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Cysteine-rich 61-Connective Tissue Growth Factor-nephroblastoma-overexpressed 5 (CCN5)/ Wnt-1-induced Signaling Protein-2 (WISP-2) Regulates MicroRNA-10b via Hypoxia-inducible Factor-1α-*TWIST* Signaling Networks in Human Breast Cancer Cells[S]

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Background: Because CCN5 is an anti-invasive gene, present studies were designed to determine whether CCN5 exerts its anti-invasive function through controlling microRNA-10b expression.
Results: Up-regulation of TWIST1, a miR-10b activator, can be achieved by CCN5 silencing through the activation of HIF-1α JNK signaling.
Conclusion: CCN5 is a negative regulator of miR-10b in breast cancer cells.
Significance: The reactivation of CCN5 could be a unique therapeutic strategy for Triple negative breast cancer.

MicroRNAs (miRNAs) are naturally occurring single-stranded RNA molecules that post-transcriptionally regulate the expression of target mRNA transcripts. Many of these target mRNA transcripts are involved in regulating processes commonly altered during tumorigenesis and metastatic growth. These include cell proliferation, differentiation, apoptosis, migration, and invasion. Among the several miRNAs, miRNA-10b (miR-10b) expression is increased in metastatic breast cancer cells and positively regulates cell migration and invasion through the suppression of the homeobox D10 (HOXD10) tumor suppressor signaling pathway. In breast metastatic cells, miR-10b expression is enhanced by a transcription factor *TWIST1*. We find that miR-10b expression in breast cancer cells can be suppressed by CCN5, and this CCN5 effect is mediated through the inhibition of *TWIST1* expression. Moreover, CCN5-induced inhibition of *TWIST1* expression is mediated through the translational inhibition/modification of hypoxia-inducible factor-1α via impeding JNK signaling pathway. Collectively, these studies suggest a novel regulatory pathway exists through which CCN5 exerts its anti-invasive function. On the basis of these findings, it is plausible that reactivation of CCN5 in miR-10b-positive invasive/metastatic breast cancers alone or in combination with current therapeutic regimens could provide a unique, alternative strategy to existing breast cancer therapy.

Breast cancer is the most common malignancy and second most common cause of cancer death in women in the United States. It develops in one in seven women, impacting nearly every family in the world. Approximately 60% of these women will eventually develop an invasive form of the disease (1), which can be deadly and non-curable. Therefore, it is a major health issue for women. Accordingly, identification of the molecular signature(s) involved in the transition of breast cancer from a non-invasive to an invasive phenotype is a high priority in the management of the disease to develop better therapies for clinical testing.

CCN5,[2] which is also known as *WISP-2*, is a member of the CCN (cysteine-rich 61-connective tissue growth factor-nephroblastoma-overexpressed) family of growth factors (2, 3). It is a 29–31-kDa secreted protein and is structurally similar to other members of the CCN family, except the carboxyl-terminal (CT) domain, which is absent in this protein (3–5). *CCN5/WISP-2* is constitutively expressed in less aggressive human breast cancer cells (*i.e.* MCF-7 and ZR-75-1), whereas its expression is minimally detected in moderately aggressive breast cancer cell lines (*i.e.* SKBR-3), and it is undetected in the highly aggressive breast cancer cell line (*i.e.* MDA-MB-231) (6, 7). There is significant experimental evidence that CCN5 plays an anti-invasive role in breast carcinogenesis through controlling adhesion and cell motility (8, 9). Moreover, provocative studies have found CCN5 reverses epithelial-mesenchymal transition (EMT) processes as well as the gain-of-function of

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

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[2] The abbreviations used are: CCN5, cysteine-rich 61-connective tissue growth factor-nephroblastoma-overexpressed 5; CT, carboxyl-terminal; EMT, epithelial-mesenchymal transition; LCM, laser capture microdissection; DCIS, ductal carcinoma *in situ*; IC, invasive cancer; Dox, doxycycline; miR-10b, microRNA-10b.

mutant p53 (10), which are important in the invasion and metastatic growth of breast cancer cells (11–18). However, the mechanism by which CCN5 deficiency enhances the invasiveness of breast cancer cells has not been elucidated and remains poorly understood.

One critical challenge to understanding the anti-invasive activity of CCN5 is to identify the executing molecules regulated by CCN5 that promote invasion and/or metastasis. Over the last decade multiple studies have enhanced our understanding of the molecular taxonomy of cancers (19, 20). The studies have identified thousands of protein-coding transcripts (mRNA) that can be used to classify or grade different human cancers (21). Several non-coding microRNAs have been identified with both biological functions (22) as well as pathobiological functions including regulation of tumorigenesis in various organs through modulation of both oncogenic and tumor suppressor pathways (23–36). Recent studies have demonstrated microRNA-10b (miR-10b), which is highly expressed in metastatic breast cancer cells, can cause breast cancer cells to invade and metastasize (30, 37). MiR-10b is induced by a transcription factor, *TWIST*, a pro-metastatic gene (38), through direct binding to the putative promoter of miR-10b. *TWIST*-induced miR-10b inhibits translation of homeobox D10 (HOXD10), resulting in the increased expression of pro-metastatic gene RHOC (30, 39, 40). However, the upstream regulators of *TWIST* in breast cancer cells have not yet been detected. The hypoxia-inducible factor-1α (HIF-1α) transcription complex positively regulates the expression of *TWIST* by binding directly to the hypoxia response element in the *TWIST* proximal promoter region (41). Therefore, one could hypothesize that *HIF-1α* is a primary regulator of miR-10b in human breast cancer cells and this could be regulated through *TWIST*.

Considering this background information, our studies were designed to determine whether CCN5 exerts its anti-invasive function through controlling miR-10b expression. The experiments detailed herein found suppression of CCN5 in breast cancer cells augments miR-10b expression, which may trigger epithelial to mesenchymal transition and invasiveness by down-regulating epithelial markers. The studies further show induction of *TWIST* at the transcription level can be achieved by RNA interference (RNAi)-based silencing of CCN5, and this induction is mediated through the activation of *HIF-1α*. Based on these unique findings, we propose a model in which CCN5 signaling has a central role in non-invasive to invasive transition followed by metastasis.

EXPERIMENTAL PROCEDURES

Human Tumor Tissue Samples—Snap-frozen tumor tissues with different stages ($n = 25$) were obtained from the University of Kansas Medical Center Tissue Repository Bank. The study was approved by the Kansas City Veterans Affairs Medical Center Institutional Review Board.

Cell Lines and Culture Condition—Human breast tumor-derived endoplasmic reticulum (ER)-positive MCF-7, ZR-75-1, SKBR-3, and ER-negative MDA-MB-231 cells were purchased from American Type Culture Collections (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (Sigma) containing 10% FBS (Hyclone, Road Logan, UT) at 37 °C in a humidified chamber.

Laser Capture Microdissection—The laser capture microdissection (LCM) was carried out as described (8, 42). Briefly, paraffin-embedded breast tissue sections were stained using an RNase-free abbreviated Papanicolaou staining protocol and ductal carcinoma *in situ* (DCIS), and tumor cells were captured by an Arcturus AutoPixCell instrument and macroLCM caps (Arcturus, Mountain View, CA). DCIS ($n = 15$) and invasive cancer (IC) samples ($n = 15$) were microdissected. RNA was isolated from microdissected specimens using the Recover All Total Nucleic Acid Isolation kit (Ambion, Inc) as per the manufacturer's instructions. Briefly, microdissected cells were placed in digestion buffer and protease for 3 h at 50 °C. RNA was purified using a filter cartridge. Samples were treated with DNase to remove any genomic DNA, whereas RNA was bound to the filter. RNA was eluted with elution buffer. Isolated and purified RNA from these LCM specimens was reverse-transcribed to cDNA, and real-time PCR for CCN5 and miR-10b were performed according to the method described above.

Synthesis and Cloning of CCN5 in pSilencer Vectors—*CCN5/WISP-2*-specific short-hairpin RNA (shRNA) were designed and cloned into the pSilencer vectors according to our previous method (7, 43). RNA interference sequences for *CCN5/WISP-2* were previously described in detail (6, 7, 43) and are outlined in supplemental Fig. 1.

Transfection—For transient transfection experiments, breast tumor cells were transfected with shRNAs or expression vectors using Lipofectin reagent (Invitrogen) according to our previous method (7, 43, 44).

RNA Extraction and Real-time RT PCR—Total RNA extraction was essentially the same as previously described (7). Briefly, total RNA was extracted from different breast cancer cell lines using TRIzol (Invitrogen) as per the manufacturer's protocol. A Taqman microRNA reverse transcription kit was used to prepare cDNA from total RNA. Real-time PCR was performed from cDNA products using Taqman universal PCR and a Taqman microRNA assay kit by Applied Biosystem Step One real-time PCR system. PCR was performed for 15 s at 95 °C and 1 min at 60 °C for 40 cycles. CT values for CCN5 were determined as described earlier (8). CT values for miR-10b were normalized to control RNU6B by negating the average CT value for each sample. Relative quantification values for miR-10b in each sample were determined using the $2^{-\Delta\Delta CT}$ method (45). Each PCR reaction was performed in triplicate (mean ± S.D.).

Generation of Double Stable Tet-On MDA-MB-231 Cell Lines—MDA-MB-231 cells were transfected with the pTet-On plasmid. The transfected MDA-MB-231 cells (2×10^5 cells/ml) were grown in 25-cm^2 tissue culture dishes in the presence of 200 μg/ml G418 for the selection of pTet-On-positive cloned cells. After 4 weeks in selective culture medium, healthy and fast-growing G418-resistant cell colonies were isolated. The presence of rtTA was confirmed by Western blot analysis using VP16 polyclonal antibody (Clontech Laboratories Inc.). Colonies showing the highest expression were grown in complete media until 50–60% confluent. The cells were transfected with pTRE-pur-*ccn5*, and transfected cells were plated in 25-cm^2 culture dishes in complete media. After 48 h cells were exposed

to puromycin (2 μg/ml) for the selection of antibiotic resistant cells. After 4 weeks, the resistant colonies were tested for doxycycline (Dox)-regulated *ccn5* expression in the presence and absence of 2 μg/ml Dox using Northern blot analysis.

Mouse Xenograft Studies—All procedures involving animals followed National Institutes of Health guidelines and were approved by the Kansas City Veterans Affairs Medical Center Animal Research Committee. Athymic mice (nu/nu) ~8 weeks old were obtained from Charles River Laboratories and were maintained in a specific pathogen-free facility at Veterans Affairs Medical Center. Mice (n = 8/group) received a single subcutaneous injection with double-stable pTet-On MDA-MB-231/*ccn5* cells (2.5×10^6) resuspended in 0.1 ml of PBS and 0.1 ml of Matrigel. To induce tetracycline-response element-dependent WISP-2/CCN5 expression, Dox was given to the nude mice in their drinking water at a concentration of 2 mg/ml supplemented with 1% (wt:vol) sucrose and monitored for tumor growth. After 45 days, when tumors reached a volume of ~300 mm^3, mice were euthanized, and tumors were collected for RNA extraction. Tumor volume was calculated by formula $0.5 \times w^2 \times l$ (where w = width and l = length).

Western Immunoblot Analysis—The Western blot analysis was performed in the MCF-7 cell line with or without CCN5-shRNA-containing vector according to the previous method (46). Cell lysates were prepared by adding lysis buffer containing 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, and a protease inhibitor mixture including 1 μg/ml aprotinin, 1 μg of leupeptin, and 1.0 mM PMSF. Equal amounts of protein were loaded to 7.5–10% SDS-PAGE and blotted onto a nitrocellulose membrane. Membranes were incubated with specific primary antibodies overnight followed by secondary antibodies. Super Signal ULTRA Chemiluminescent Substrate (Pierce) quantitated the signals by using ID Image Analysis Software Version 3.6 (Eastman Kodak Co).

Immunohistochemistry—Protein expressions were evaluated by immunostaining using Zymed Laboratories Inc. (San Francisco, CA) broad range immunohistochemical kits as previously described (47). Briefly, after microwave treatment of deparaffinized sections in citrate buffer and endogenous peroxide blocking, the sections were incubated in specific antibody solution overnight at 4 °C in a moist chamber. The immunoreactivity was detected by conjugated streptavidin, and the sections were counterstained with hematoxylin.

Migration Assay—In vitro migration assays were performed as previously described (46). Briefly, mismatched shRNA, CCN5-shRNA, and CCN5-shRNA plus anti-miR-10b-transfected MCF-7 cells were seeded on a Transwell filter (8-μm pore size) insert (Boyden chamber) containing appropriate culture media. Cells adherent to the upper surface of the membranes, which had not migrated, were removed after 24 h, and cells that had migrated to the bottom surface were fixed with methanol, stained with Giemsa, and counted under a microscope.

Luciferase Activity—MCF-7 cells were seeded in 96-well tissue culture plates containing appropriate media. Confluent cells (~70%) were transfected with pSGG reporter plasmids containing *TWIST1* promoter (SwitchGear Genomics, Menlo Park, CA), empty promoter, endogenous pRL-TK promoter (Promega, Madison, WI), 1.0-U6 vector alone, and *ccn5/wisp-2* using FuGENE-HD (Promega) according to manufacturer's instructions. Cells were cultured for 48 h, and luciferase assay

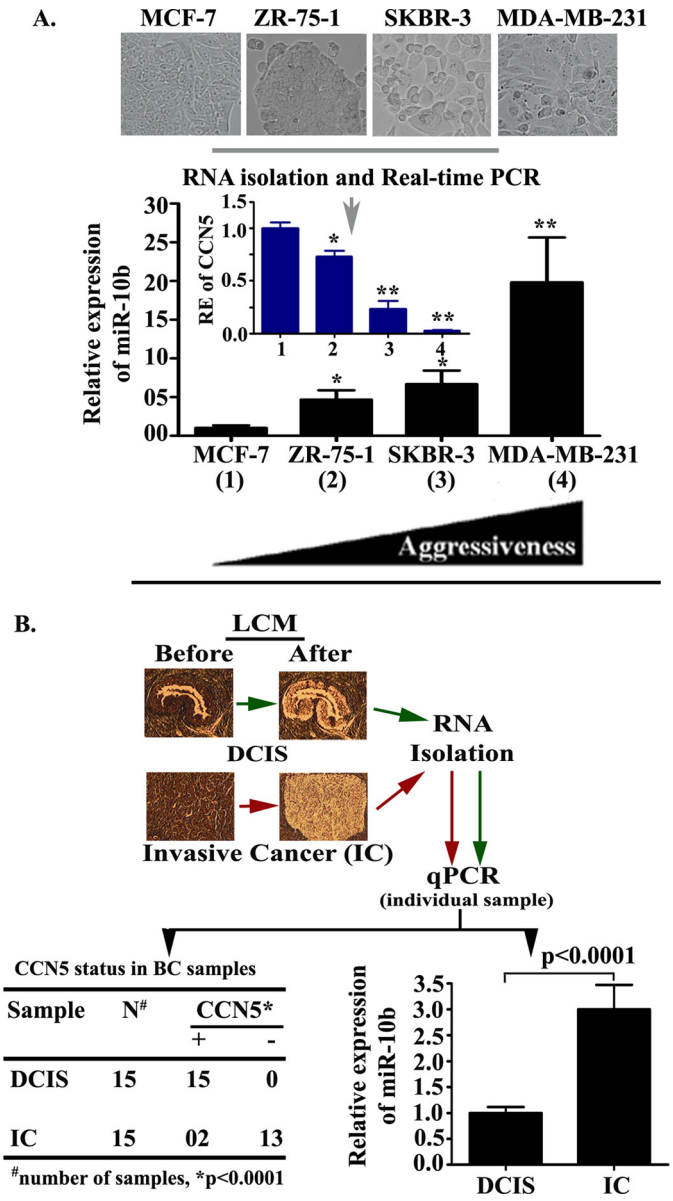

FIGURE 1. **Real-time quantitative PCR analysis of CCN5 and miR-10b in different breast cancer cell lines and tissues.** *A*, *upper panel*, microscopic images of different breast cancer cell lines show morphological differences of these cells. Total RNA was isolated from these breast cancer cell lines, cDNA was prepared, and quantitative RT-PCR analysis was performed (for detailed protocol see "Experimental Procedures"). *Lower panel*, shown is quantitative RT-PCR of miR-10b and CCN5 (*inset*) in different breast cancer cell lines. The *bar graph* shows Ct values normalized to the Ct values of GAPDH as -fold change compared with MCF-7 cell line-overexpressed CCN5. *Error bars* represent S.D. from the mean of triplicate reaction. *, $p < 0.05$ *versus* MCF-7; $p < 0.002$ *versus* MCF-7. *B*, real-time quantitative RT-PCR was performed on mRNA from LCM non-invasive (*i.e.* DCIS) and invasive breast cancer tissue samples. The *upper panel* shows representative images of LCM that used for quantitative RT-PCR (*qPCR*). The table represents the status of CCN5 in DCIS and IC samples. (+) indicates CCN5 mRNA expression was detected in the LCM samples, and (−) indicates CCN5 mRNA expression was undetected in the LCM samples. The *bar graph* shows Ct values on miR-10b normalized to the Ct values of GAPDH as a -fold change compared with DCIS. *Error bars* represent S.D. from the mean of 15 different reactions. *p* values are based on *t* tests or X^2 tests and linear regression analysis. + indicates CCN5 positive samples, and − indicates CCN5 negative samples.

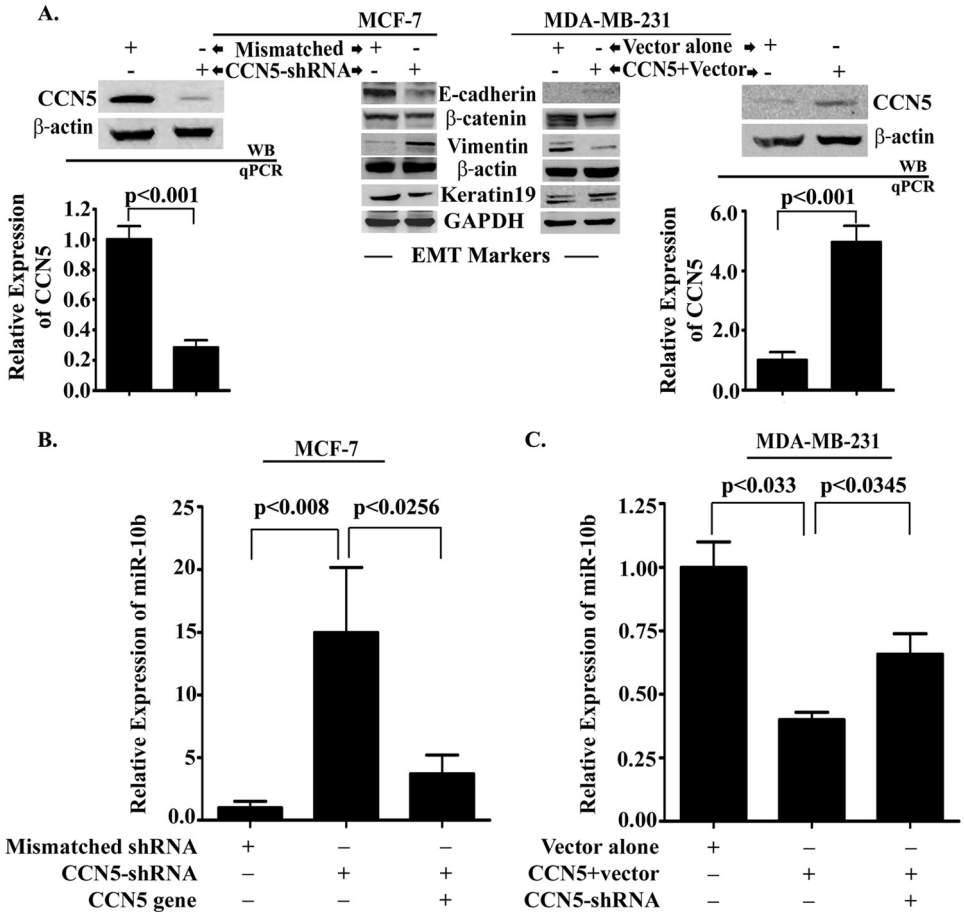

FIGURE 2. **Regulation of EMT markers and miR-10b by CCN5.** *A*, whole protein extracts of MCF-7 cells transfected with CCN5-shRNA or mismatched shRNA and MDA-MB-231 cells transfected with CCN5 gene or vector alone were analyzed via Western blot for EMT markers. Transfection efficiency of CCN5 was checked by Western blotting (*WB*) as well as real-time PCR (*qPCR*; *right* and *left panels*). The *histogram* indicates relative expression of CCN5 in MCF-7 and MDA-MB-231 cells under different experimental conditions. The *error bars* indicate S.D. of the mean of triplicate experiments. *p* values are based on *t* tests. *B* and *C*, total RNA was isolated from MCF-7 and MDA-MB-231 cells under the experimental conditions indicated, real-time quantitative RT-PCR was performed to evaluate the quantitative expression of miR-10b, and data are represented as *bar graphs*. The *error bars* indicate S.D. of the mean of triplicate experiments. *p* values are based on *t* tests.

was performed using DualGlo Luciferase assay system (Promega) in accordance with manufacturer's instructions.

Statistical Analysis—All data are expressed as the mean ± S.D. Statistically significant differences between groups were determined using χ^2 correlation coefficient simple linear regression and non-paired Student's two-tailed *t* test or Fisher's exact test and the Wilcoxon Rank-sum test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Expression of miR-10b Is Inversely Correlated with CCN5/WISP-2 Expression in Human Breast Tumor Cells and Tissue Samples—Previously, we reported that CCN5 is differentially expressed in breast tumor cell lines and human breast samples (2). Its expression is primarily detected in non-invasive breast cancer cell lines and non-invasive lesions (*i.e.* atypical ductal hyperplasia and DCIS) and almost undetectable in invasive cell lines and lesions (47). In these series of experiments the expression profile of miR-10b was initially evaluated in multiple breast tumor cell lines. These included MCF-7, ZR-75 (less aggressive and epithelial type), SKBR-3 (moderately aggressive), and MDA-MB-231 (highly aggressive and mesenchymal type). Among these cell lines CCN5 was highly expressed in MCF-7 and ZR-75-1 as compared with SKBR-3 and MDA-MB-231 where CCN5 expression was either absent or minimal (Fig. 1*A*). In contrast, miR-10b expression was significantly higher ($p < 0.001$) in MDA-MB-231 cells as compared with MCF-7 or ZR-75-1 cells (Fig. 1*A*). Together, this result indicates an inverse correlation between miR-10b and CCN5 expression in these cell lines.

We deduced that, if the *in vitro* data are biologically meaningful, this correlation should also exist in human breast tissues. Accordingly, a comparative study was performed between non-invasive breast cancer lesion (*i.e.* DCIS) and invasive breast cancer lesions. For this, total RNA was isolated from LCM samples of DCIS and invasive breast cancer tissues from multiple tissue samples ($n = 15$/category), and CCN5 and miR-10b expression were determined by real-time quantitative PCR using CCN5 or miR-10b-specific primers. All DCIS samples ($n = 15$) and two IC samples show high levels of CCN5 expression, whereas no or minimal CCN5 expression was detected in the other IC samples ($n = 13$) (Fig. 1*B*). Given the limited sample size and the small number of expected positives values, hypothesis testing is not

prudent. However, we can look at the distribution of our data, and we have two values of IC CCN5 that are at least 1 S.D. above the mean of the entire 15 observations, indicating potential positivity for CCN5 (supplemental Fig. 2). Although to identify a true cut point would require larger sample sizes and replication, the statistical analysis indicates that less than 1.4 (relative expression value of quantitative RT-PCR) can be considered the cutoff value for stating CCN5 negative (CCN5−). Therefore, present samples are divided into two categories; these are: 1) CCN5-positive (CCN+) samples and 2) CCN5 negative (CCN5−) samples. These findings are consistent with our previous immunohistochemical studies (8).

The expression of miR-10b was significantly higher in IC samples (CCN5−) as compared with DCIS samples (CCN5+) (Fig. 1*B*). The IC samples with detectable CCN5 expression showed markedly less miR-10b expression as compared with IC samples without detectable CCN5 expression (data not included in this study). The relative expression profiles of CCN5 and miR-10b demonstrate an inverse correlation between miR-10b expression and CCN5 expression in human breast tumor tissue samples ($p < 0.007$; χ^2 distribution correlation coefficient and simple linear regression analysis ($r = -0.99431$)).

CCN5/WISP-2 Is a Negative Regulator of miR-10b in Breast Cancer Cells—The objective of this series of experiments was to evaluate whether CCN5 regulates miR-10b expression in human breast cancer. To do so RNAi and CCN5-enforced ectopic expression strategies were used to evaluate the effect of CCN5 on miR-10b expression in different breast tumor cell lines. To begin, three different CCN5-directed shRNA sequences were identified with varying degrees of CCN5 expression silencing in MCF-7 breast cancer cells (supplemental Fig. 1). The shRNA (W2shRNA-3) had the greatest impact in knocking down CCN5 expression in MCF-7 cells (Fig. 2*A*, *left panel*) and was, therefore, used for subsequent studies.

To verify the functional impact of CCN5 on breast cancer cells, the effects of CCN5 ablation on cell proliferation/viability and EMT were assessed in MCF-7 and ZR-75-1 cell lines. With CCN5 depletion, the growth of these two cell lines was not significantly altered (supplemental Fig. 3). However, consistent with our previous work (8), CCN5 was found to regulate the EMT process. For example, depletion of CCN5 in MCF-7 cells (Fig. 2*A*, *left panel*) markedly enhanced the EMT process by inhibiting the expressions of epithelial markers (*i.e.* E-cadherin and keratin-19) and inducing mesenchymal marker (*i.e.* Vimentin) (Fig. 2*A*, *middle panel*), whereas enforced expression of CCN5 in MDA-MB-231 cells reversed the EMT event (Fig. 2*A*, *middle* and *right panels*).

After completing the studies confirming the roles of CCN5 in breast cancer cells, the effect of CCN5 on miR-10b expression was evaluated under tissue culture conditions. The *in vitro* studies showed markedly elevated miR-10b expression in CCN5 knock-out MCF-7 cells as compared with mismatched transfected cells, whereas miR-10b expression was decreased significantly in ectopically CCN5-overexpressed MDA-MB-231 cells (Fig. 2*B*). We noted that both effects could be rescued by either introduction of the CCN5 gene into the CCN5 knock-out MCF-7 cells or CCN5-specific shRNA (W2shRNA-3) into CCN5-overexpressed MDA-MB-231 cells (Fig. 2, *B* and *C*).

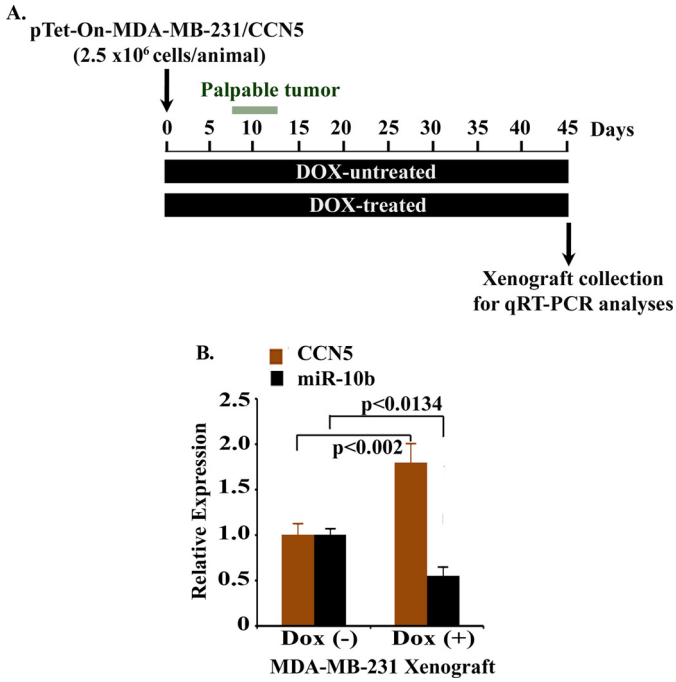

FIGURE 3. **Regulation of miR-10b by CCN5 in MDA-MB-231 xenograft in nude mice.** *A*, shown is a schematic outline of MDA-MB-231-xenograft tumor nude mice experiment in which CCN5 is regulated under the influence of Dox. Double stable Tet-On MDA-MB-231 (2×10^6) cells were injected into athymic mice ($n = 8$/group) for development of tumor. Dox was administered to induce TRE-dependent CCN5 expression. After 45 days, mice were sacrificed, and tumors were collected for RNA extraction. *B*, real-time quantitative RT-PCR (*qPCR*) analysis of CCN5 and miR-10b in Dox-treated and untreated tumors is shown. The *bar graph* shows Ct values normalized to the Ct values of GAPDH as -fold change compared with Dox-treated samples. The *error bars* indicate S.D. of the mean of eight mice data. *p* values are based on *t* tests.

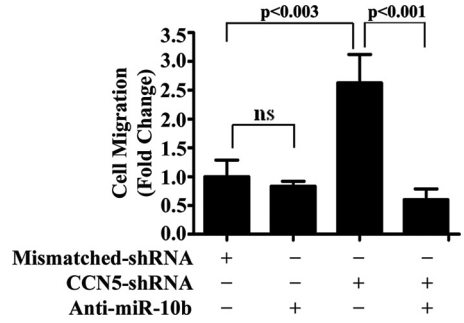

FIGURE 4. **CCN5-shRNA-induced migration of MCF-7 is blocked by anti-miR-10b.** The *histogram* shows the *in vitro* migration rate of MCF-7 cells transfected with mismatched shRNA, CCN5-shRNA, anti-miR-10b, or CCN5-shRNA and anti-miR-10b together. *Error bars* indicate S.D. of the mean of triplicate experiments. *p* values are based on *t* tests. *ns*, non-significant.

As a surrogate assay for determining the negative regulatory role of CCN5 on miR-10b expression in breast cancer cells, a MDA-MB-231 xenograft tumor was used in athymic nude mice model where CCN5 expression was conditionally up-regulated in xenograft tumors by Dox, a derivative of tetracycline. Similar to the *in vitro* results, in the *in vivo* studies Dox-induced activation of CCN5 in MDA-MB-231 tumors markedly reduced miR-10b expression compared with Dox untreated MDA-MB-231 xenograft tumors (Fig. 3). Together, these studies imply

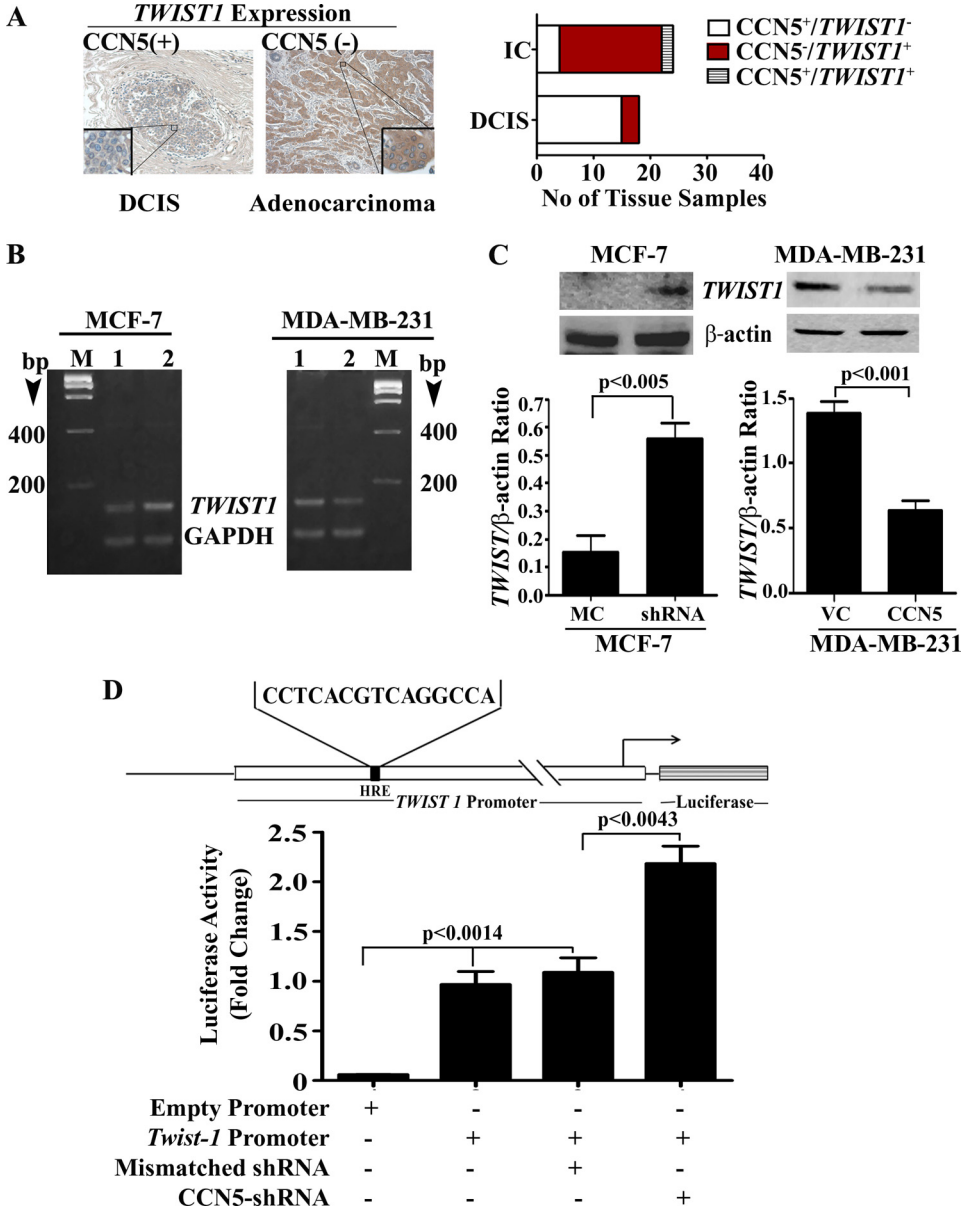

FIGURE 5. **CCN5 represses *TWIST1* in breast tumor cells.** *A*, shown are immunohistochemical analyses of *TWIST1* in DCIS and IC tissue samples (*left panel*). The *histogram* represents the status of CCN5 and *TWIST1* proteins in different breast cancer tissue samples (*right panel*). *B*, shown is RT-PCR analysis of *TWIST1* mRNA levels in MCF-7 cells transfected with mismatched-shRNA (*lane 1*) or CCN5-shRNA (*lane 2*) and in MDA-MB-231 cells transfected with pZsGreen1-C1 vector containing CCN5 gene (*lane 2*) or vector alone (*lane 1*). *M*, molecular markers; *1*, mismatched transfected MCF-7 cells or vector-transfected MDA-MB-231 cells; *2*, CCN5-shRNA-transfected MCF-7 cells or CCN5 transfected MDA-MB-231 cells. *C*, *TWIST1* protein levels in MCF-7 and MDA-MB-231 cells were evaluated under different experimental conditions by Western blot analysis using a specific antibody. *Histograms* show -fold changes of *TWIST1* expression in CCN5 positive and negative breast cancer cell lines. Expression is normalized to β-actin. *Error bars* indicate S.D. of the mean of triplicate experiments. *p* values are based on *t* tests. MC, mismatched control; *VC*, vector control. *D*, the *upper panel* shows the schematic representation of *TWIST1* promoter sequence. The *TWIST1* promoter region contains a consensus sequence of hypoxia response element (*HRE*), as shown by a *black box*. The *lower panel* represents the *histogram* of -fold change of *TWIST1* promoter activity under the experimental conditions indicated. *Error bars* indicate S.D. of the mean of triplicate experiments. *p* values are based on *t* tests.

that CCN5 is an upstream regulator of miR-10b expression in breast cancer cells.

CCN5 Ablation-induced Migration of MCF-7 Is Inhibited by Anti-miR-10b—EMT induces transition from an epithelial and non-motile morphology to a migratory phenotype with the ability to invade other tissues (48). Our present data (Fig. 2*A*) as well as previous studies have shown depletion of CCN5 in breast cancer cells enhances the EMT process and augments migration and invasion (8, 10). In contrast, miR-10b overexpression is directly linked with EMT and the process of invasion. Therefore, in this study we investigated whether the CCN5-induced inhibition of migration and invasion is mediated through down-regulation of miR-10b expression. To evaluate this mechanism, MCF-7 cells were transfected with pSilencer expression vector containing CCN5-shRNA or mismatched-shRNA or were co-transfected with pSilencer expression vectors containing CCN5-shRNA and anti-miR-10b, an inhibitor of miR-10b and anti-miR-10b alone. Transfected cells were seeded in a Transwell filter inserts (supplemental Fig. 4). After 24 h, migrated cells were stained and counted. We observed an approximate 3.9-fold increase in the migration toward serum of CCN5-shRNA-transfected MCF-7 cells com-

pared with mismatched-MCF-7 cells (Fig. 4). This induction, however, was completely abolished in anti-miR-10b-transfected cells (Fig. 4). The anti-miR-10 alone had no significant impact on the migration of MCF-7 cells. Finally, in this study we also tested if CCN5 is able to regulate miR-210 in MDA-MB-231 cells in which this microRNA plays a critical role in proliferation, migration, and invasion (49). We found miR-210 is an irrelevant microRNA species in the downstream of CCN5 signaling, as ectopic expression of CCN5 in MDA-MB-231 cells is incapable of regulation of miR-210 (data not shown).Together, these studies support the hypothesis that the CCN5 accomplishes its anti-migratory and anti-invasive functions through suppression of miR-10b expression in breast cancer cells.

Down-regulation of miR-10b by CCN5 Is Mediated through Blocking TWIST1 at the Transcription Level—The transcriptional activation of miR-10b is achieved by a prometastatic transcription factor *TWIST1* (30, 39, 50). This activation is essential for *TWIST1*-induced EMT that eventually promotes invasive phenotypes (50). The objective of this study was to evaluate whether CCN5 blocks *TWIST1* to prevent miR-10b expression and activity in breast cancer cells. To do so, the status of the *TWIST1* protein level was initially evaluated in CCN5-positive and CCN5-negative breast cancer tissue sections using immunohistochemical analysis (Fig. 5*A*). The studies showed that *TWIST1* protein level was significantly higher (computed X^2 value is considered significant, $p < 0.001$) in CCN5-negative sections.

Next, the *TWIST1* mRNA and protein expression profiles were evaluated in CCN5-depleted MCF-7 cells and CCN5 ectopically overexpressed MDA-MB-231 cells. The studies showed both mRNA and protein levels of *TWIST1* were significantly increased in CCN5-depleted MCF-7 cells as compared with mismatched-controlled MCF-7 cell but markedly decreased in CCN5-overexpressed MDA-MB-231 cells as compared with vector alone-transfected cells (Fig. 5, *B* and *C*).

The next series of experiments was designed to evaluate whether CCN5 loss enhances the transcriptional response of *TWIST1* under normal culture conditions. To assess this, shRNA targeting CCN5 or mismatched vectors were co-transfected with a reporter gene containing the *TWIST* promoter region linked to luciferase in MCF-7 cells. The studies showed the shRNA against CCN5 enhanced luciferase activity by 2-fold compared with mismatched shRNA or *Twist1* promoter-transfected MCF-7 cells (Fig. 5*D*). The same trend was obtained in ZR-75-1 cell line (data not shown).

Finally, we determined whether CCN5-mediated *TWIST1* regulation is required to maintain miR-10b expression and activity in breast cancer cells. To do so, we addressed the involvement of *TWIST1* in CCN5-mediated regulation of miR-10b by inhibiting *TWIST*1 up-regulation in the CCN5-depleted environment using *TWIST*1-RNAi. Silencing of CCN5 in MCF-7 cells significantly induced both *TWIST1* and miR-10b mRNA expression (Fig. 6, *lane 4*), and these inductions were markedly abolished by siRNA-based knockdown of *TWIST1* expression in MCF-7 cells but had no effect on CCN5-shRNA-induced inhibition of CCN5 (Fig. 6, *lane 5*). Lipofectin or control siRNA had no effect on *TWIST1* and miR-10b (Fig. 6, *lanes 1* and *2*). Thus, these studies suggest CCN5 represses miR-10b

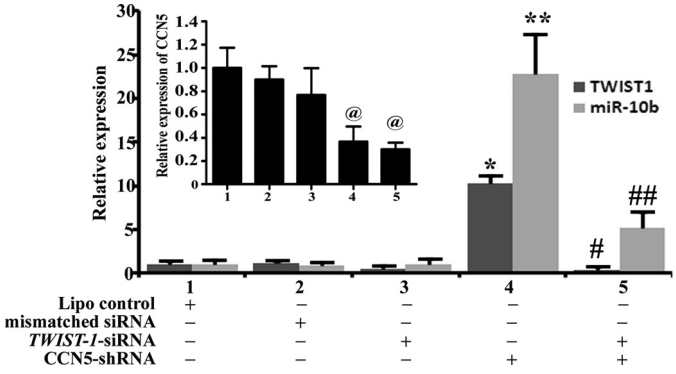

FIGURE 6. **CCN5-mediated *TWIST1* regulation is required to maintain miR-10b expression.** Real-time quantitative RT-PCR analysis of *TWIST* 1, miR-10b, and CCN5 (*inset*) in MCF-7 cells transfected with indicated RNAi is shown. The *bar graph* shows Ct values of *TWIST1*, miR-10b, or CCN5 normalized to the Ct values of GAPDH as a -fold change compared with mismatched-siRNA transfected control (*lane 2*). *Error bars* indicate S.D. of the mean of triplicate experiments. *p* values are based on *t* tests. *, $p < 0.01$ *versus* lipo control, **, $p < 0.001$ *versus* lipo control; #, $p < 0.005$ *versus* CCN5-shRNA transfected cells; ##, $p < 0.001$ *versus* CCN5-shRNA transfected cells; @, $p < 0.001$ *versus* lipo control.

expression in breast tumor cells through the inhibition of transcription of *TWIST1.*

CCN5 Is a Negative Regulator of HIF-1α in MCF-7 Cells—In light of the preceding data, we sought to identify the downstream molecule that participates in *TWIST* transcription regulation by the secretory protein CCN5. Because *HIF-1α* is a critical factor involved in regulating *TWIST* transcriptional activity in cancer cells (41), we assayed whether loss or gain of CCN5 activity impacted *HIF-1α* expression in breast cancer cells. Initially, the *HIF-1α* expression profile in different breast tumor cell lines was evaluated. *HIF-1α* expression was detected in both less aggressive (*i.e.* MCF-7 and ZR-75-1) and aggressive (MDA-MB-231) cell lines, but the expression was markedly higher in MDA-MB-231 cells (Fig. 7*A*). Next, we determined the impact of loss and gain of CCN5 on *HIF-1α* mRNA and protein levels in breast tumor cell lines. We found that when CCN5 expression was silenced in MCF-7 cells, *HIF-1α* increased significantly in these cells at the protein level (Fig. 7*C*), but it was unchanged at mRNA level (Figs. 7*B* and supplemental Fig. 5). This up-regulation in the protein level could be due to the stability or synthesis of the protein. Conversely, ectopic expression of CCN5 in MDA-MB-231 cells repressed *HIF-1α* at the protein level without affecting the expression of mRNA, as insignificant change was observed at mRNA level in CCN5-transfected MDA-MB-231 cells compared with vector-alone-transfected cells (Fig. 7, *B* and *C*). Together, these studies suggest that *HIF-1α* is a downstream effector of CCN5, and the up-regulation of HIF-1α protein by inactivation of CCN5 is probably due to the induction in the stability of this protein or overproduction of this protein. However, further studies are warranted.

To address whether activation of *HIF-1α* is required for miR-10b expression via *TWIST1* in CCN5 knock-out cells, *HIF-1α* expression was suppressed in shCCN5-MCF-7 cells by *HIF-1α* RNA*i*, and *TWIST1* expression was evaluated. CCN5 knock-out cells, which exhibited significant amounts of *TWIST1* mRNA expression (Fig. 7*D*), were unable to activate *TWIST1* in

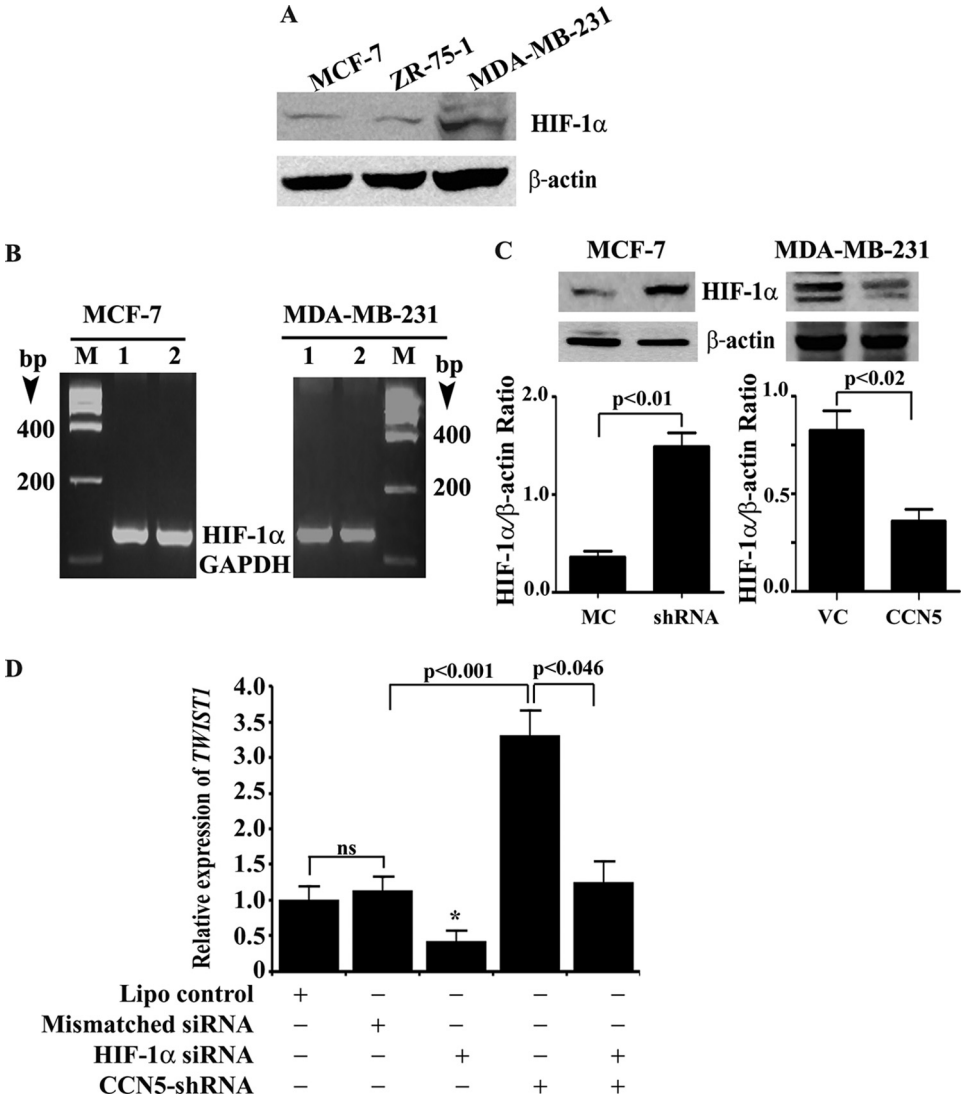

FIGURE 7. **CCN5 is a negative regulator of *HIF-1α* in breast cancer cells.** *A*, Western blot analysis of *HIF-1α* in MCF-7, ZR-75-1, and MDA-MB-231 cells. Expression was normalized to *β*-actin. *B*, RT-PCR analysis of *HIF-1α* mRNA levels in MCF-7 cells transfected with mismatched-shRNA (*lane 1*) or CCN5-shRNA (*lane 2*) and in MDA-MB-231 cells transfected with pZsGreen1-C1 vector containing CCN5 gene (*lane 2*) or vector alone (*lane 1*). *M*, molecular weight markers. *C*, Western blot analysis of *TWIST1* in MCF-7 (*left panel*) and MDA-MB-231 (*right panel*) cells under experimental conditions indicated were evaluated by Western immunoblot analysis using specific antibody. Protein expression is normalized to *β*-actin protein. *Error bars* indicate S.D. of the mean of triplicate experiments. *p* values are based on *t* tests. *MC*, mismatched control; *VC*, vector control. *D*, real-time quantitative RT-PCR analysis of *TWIST* 1 in MCF-7 cells transfected with indicated RNAi is shown. The *bar graph* shows Ct values of *TWIST1*, normalized to the Ct values of GAPDH as -fold change compared with mismatched siRNA-transfected control (*second lane*) *Error bars* indicate S.D. of the mean of triplicate experiments. *p* values are based on *t* tests.

an *HIF-1α* silenced environment (Fig. 7*D*, *fifth lane*). Thus, these results demonstrate *HIF-1α* is a prime CCN5 downstream component involved in regulating *TWIST-1*-miR-10b expression in breast cancer cells.

As CCN5 loss causes *HIF-1α* activation to activate *TWIST* followed by miR-10b, the next goal was to determine the possible mechanism of activation of *HIF-1α* expression in breast cancer cells. Previously, we found that CCN5 regulates multiple signal transduction pathways to regulate target genes (8). Therefore, we intended to investigate whether any signaling molecule was associated with CCN5 dependence. To achieve this goal, mismatched-MCF-7 and CCN5-shRNA-MCF-7 cells were grown to ~70% confluency. Cell lysates were analyzed for the activities and expressions of extracellular signal-regulated protein kinase 1 and 2 (ERK1/2), Jun-*N*-terminal kinase (JNK); p54/p46), and p38 by immuno-Western blot analyses using specific antibodies (Fig. 8*A*). Up-regulation of activated forms of ERK1/2 and JNK by silencing CCN5 was identified in MCF-7 cells because it reflected an increase in phosphorylation of ERK1/2 and JNK, whereas the phospho-p38 protein was absent in mismatched and CCN5-silenced MCF-7 cells (Fig. 8*A*). These studies indicated that in MCF-7 cells, CCN5 silencing activates MAPK pathways, specifically ERK1/2 and JNK pathways. This result persuaded us to define if ERK1/2 or JNK activation or both are required for the overexpression of HIF-1α in CCN5-silenced MCF-7 cells. To do so, CCN5-positive and -negative MCF-7 cells were treated with either 50 μM PD98059, a specific ERK1/2 inhibitor, or 20 μM SP600125, a specific inhibitor of JNK and HIF-1α and *TWIST1* expression was determined As indicated in Fig. 8*B*, the overexpression of *HIF-1α* as well as *TWIST 1* in CCN5-negative cells was inhibited by SP600125 but not by PD98059. Therefore, our results

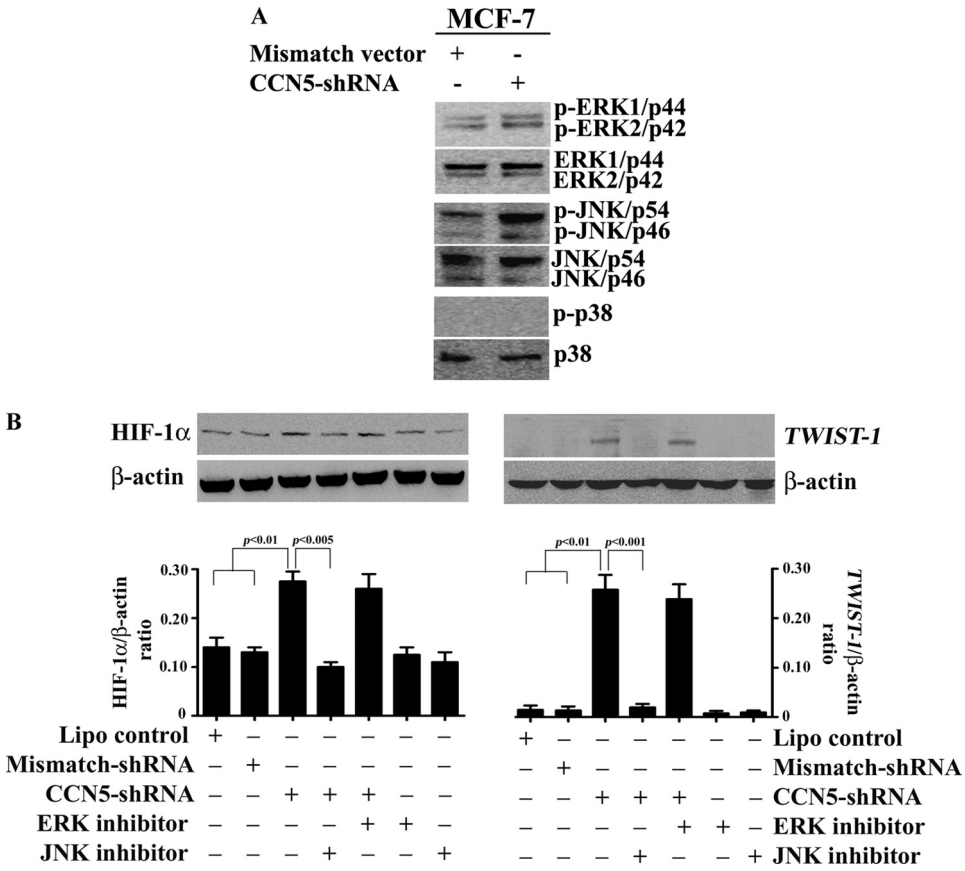

FIGURE 8. **CCN5-shRNA-induced activation of ERK1/2 and JNK in MCF-7 cells.** *A*, Western blot analysis of phosphorylated and nonphosphorylated forms of ERK1/2 (p44/p42), JNK (p54/p46), and p38 in MCF-7 cells transfected with CCN5-shRNA or mismatched shRNA is shown. *B*, Western blot analysis of HIF-1α and *TWIST1* in MCF-7 cells, which express CCN5-shRNA or mismatched-shRNA, were treated with ERK inhibitor or JNK inhibitor for 1 h. *Error bars* indicate S.D. of the mean of triplicate experiments. *p* values are based on *t* tests.

strongly suggest the JNK pathway may play a critical role in *HIF-1α* activation in CCN5-silenced cells.

DISCUSSION

The miR-10b, which was originally found to be down-regulated in a group of breast cancer samples (50), is known to overexpress in 50% or more metastatic breast cancer specimens (30, 50). The up-regulation of miR-10b in non-invasive breast cancer cells promoted EMT, migration, invasion, and metastatic growth of these cells under tissue culture and *in vivo* conditions (30, 39, 51). Moreover, therapeutic inhibition of miR10b blocks metastatic growth in mouse model (52). The present study reveals that miR-10b expression in breast cancer cells is regulated by extracellular protein CCN5/WISP-2. We found that CCN5/WISP-2 activation in breast cancer cells suppresses miR-10b expression through the inhibition of *HIF-1α-TWIST1* signaling cascades (Fig. 9) and thus supports the existing hypothesis that *ccn5/wisp-2* is an anti-invasive gene (8–10), at least in breast cancer cells.

In this study we show for the first time that CCN5 expression is inversely correlated with miR-10b expression in breast cancer cell lines and tissue samples (Fig. 1). We also show that CCN5, which reversed the EMT process in breast cancer cells (Fig. 2*A*) (8, 9) as well as in pancreatic cancer cells (43) and suppressed *in vitro* migration and invasion (8, 10), is able to modulate miR-10b expression in breast cancer cells under tissue culture and xenograft model (Figs. 2 and 3). Moreover, our data establish that CCN5-induced prevention of the accruing ability of the breast cancer cells to migrate, which is a hallmark

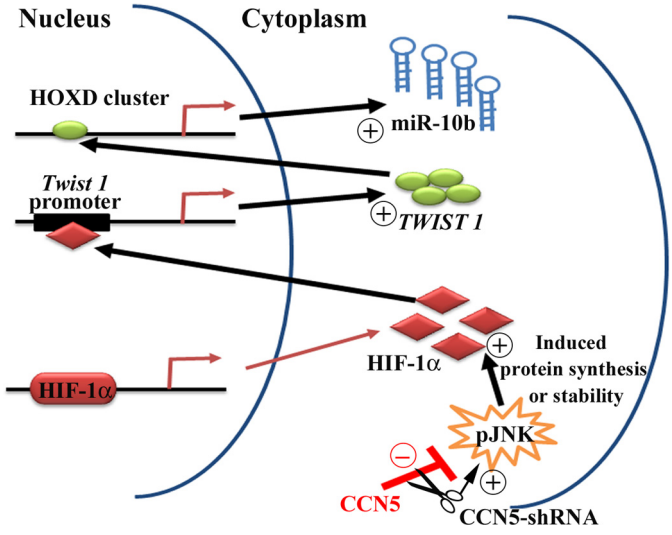

FIGURE 9. **Diagrammatic illustration of signaling pathway involved in the regulation of miR-10b by CCN5 in breast cancer cells.** Under CCN5-free microenvironment, phosphorylates JNK resulting in activation of JNK pathway inducing HIF-1α synthesis or stability, which in turn up-regulates *TWIST1*, resulting in up-regulation of miR-10b from HOXD cluster (30), promoting migration of breast cancer cells. All the cascades are absent or minimal in CCN5-positive breast cancer cells or may be reversed if CCN5 is activated in CCN5-negative breast cancer cells. (+) indicates positive effect, and (−) indicates negative effect.

of invasion and metastasis growth (53), is regulated by the suppression of miR-10b expression (Fig. 4).

Mechanistically, *TWIST1*, a pro-metastatic transcription factor, augments miR-10b transcriptional activity, which in turn activates Rho GTPase RHOC by inhibiting its transcriptional repressor homeobox protein D10 (HOXD10) (30, 50). This sequence of events is essential for *TWIST1*-induced EMT to promote migration and invasiveness of cancer cells (30, 50, 54). Notably, we uncovered CCN5/WISP-2 as a negative regulator of *TWIST1* in breast cancer cells. CCN5/WISP-2 inhibition in MCF-7 cells augmented *TWIST1* mRNA and protein levels, whereas CCN5/WISP-2-enforced ectopic expression in MDA-MB-231 cells abrogated *TWIST1* mRNA and protein levels (Fig. 5). CCN5-mediated suppression of *TWIST1* expression is achieved at the transcriptional level as *TWIST1* promoter activity was markedly elevated in CCN5 knock-out MCF-7 cells (Fig. 5*D*). Moreover, the current studies also showing miR-10b up-regulation in CCN5-silenced breast tumor cells are mediated through up-regulation of *TWIST1*, as removal of *TWIST1* expression *in vitro* by RNAi abolishes miR-10b expression in CCN5 knock-out breast cancer cells (Fig. 6). However, the molecular mechanism by which CCN5 suppresses transcriptional activity of *TWIST1* is unclear. Based on the molecular structure of CCN5 protein (3, 5), it would be premature to conclude, but one could speculate that CCN5 protein cannot appear to function as a dedicated transcriptional repressor. Therefore, we assumed that CCN5 might exert its repressor activity through an indirect pathway. Importantly, the subsequent studies support our proposition and indicate that the transcriptional repressor effect of CCN5 on *TWIST1* is exerted through the translational inhibition of *HIF-1α* (Fig. 7), a transcription factor already known for its role in transcriptional regulation of *TWIST1*. Additionally, these studies show that the regulation of *HIF-1α* by CCN5 is mediated through JNK signaling pathway.

Overall, the current studies unveil a molecular mechanism of regulation of miR-10b expression in breast carcinogenesis. By using a loss of function of CCN5 (inhibition of CCN5 expression) and a gain of function (enforced ectopic expression of CCN5) approach postulate that *CCN5* is a critical negative regulator of miR-10b expression in breast cancer cells, this could be performed through a key-signaling pathway involving *HIF-1α* through regulation of *TWIST1* (Fig. 9). On the basis of these novel observations, it is plausible that the reactivation of CCN5 into miR-10b-positive invasive/metastatic breast cancers alone or in combination of current therapeutic regimens may provide a unique alternative strategy to existing breast cancer therapy.

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