-W1/W2 cell lines and their conditioned media (Fig. 3B). Conversely, adenovirus-mediated reexpression of NCOA1 in both PyMT × Ncoa1-K1/K2 cell lines significantly increased CSF1 protein concentration in their conditioned media (Fig. 3C). More importantly, knockdown of NCOA1 in MCF-7 and MDA-MB-231 human breast cancer cells also significantly reduced CSF1 mRNA expression (Fig. 3D and E). These results, together with those shown in Fig. 2C and D, indicate that NCOA1 expression levels are tightly associated with CSF1 expression levels in both mouse and human breast cancer cells.

**NCOA1 associates with the proximal region of the CSF1 promoter in breast cancer cells**

To examine the association of NCOA1 with the CSF1 promoter in breast cancer cells, we designed six pairs of PCR primers for amplifying DNA fragments a to f and performed ChIP assays to identify specific NCOA1-associating chromatin regions near the promoter from bp –1956 to 284 (Fig. 4A). We performed qPCR to measure the eluted DNA immunoprecipitated by NCOA1 or c-Fos antibody from the protein–DNA
Figure 2. NCOA1 overexpression in mammary tumor cells enhances macrophage recruitment and CSF1 expression. A, immunostaining of macrophages (brown) with F4/80 antibody in Tg(Neu) (Neu) and Tg(NCOA1) × Tg(Neu) (NCOA1 × Neu) mammary tumors. T, tumor area; M, macrophage. B, macrophage numbers in Tg(Neu) and Tg(NCOA1) × Tg(Neu) tumors at early and late stages. Macrophages in 10 viewing fields at ×400 magnification were counted on each tumor section. Five tumors were analyzed for each group. Data, mean ± SD. *P < 0.05.

NCOA1 serves as a coactivator for AP-1 to promote the CSF1 promoter activity

To determine the role of NCOA1 in regulation of CSF1 transcription, two luciferase reporters with 5′ CSF1 DNA sequences from bp −1596 to 284 (pGL3-F1) and from bp −790 to 284 (pGL3-F2) were constructed. In agreement with the presence of NCOA1-associated regions in both reporters, the luciferase activities derived from both reporters in transfected HeLa cells were significantly enhanced by NCOA1 expression in a dose-dependent manner (Fig. 5A). These results suggest that NCOA1 overexpression potentiates the transcriptional activity of the 5′ CSF1 promoter region from bp −790 to 284 in the pGL3-F2 reporter, which contains the NCOA1-associated regions d and e (bp −600 to −98 in Fig. 4A). Thus, we proposed that NCOA1 promotes CSF1 promoter activity mainly through its recruitment to the region from bp −600 to −98.

NCOA1 and NCOA1-interacting proteins have been reported to associate with multiple TFs, including NF-κB, PEA3, TCF-4, and AP-1 (c-Jun/c-Fos), and coactivate their transcriptional activities in cancer cells (18, 19, 39, 40). Therefore, we tested whether NCOA1 overexpression could coactivate any of these TFs to enhance the CSF1 promoter activity as reflected by luciferase activity from the pGL3-F2 reporter. Transfection with NF-κB, PEA3, or TCF-4 expression plasmid, respectively, activated their cognate responsive reporters, indicating these TFs expressed well in these transfected cells (Supplementary Fig. S3). However, coexpression of NCOA1 with NF-κB, PEA3, or TCF-4 did not or only slightly increase the luciferase activity of the pGL3-F2 reporter containing the 5′ CSF1 regulatory sequence associated with NCOA1. Interestingly, coexpression of NCOA1 with c-Jun and c-Fos dramatically increased the luciferase activity of the pGL3-F2 reporter in a NCOA1 dose-dependent manner, showing a 6-fold induction at the highest level of NCOA1 expression (Fig. 5B). There are three putative AP-1-binding sites at bps −614 (TGATATCA), −300 (TGACTCA) and −106 (TGAATCA; Fig. 5C) of the 5′ CSF1 promoter region tested in the pGL3-F2 reporter. Deletion of the −614 site (pGL3-M1), the −300 site (pGL3-M2), or both of these sites (pGL3-M12) in the pGL3-F2 reporter did not significantly...
Knockdown of either NCOA1 or CSF1 in Tg(NCOA1)×Tg(Neu) mouse MG tumor cells reduces macrophage recruitment and tumor cell invasion

To determine whether NCOA1 overexpression in mouse mammary tumor cells is responsible for the increased macrophage recruitment, we assessed macrophage invasion attracted by NCOA1-overexpressed mammary tumor cells in a Matrigel layer–based Transwell assay. We found that Tg(NCOA1)×Tg(Neu) tumor cells seeded in the lower chambers were able to attract nearly two times more macrophages from the upper chamber than the same number of WT Tg(Neu) tumor cells. Knockdown of either NCOA1 or CSF1 in Tg(NCOA1)×Tg(Neu) tumor cells by siRNAs reduced more than 65% and 75% of their macrophage recruitment capability, respectively (Fig. 6A, left). Addition of recombinant CSF1 protein to the lower chambers abolished the effect of either NCOA1 or CSF1 knockdown in Tg(NCOA1)×Tg(Neu) cells on macrophage recruitment (Fig. 6A, middle). Furthermore, addition of neutralizing CSF1 antibody to the lower chambers with WT Tg(Neu) or Tg(NCOA1)×Tg(Neu) cells also significantly reduced the number of recruited macrophages (Fig. 6A, right). These results indicate that NCOA1-upregulated CSF1 expression in breast tumor cells plays a crucial role in macrophage recruitment.

Knockdown of CSF1 expression in human breast cancer cells with NCOA1 overexpression reduces macrophages in the xenograft tumors and decreases lung metastases in SCID mice

The MDA-231-LM3.3 human breast cancer cell line has a much stronger lung metastasis potential than its original MDA-MB-231 parent cell line. We found that the expression levels of both NCOA1 and its target CSF1 are significantly increased in MDA-231-LM3.3hCFL cells versus MDA-MB-231hCFL cells harboring a nontargeting control shRNA (Fig. 6B). We also generated MDA-231-LM3.3hCFL-1 and MDA-231-LM3.3hCFL-2 cells with stable knockdown of CSF1 mRNA using two different lentiviral shRNAs (Fig. 6B). We injected these cells into the mammary fat pads of SCID mice, and compared their tumor growth rates and metastases. SCID mice are devoid of functional T and B cells but still retain macrophages that could be activated by a T-cell–independent mechanism (41). The xenograft tumors derived from all four groups of cells became palpable within a week after injection and these tumors also grew comparably (data not shown). However, the average numbers of F4/80-positive macrophages in MDA-MB-231hCFL-1 and MDA-231-LM3.3hCFL-1 tumors were significantly less than the number of macrophages in MDA-231-LM3.3hCFL tumors (Fig. 6C). Accordingly, the extent of lung metastases derived from MDA-MB-231hCFL, MDA-231-LM3.3hCFL-1, and MDA-231-LM3.3hCFL-2 tumors was much smaller than that derived from MDA-231-LM3.3hCFL tumors.
tumors (Fig. 6D). These results suggest that the upregulated CSF1 expression in MDA-231-LM3.3 cells with NCOA1 overexpression mediates, at least in part, the NCOA1-promoted breast cancer metastasis.

The coupled high expression of both NCOA1 and CSF1 in human breast tumors positively correlates with lymph node metastasis, high tumor grade, and poor prognosis

To examine the expression association between NCOA1 and CSF1 in human breast cancer and to address the role of their coexpression in metastasis development, we examined the expression of NCOA1 and CSF1 in 453 breast tumors by IHC. In positively stained tumor cells, NCOA1 immunoreactivity is mainly located in the nucleus, whereas CSF1 immunoreactivity is distributed both intra- as well as extracellularly (Fig. 7A). Medium to high levels (3< scores ≤8, designated as high-expression groups) of NCOA1 and CSF1 proteins were detected in 56.3% and 58.5% tumor samples, respectively. Importantly, as many as 42.4% of tumors showed high expression of both NCOA1 and CSF1, but only 13.9% and 16.1% of tumors expressed high NCOA1 with low CSF1 (0≤ scores 3) and low NCOA1 with high CSF1, respectively. This indicates a positive association of NCOA1 expression with CSF1 expression in these breast tumors (Fig. 7B). Furthermore, only 16.1% (73/240) of lymph node–negative tumors showed high NCOA1 and CSF1 expression, whereas as many as 27.2% (123/213) of lymph node–positive tumors had high NCOA1 and CSF1 expression. However, node-positive status was not associated with either NCOA1 or CSF1 high expression alone (Fig. 7B). These results suggest that tumors with high-expression levels of both NCOA1 and CSF1 have greater metastatic potentials. Moreover, a significantly higher number of grade 3 tumors expressed both NCOA1 and CSF1 at higher levels versus grades 1 and 2 tumors, suggesting a positive correlation between high tumor grade and high NCOA1 and CSF1 expression (Fig. 7B). Finally, patients with high expression of both NCOA1 and CSF1 demonstrated a significantly worse disease-free survival than patients with low expression of both NCOA1 and CSF1 or with high NCOA1 expression alone. There were no significant differences in disease recurrence among other patient groups (Fig. 7C). These results suggest that the coupled overexpression of NCOA1 and CSF1 in breast cancer plays a crucial role in disease recurrence and, therefore, serves as a marker of poor prognosis.

Discussion

Recent studies have suggested many detrimental roles of altered expression of transcriptional coactivators in development of human diseases, including cancers (1, 42, 43). Thus, there is an urgent need to define the exact roles of coactivators
in carcinogenesis and their underlying molecular mechanisms using animal models and molecular approaches. In this study, we investigated mammary tumorigenesis and metastasis in Tg(NCOA1)×Tg(Neu) mice and RCAS-PyMT retrovirus–infected Tg(NCOA1)×Tg(TVA) mice with hNCOA1 overexpression in their MECs. NCOA1 overexpression itself did not affect MG morphogenesis in WT and Tg(NCOA1) mice. Nor did its overexpression affect oncogene-induced mammary tumor initiation and growth. Moreover, our previous study showed that NCOA1 knockout did not affect mammary tumor formation and growth in Tg(PyMT) mice (16). These results suggest that deregulated NCOA1 expression itself is not oncogenic, which is different from the oncogenic capability of overexpressed NCOA3 (9–11). Therefore, the p160 coactivator family members may have distinct contributions to breast cancer initiation and growth in addition to their possible redundant functions.

NCOA1 expression is low in normal human MECs, but high in metastatic breast cancer exhibiting early recurrence, resistance to endocrine therapy, and poor disease-free survival (15, 44). Our study further demonstrates that NCOA1 overexpression significantly promotes mammary tumor cell dissemination into the blood circulation, followed by a significantly increased incidence of lung metastasis in both Tg(NCOA1)×Tg(Neu) and Tg(NCOA1)×Tg(TVA)×RCAS-PyMT breast cancer mouse models, indicating NCOA1 overexpression indeed drives breast cancer metastasis in vivo. On the other hand, it has been shown that knockout of NCOA1 drastically reduced breast cancer metastasis in Tg(PyMT) and Tg(Neu) mice (16, 17). Together, these clinical and experimental findings suggest NCOA1 as a potential target for controlling breast cancer metastasis.

We found that NCOA1 overexpression in mouse mammary tumors and human breast cancer cells positively correlates with CSF1 expression, and NCOA1 knockout or knockdown in these cells reduces CSF1 expression. We further showed that both NCOA1 and c-Fos associate with a previously known AP-1 site (45), and this association activates the CSF1 promoter. These results clearly identified CSF1 as a direct novel target gene of NCOA1 and c-Jun/c-Fos in breast cancer cells. Recent studies have also demonstrated that NCOA1 serves as a coactivator for PEA3, c-Jun/c-Fos, Ets-2, and HOXC11 to upregulate Twist1, integrin α5 (ITGA5), c-Myc, and S100β expression, respectively, which in turn promotes breast cancer cell EMT, migration, invasion, and/or resistance to endocrine therapies (18, 19, 46, 47). Together, these findings indicate that NCOA1 can coactivate different TFs to regulate multiple target genes important for breast cancer.

The breast cancer cell–produced CSF1 plays a crucial role to stimulate cancer cell invasion and metastasis through both paracrine and autocrine pathways (48). The paracrine pathway consists of CSF1 from breast cancer cells, CAMs recruited by CSF1, EGF from CAMs, and EGF-stimulated breast cancer cell invasion and metastasis (28–31). The recruited CAMs can also induce immunosuppression against tumors to accelerate cancer progression (49), produce more urokinase-type plasminogen activator to augment tumor cell invasion (50), and secrete proangiogenic factors such as IL-6, VEGF, MCP-1, and TGFβ to stimulate angiogenesis (28). The autocrine pathway also stimulates breast cancer cell invasion and this is mediated by the CSF1 receptor, a transmembrane tyrosine kinase receptor in breast cancer cells (48). Our study further revealed an important role of...
NCOA1-upregulated CSF1 in promotion of breast cancer metastasis through its paracrine pathway. Specifically, we demonstrated that Tg(NCOA1)×Tg(Neu) (NCOA1×Neu) tumor cells, NCOA1, and Csfn siRNA-transfected Tg(NCOA1)×Tg(Neu) (NCOA1×Neu) tumor cells, and untransfected Tg(Neu) (Neu) tumor cells. Cells were treated with or without recombinant Csf1 (rCsf1) protein or Csf1 antibody (a-Csf1) as indicated. The assay was performed using a Transwell coculture system with the indicated tumor cells in the lower chambers and macrophages above a Matrigel layer in the upper chambers. The knockdown efficiency of NCOA1 and Csf1 mRNAs was evaluated by qPCR (Supplementary Fig. S4). B, qPCR measurement of relative NCOA1 and Csf1 mRNA levels in MDA-MB-231shCtrl, MDA-231-LM3.3shCtrl, MDA-231-LM3.3shCSF1-1, and/or MDA-231-LM3.3shCSF1-2 cells. These cells were generated by lentiviral expression of control shRNA (shCtrl) and Csf1 shRNAs (shCSF1-1 and shCSF1-2). The expression levels of these mRNAs were normalized to 18S rRNA. C and D, MDA-MB-231shCtrl, MDA-231-LM3.3shCtrl, MDA-231-LM3.3shCSF1-1, and MDA-231-LM3.3shCSF1-2 cells (2×10⁶) were injected into the fourth pair of mammary fat pads of SCID mice (n=5). Mice were sacrificed 30 days after injection. F4/80 immunostaining was performed to identify macrophages (brown) on the xenograft tumor (T) sections. F4/80-positive cells were counted as described in Fig. 2B. Data, mean±SD (C). Hematoxylin and eosin staining of nonadjacent lung sections was performed to determine the metastasis index, which was presented as the average ratio of lung metastasis (M) area to the total metastasis, and lung (L) area (D). *P<0.05 by the Student t test.

Figure 6. Knockdown of Csf1 blocks NCOA1-promoted macrophage recruitment in culture and in mice and inhibits metastasis. A, the numbers of macrophages recruited by nontargeting siRNA-transfected Tg(Neu) (Neu-siCtrl) and Tg(NCOA1)×Tg(Neu) (NCOA1×Neu-siCtrl) tumor cells, hNCOA1, and Csfn siRNA-transfected Tg(NCOA1)×Tg(Neu) (NCOA1×Neu-siNCOA1 and NCOA1×Neu-siCsf1) tumor cells, and untransfected Tg(Neu) (Neu) and Tg (NCOA1)×Tg(Neu) (NCOA1×Neu) tumor cells. Cells were treated with or without recombinant Csf1 (rCsf1) protein or Csf1 antibody (a-Csf1) as indicated. The assay was performed using a Transwell coculture system with the indicated tumor cells in the lower chambers and macrophages above a Matrigel layer in the upper chambers. The knockdown efficiency of hNCOA1 and hCsf1 mRNAs was evaluated by qPCR (Supplementary Fig. S4). B, qPCR measurement of relative NCOA1 and Csf1 mRNA levels in MDA-MB-231shCtrl, MDA-231-LM3.3shCtrl, MDA-231-LM3.3shCSF1-1, and/or MDA-231-LM3.3shCSF1-2 cells. These cells were generated by lentiviral expression of control shRNA (shCtrl) and Csf1 shRNAs (shCSF1-1 and shCSF1-2). The expression levels of these mRNAs were normalized to 18S rRNA. C and D, MDA-MB-231shCtrl, MDA-231-LM3.3shCtrl, MDA-231-LM3.3shCSF1-1, and MDA-231-LM3.3shCSF1-2 cells (2×10⁶) were injected into the fourth pair of mammary fat pads of SCID mice (n=5). Mice were sacrificed 30 days after injection. F4/80 immunostaining was performed to identify macrophages (brown) on the xenograft tumor (T) sections. F4/80-positive cells were counted as described in Fig. 2B. Data, mean±SD (C). Hematoxylin and eosin staining of nonadjacent lung sections was performed to determine the metastasis index, which was presented as the average ratio of lung metastasis (M) area to the total metastasis, and lung (L) area (D). *P<0.05 by the Student t test.
In human breast tumors, NCOA1 expression has been shown to be an independent marker for predicting disease recurrence and endocrine therapy resistance (15, 44). However, the expression and functional relationship between NCOA1 and CSF1 in human breast cancer has not been previously investigated. In this study, we identified a positive correlation between NCOA1 and CSF1 protein expression in human breast tumors. More importantly, we found this correlation is associated with lymph node metastasis, tumor grade, and recurrence. These results suggest that NCOA1-induced CSF1 expression in human breast cancer also plays an important role in driving breast cancer progression and metastasis. Therefore, targeting NCOA1 could inhibit CSF1 expression and CSF1 expression–induced breast cancer cell invasion and metastasis. Given that NCOA1 also upregulates other genes important for breast cancer cell survival, invasion, and metastasis, such as Twist1, ITGA5, c-Myc, and S100j1 (18, 19, 46, 47), NCOA1 may be a potential molecular target for inhibiting breast cancer progression and metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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