Excessive Hyaluronan Production Promotes Acquisition of Cancer Stem Cell Signatures through the Coordinated Regulation of Twist and the Transforming Growth Factor β (TGF-β)-Snail Signaling Axis**

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Background: Hyaluronan overproduction is implicated in breast cancer progression.
Results: Hyaluronan overproduction expands cancer stem-like cells through the up-regulation of Snail and Twist expression.
Conclusion: Hyaluronan overproduction allows plastic cancer cells to revert to stem cell states via Twist and the transforming growth factor β-Snail signaling axis.
Significance: These findings will help to understand the unique hyaluronan-dependent mechanisms that govern cancer cell plasticity and cancer stem cell expansion.

The cancer stem cell (CSC) model suggests that a small subpopulation of cancer cells possesses the ability to self-renew and give rise to malignant progeny that drive cancer progression. Recent reports have also proposed the existence of certain extracellular or intracellular signals that allow cancer progenitors to dynamically revert to a stem cell state. However, the mechanisms underlying cancer cell plasticity and CSC expansion are not entirely clear. Our previous studies using a hyaluronan synthase 2 (Has2) transgenic mouse model demonstrated that hyaluronan overproduction caused rapid development of aggressive breast carcinoma at a high incidence. Thus, we hypothesize that hyaluronan overproduction may accelerate cancer progression by expanding CSC subpopulations during cancer development. Primary cancer cells were established from mammary tumors developed in the transgenic mice and subjected to the Hoechst 33342 dye exclusion assay to sort side population (SP) from non-side population (non-SP) cells. Flow cytometric analysis demonstrated the enrichment of CD44high/CD24low CSC-like cells in the SP fraction of hyaluronan-overproducing cancer cells. This subpopulation exhibited several characteristics that were similar to CSCs, including cancer-initiating and mammosphere-forming abilities. Excess hyaluronan production drove the epithelial-to-mesenchymal transition process defined as the loss of epithelial phenotypes, up-regulation of transforming growth factor β (TGF-β), and induction of the epithelial-to-mesenchymal transition-related transcriptional factors Snail and Twist. Inhibition of TGF-β-Snail signaling or silencing of Twist expression abrogated the entrance into a stem cell state. Taken together, our findings suggest that hyaluronan overproduction allows plastic cancer cell populations to revert to stem cell states via Twist and the TGF-β-Snail signaling axis.

Cancer-initiating cells, also referred to as cancer stem cells (CSCs), are believed to drive cancer growth and progression through aberrant self-renewal and generation of heterogeneous cancer cell lineages (1, 2). The existence of CSCs was first demonstrated in solid tumors by Al-Hajj et al. (3), who identified these cells as a minor subpopulation of CD44high/CD24low lineage cells in breast cancer. They found that this subpopulation only was highly tumorigenic when injected into immunocompromised NOD/SCID mice, whereas the remaining bulk of cancer cells had no such ability. Although recent technological advancements and putative surface markers have enabled us to identify and characterize CSCs, the fundamental aspects of the mechanisms that govern the conversion of malignant cells into CSCs are still poorly understood for most types of cancers.

**The abbreviations used are: CSC, cancer stem cell; SP, side population; EMT, epithelial-to-mesenchymal transition; ECM, extracellular matrix; HA, hyaluronan; Ctg mice, conditional transgenic mice; qRT-PCR, quantitative RT-PCR; 4-OHT, 4-hydroxytamoxifen; DLT, delta Neo-like cell; MMTV, mouse mammary tumor virus; 4-MU, 4-methylumbelliferone; HMW, high molecular weight.
Increasing evidence has suggested that cancer cells undergoing epithelial-to-mesenchymal transition (EMT) acquire stem-like cell signatures, such as self-renewing ability (4, 5). EMT is a key biological process during embryonic morphogenesis in which cells undergo a developmental switch from a polarized epithelial phenotype to a mesenchymal phenotype (6). The onset of EMT is typically associated with the acquisition of spindle cell morphology in combination with the down-regulation of the epithelial marker E-cadherin. Recent studies have identified several transcriptional factors as capable of regulating this process. Among them, Twist and Snail have emerged as the most promising candidates of EMT "master genes" (7, 8). Microenvironmental signals provoke EMT as well, and transforming growth factor β (TGF-β), whose activities are dysregulated during malignant cancer progression, has also been shown to play an important role in EMT (9).

Like normal stem cells, CSCs rely on a specialized microenvironment called a CSC niche wherein they retain their exclusive abilities to self-renew and give rise to differentiated progenitor cells. The complex interplay between the cancer and host cells comprising the cancer microenvironment is orchestrated by a multitude of complex signaling networks that are mediated by cytokines, growth factors, and extracellular matrix (ECM). Accordingly, the CSC niche is believed to play a crucial role in controlling the molecular and biological CSC profiles, and its malignant alterations have been implicated in expansion of the CSC subpopulation and cancer propagation (10).

Cancer development and progression are often accompanied with extensive remodeling of the ECM in the cancer microenvironment. Hyaluronan (HA) is a major constituent of ECM whose increased deposition within cancers has been correlated with cancer aggressiveness and adverse clinical outcome in humans (11–14). HA biosynthesis, which is critical in establishing its biological function, is regulated by three mammalian HA synthases as follows: Has1, Has2, and Has3. Accumulating evidence has demonstrated the up-regulation of Has gene expression in aggressive and metastatic cancers (15, 16). Furthermore, our in vivo study using a conditional transgenic (cTg) mouse model allowing Has2 overexpression in breast cancer has demonstrated that HA overproduction by malignant cells caused rapid development of aggressive breast carcinoma at a high incidence (17). In Has2-overexpressing tumors, cancer cells acquired an EMT phenotype characterized by the down-regulation of E-cadherin and increased nuclear translocation of β-catenin. Given the potential importance of EMT in CSC conversion, it would be of particular interest to study whether HA overproduction gave rise to CSCs by inducing EMT in cancer cells. Here, we utilized Has2 cTg mice to elucidate the unique HA-dependent mechanisms that govern CSC conversion.

**EXPERIMENTAL PROCEDURES**

**Transgenic Mouse Lines and Establishment of Primary Breast Carcinoma Cells**—Has2 cTg breast cancer model mice were generated as described previously (17). Mouse mammary tumor virus-Neu (MMTV-Neu) model mice were obtained from Charles River Laboratories International, Inc. (Wilmington, MA). Animal care and experimentation were performed according to the study guidelines established by the Kyoto Sangyo University ethics committee for animal care, handling, and termination. Primary cancer cells were established from primary mammary tumors that had developed spontaneously in Has2 cTg and MMTV-Neu Tg mice as described previously (18).

**Isolation of Side Population (SP) and Non-side Population (non-SP) Cells**—Breast cancer cells were suspended at 1 × 10^6 cells/ml in DMEM containing 10% fetal bovine serum (FBS). Cells were incubated with 5 μg/ml Hoechst 33342 dye at 37 °C for 90 min in the dark. The specific ABCG2 transporter inhibitor, fumitremorgin C (Sigma), was added prior to the addition of Hoechst dye to inhibit dye efflux. Cells were washed, resuspended in cold PBS supplemented with 1% FBS at 1 × 10^6 cells/ml, and then filtered through cell strainers (70-μm pore size, BD Biosciences). Cell analysis and sorting were performed on a FACS Vantage cell sorter (BD Biosciences). The Hoechst dye was excited at a wavelength of 355 nm, and a fluorescence profile was generated for dual-wavelength analysis (450/50 and 675/20 nm).

**HA Measurement**—HA concentrations were determined by a competitive ELISA-like assays, as described previously (17).

**Flow Cytometric Analysis**—Breast carcinoma cells were suspended in cold PBS supplemented with 1% FBS, and 50 μl of cell suspension (5 × 10^5 cells) was incubated with phycoerythrin-conjugated anti-CD44 (1:160 dilution, eBioscience, San Diego) and FITC-conjugated anti-CD24 antibodies (1:50 dilution, eBioscience) for 60 min at 4 °C. The cells were washed, resuspended in cold PBS supplemented with 1% FBS, and then filtered through cell strainers. A total of 10,000 viable cells were analyzed with a FACS Calibur (BD Biosciences) and CellQuest software version 5.1.

**Quantitative RT-PCR (qRT-PCR)**—Total RNA from breast carcinoma cells was isolated using the Qiagen RNeasy mini kit (Qiagen, Germantown, MD), and cDNA was synthesized with the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. Quantitative real-time PCR for the murine Has2 gene was performed as described previously (17). TaqMan gene expression assays (Applied Biosystems, Foster City, CA) were used for the gene expression analyses of TGF-β (Mm03024053_m1), TNF-α (Mm00443260_g1), Snail (Mm00441533_g1), and Twist1 (Mm00442036_m1). The real-time PCR condition for gene expression was as follows: one cycle at 94 °C for 30 s; 40 cycles at 94 °C for 3 s, and 60 °C for 30 s. Relative mRNA expression was normalized using GAPDH expression.

**Immunofluorescence**—Breast carcinoma cells were seeded into a 4-well Nunc Lab-TekII Chamber Slide (5 × 10^4 cells/well, Thermo Scientific, Rockford, IL) and cultured for 3 days. The cells were fixed with 4% paraformaldehyde in PBS, followed by permeabilization with PBS containing 0.2% Triton X-100, blocked with PBS containing 3% BSA, and incubated with an anti-E-cadherin antibody (1:200 dilution, Cell Signaling Technology, Danvers, MA) in PBS containing 1% BSA for 60 min. The specimens were then incubated with Alexa Fluor 488 anti-rabbit IgG (1:500 dilution, Invitrogen) in PBS containing 1% BSA for 60 min. Each sample was mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing DAPI. Images were captured using a Leica TCS SPE confocal microscope (Leica Microsystems CMS GmbH, Jena, Germany).
Mammosphere Formation Assay—The mammosphere formation assay was performed as described previously (19). Briefly, aliquots of 5,000 cells were seeded into 24-well ultralow attachment plates (Corning Glass) and cultured in serum-free minimal essential medium/Ham’s F-12 supplemented with 20 ng/ml bFGF (Wako Pure Chemical Industries, Osaka, Japan), 20 ng/ml EGF (Miltenyi Biotec, Bergisch Gladbach, Germany), and B27 (Invitrogen). The treated cells were replenished with fresh medium every 3 days and cultured for 7 days. At the end of culturing, the number of spheres with a size of 75 μm or more was counted under a phase contrast microscope.

Tumorigenicity Assay—Has2^Neo and Has2^SP cells were suspended in Hanks’ balanced salt solution and injected subcutaneously into 6-week-old female BALB/c nude mice (n = 6 per group, CLEA Japan, Inc., Tokyo, Japan) at 10-fold serial dilutions from 1 × 10^5 to 1 × 10^2 cells. Tumor incidences were calculated 22 days after inoculation. Tumor diameters were measured every 3 days with digital calipers, and the tumor volume was calculated by the formula: Volume = (width)^2 × length/2.

Treatment of Cancer Cells with Exogenous HA Molecules—Control Has2^Neo and Has2^SP cells were seeded into 6-well plates (2 × 10^5 cells/well) and cultured for 24 h. The cells were treated with 100 μg/ml high molecular weight (HMW)-HA, HA oligosaccharides of a molecular size of 8 kDa, and tetrasaccharides for 7 and 30 days. After the treatment, the cells were analyzed for expression of EMT-related genes and CSC numbers.

Inhibitor Treatments—Has2^Neo parental and SP cells were seeded into 6-well plates (2 × 10^5 cells/well) and cultured for 2 days. The medium was replaced with DMEM containing 0.2% FBS, and the cells were treated with either 0–20 μM SB202190 (p38 MAPK inhibitor, Wako Pure Chemical Industries) for 30 min or 0–20 μM SB431542 (TGFB-β type I receptor (TGFB receptor inhibitor, Sigma) for 6 h. After the preparation of whole cell lysates, protein samples were analyzed for the expression and phosphorylation of Smad2 and p38 MAPK by Western blot analysis. For qRT-PCR and immunofluorescence, Has2^Neo parental and SP cells were treated with 10 μM SB431542 or SB202190 for 2 days. For flow cytometric analysis, Has2^Neo parental and SP cells were treated with 10 μM SB431542 or SB202190 for 7 days. Has2^Neo cells were treated with 20 μM GN25 (p53-Snail binding inhibitor, Calbiochem) for 2 days and analyzed for E-cadherin localization by immunofluorescence. qRT-PCR was performed to analyze Snail and Twist expression. For mammosphere formation assay, Has2^Neo cells were pretreated with 20 μM GN25 for 2 days and cultured for an additional 7 days. Has2^Neo cells were treated with 20 μM GN25 for 7 days and then analyzed for CD24 and CD44 expression by flow cytometry. Has2^Neo cells were treated with 4-methylumbelliferone (4-MU, Wako Pure Chemical Industries) at 300 or 600 μM for 7 days and analyzed for CD44 and CD24 expression by flow cytometry. For HA measurement, Has2^Neo cells were seeded into 96-well plates at 1 × 10^4 cells/well and cultured for 24 h. Cells were pretreated with 4-MU (300 or 600 μM) for 24 h to eliminate pre-existing HA and then cultured into fresh medium containing 4-MU for an additional 2 days. HA concentrations in the conditioned media were measured by a competitive ELISA-like assay.

Western Blot Analysis—Breast carcinoma cells were lysed in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) containing a protease and phosphatase inhibitor mixture (Nacalai Tesque, Osaka, Japan). Protein concentration was determined with a Pierce Microplate BCA protein assay kit (Thermo Scientific). Equal amounts of protein samples (2 μg) were then subjected to SDS-PAGE on 12% polyacrylamide gels. After protein transfer to PVDF membranes (Millipore, Billerica MA), the membranes were blocked with blocking-one solution (Nacalai Tesque) at 4 °C overnight, and then incubated with primary antibodies against phospho-Smad2, Smad2, phospho-p38 MAPK, or p38 MAPK (1:1000 dilution, Cell Signaling Technology) at room temperature for 60 min. Afterward, the membranes were washed with TBST (50 mM Tris-HCl, pH 7.4, 138 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20) and incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution, Cell Signaling Technology) for 60 min. The immunocomplexes were visualized with Western blotting detection reagents (Wako Pure Chemical Industries). Detection of chemiluminescent signals was performed on an ImageQuant LAS4000 Mini Luminescence Image Analyzer (GE Healthcare).

TGF-β and TNF-α Measurement—Breast carcinoma cells were seeded into 6-well plates (3 × 10^5 cells/well) and cultured for 24 h in DMEM supplemented with 10% FBS. The medium was then replaced with DMEM containing 0.2% FBS. Twenty-four hours later, the condition medium was collected, centrifuged at 13,000 rpm for 15 min to pellet debris, and stored at −80 °C. TGF-β and TNF-α were measured using mouse TGF-β and TNF-α FlowCytomix Simplex kits (eBioscience) according to the manufacturer’s instructions.

Viral Preparation and Transduction—Recombinant lentiviruses were produced by co-transfecting the Lenti-X 293T cell line (Takara Bio) with Twist-siRNA3 (Addgene plasmid 1784) which carries the shRNA of murine Twist, together with packaging constructs (Lentiviral Packaging Systems, Takara Bio) according to the manufacturer’s instruction. Control lentiviruses composed of nontargeting shRNA (Santa Cruz Biotechnology, Santa Cruz, CA) were used as a negative control. For viral infection, regular medium was replaced with culture medium containing 4 μg/ml Polybrene. Has2^Neo cells were then exposed to lentiviruses for 8 h and further cultured for 2 days. Stable clones were selected in the presence of 2.5 μg/ml puromycin dihydrochloride. Recombinant retrovirus particles were produced by co-transfecting a highly transfectable GP2-293 cell line with the MSCV CreERT2 puro (Addgene plasmid 22776) retroviral vector, together with packaging constructs (Retroviral Packaging Systems, Takara Bio) according to the manufacturer’s instruction. Has2^Neo parental and SP cells were then exposed to retroviruses for 8 h and further cultured for 2 days. Stable clones were selected in the presence of 2.5 μg/ml puromycin dihydrochloride.

Plasmid Transfection—Neu carcinoma cells were seeded into 6-well plates (2 × 10^5 cells/well) and cultured in DMEM containing 10% FBS for 24 h. Cells were transfected with the pEXneo, pEExneo mHas2, or pEExneo mHas2 (W354Y) plasmids, as described previously (20). Plasmid transfection was performed with FuGENE 6 transfection reagent (Promega, Madison, WI)
RESULTS

Expansion of CSC-like Cells in Has2-overexpressing Breast Carcinoma—We have previously established Has2 cTg mice that allow Cre recombinase-dependent Has2 overexpression (17). In Has2ΔNeo mice expressing Cre recombinase under the control of the MMTV promoter, deletion of the Neo cassette and subsequent Has2 overexpression were successfully achieved by Cre-mediated recombination of the transgene in spontaneous mammary tumors (Fig. 1A). In contrast, Has2 gene expression from the transgene was silent in control Has2+Neo transgenic mice due to a lack of Cre recombinase expression. We observed that HA production in mammary tumors was 6-fold greater in Has2ΔNeo mice than in control Has2+Neo mice. Our in vivo studies using these transgenic mice further demonstrated that HA overproduction by cancer cells caused rapid development of aggressive breast carcinoma (17). We therefore speculated that HA overproduction may expand CSCs during cancer development. To assess this notion, we isolated Has2-overexpressing Has2ΔNeo and control Has2+Neo cancer cells from primary mammary tumors (18, 21). The primary cultures of these cells were then examined for breast cancer initiating CD44high/CD24low subpopulations by flow cytometric analysis. The CD44high/CD24low subpopulation accounted for 30.4 and 0.9% of whole Has2ΔNeo and Has2+Neo cancer cells, respectively (Fig. 1B).

Like CD44high/CD24low cells, SP cells that have the unique capacity to efflux lipophlic fluorescein dyes have been shown to represent primitive CSC-like populations (22, 23). Tanaka et al. (24) have demonstrated that the CD44high/CD24low and SP cells partially overlap in human breast adenocarcinoma cells. Here, we isolated SP and non-SP from parental Has2ΔNeo and Has2+Neo cells using the Hoechst efflux assay to further characterize CSCs. SP cells were defined as cells that lost dye efflux ability after treatment with fumitremorgin C, a specific ABCG2 transporter inhibitor (Fig. 1C). The non-SP cells were isolated as a cell population that was strongly stained with the Hoechst dye. The proportion of the SP in Has2ΔNeo cancer cells was higher than that in Has2+Neo cancer cells (Fig. 1C). We next examined Has2 expression and HA production in these fractions. Both Has2ΔNeo SP and non-SP cells expressed higher levels of Has2 mRNA and produced a greater concentration of HA compared with control Has2+Neo SP and non-SP cells (Fig. 1D).

To define the CSC characteristics of SP cells, we examined the cell surface expression of CD44 and CD24. CD44high/CD24low cells were enriched in the SP fractions of both Has2ΔNeo and Has2+Neo cells as compared with non-SP fractions (Fig. 2A). Interestingly, the initially negligible proportion of CD44high/CD24low cells gradually increased after several passages in the non-SP fraction of Has2ΔNeo cells (Fig. 2B). Because phenotypic plasticity has been demonstrated to allow cancer cells to dynamically enter into stem cell states, our observations suggested high plasticity of Has2-overexpressing Has2ΔNeo cells. The hallmarks of breast CSCs are anchorage-independent growth and mammosphere formation under serum-free culture conditions. In line with this definition, the SP fraction of Has2ΔNeo cells was capable of forming numerous and large mammospheres, whereas the SP fraction of control Has2+Neo cells formed mammospheres with decreased efficiency (Fig. 2C). The non-SP fractions of both Has2ΔNeo and Has2+Neo cells could only form small mammospheres with low efficiency (Fig. 2C). We next evaluated the tumorigenicities of SP cells by limiting dilution transplantation. The tumorigenic ability of the Has2ΔNeo SP cells was higher than that of Has2+Neo SP cells, which was consistent with mammosphere-forming ability (Fig. 2D). Taken together, Has2-overexpressing Has2ΔNeo SP cells appeared to contain relatively higher proportions of functional CSCs.

Has2 Overexpression Induces EMT in Breast Carcinoma Cells—We previously observed that HA-overproducing cancer cells acquired an EMT phenotype as determined by the down-regulation of E-cadherin and increased nuclear translocation of β-catenin (17). Because EMT has been implicated in the generation of cancer cells with stem-like characteristics (4), we assessed the involvement of Has2-induced EMT processes in CSC expansion. The onset of EMT is typically associated with the acquisition of spindle cell morphology in combination with down-regulation of the epithelial marker E-cadherin. As such, SP and non-SP cancer cells were initially evaluated for morphology and the expression of this adhesion molecule. Has2ΔNeo SP and non-SP cells displayed a fibroblast-like morphology that was similar to the EMT phenotype, whereas control Has2+Neo SP and non-SP cells retained an epithelial morphology (Fig. 3A). A reduction in E-cadherin was evident at cell-cell boundaries of Has2ΔNeo SP and non-SP cells (Fig. 3B), which was consistent with our observations and other previous observations that Has2 overexpression resulted in the loss of epithelial phenotypes (17, 25). Transcriptional levels of EMT-related markers were then analyzed by qRT-PCR. The expression of Twist was 8- and 10-fold higher in Has2ΔNeo SP and non-SP cells, respectively, than that of Has2+Neo SP cells (Fig. 3C), suggesting a critical role of Has2 overexpression in Twist expression. Snail expression was up-regulated in the Has2ΔNeo SP cells compared with the non-SP cells (Fig. 3C). However, the low expression of Snail in the Has2ΔNeo non-SP gradually increased with cell passages (data not shown). This was in agreement with the altered proportions of CSCs (Fig. 2B) and was consistent with an earlier report demonstrating that Snail-induced EMT enhanced cellular plasticity in nontransformed MCF10A cells (26). Of the EMT-related transcriptional factors, Zeb1 expression was inversely correlated with EMT-related phenotypes, and Slug exhibited only negligible expression levels (data not shown), which indicated a minor involvement of these factors in the Has2-induced EMT process.

EMT can be induced or regulated by various growth factors, including TGF-β and TNF-α (27, 28). We have speculated that the beneficial effect of Has2-induced EMT may be mediated by such factors, and we therefore performed qRT-PCR and cytometric bead arrays to determine the transcriptional and translational levels of TGF-β and TNF-α. Forced expression of Has2
markedly increased the expression of TGF-β and TNF-α in both SP and non-SP fractions of Has2ΔNeo cells (Fig. 3D).

**Involvement of TGF-β/TNF-α Signaling Pathways in Has2-induced EMT**—We next focused our attention on the autocrine mechanisms of TGF-β and TNF-α as potential triggers for the EMT program. To determine whether these factors produced the same effects as Has2 overexpression on CSC conversion, Has2ΔNeo cells were treated with recombinant TGF-β and/or TNF-α, and the expressions of Snail and Twist were determined (Fig. 4A). TGF-β treatment stimulated the phosphory-
Smad2 in a time- and dose-dependent manner (Fig. 4B) and significantly induced the expression of Snail. Snail expression increased in a synergistic manner with TNF-α/H9251 (Fig. 4A). Conversely, although TNF-α/H9251 treatment stimulated the phosphorylation of p38 in time- and dose-dependent manner (Fig. 4B), it was not sufficient to stimulate Snail expression. These results were consistent with previous studies that demonstrated TGF-β/H9252 elicited EMT in a Smad-dependent manner, and TNF-α accelerated this process (29). The induction of Twist was not obvious after treatment with a combination of TGF-β and TNF-α (Fig. 4A), suggesting that Has2 overexpression transcriptionally up-regulated Twist expression, independently of these signaling molecules.

Because prolonged exposure of breast cancer cells to a combination of TGF-β and TNF-α has been shown to generate CSCs via EMT acceleration (30), Has2±Neo cells were treated with TGF-β and/or TNF-α for 30 days, and the expression profiles of CD24 and CD44 were determined by flow cytometric analysis (Fig. 4C). Prolonged treatment with TGF-β alone was sufficient for Snail up-regulation and the induction of EMT, but
it could not increase the number of CD44<sup>high</sup>/CD24<sup>low</sup> cells or Twist expression even in combination with TNF-α/H9251, which was consistent with an earlier observation that TGF-β/H9252 treatment minimally affects Twist expression (31, 32).

Requirement of the TGF-β and p38 MAPK Signaling Pathways for Has2-induced EMT and CSC Expansion—To determine whether the TGF-β and TNF-α signaling pathways played a role in Has2-induced EMT, we investigated the effects of SB431542, a pharmacological inhibitor of TGF-βRI, on Snail and Twist expression. In Has2<sup>ΔNeo</sup> SP cells, the phosphorylation of Smad2 was inhibited in a dose-dependent manner by SB431542 treatment (Fig. 5A). Because p38 MAPK is a major downstream element in the TNF-α signaling pathway, we treated Has2<sup>ΔNeo</sup> SP cells with SB202190, a p38 MAPK inhibitor, that markedly abrogated the phosphorylation of p38 (Fig. 5A). In Has2<sup>ΔNeo</sup> parental and SP cells, the inhibition of TGF-β and p38 MAPK signaling pathways significantly reduced Snail expression (Fig. 5B) and up-regulated E-cadherin (Fig. 5C), suggesting that the inhibition of these pathways could reverse the EMT phenotype.

To define the molecular basis of Has2-induced CSC expansion, we subsequently investigated whether the TGF-β and p38 MAPK signaling pathways were involved in the regulation of CSC features. Has2<sup>ΔNeo</sup> parental and SP cells were treated with TGF-βRI or p38 inhibitor for 7 days and subjected to flow cytometric analysis of CSC surface markers. In addition to the suppression of Snail expression, signaling inhibition of each pathway significantly reduced CD44<sup>high</sup>/CD24<sup>low</sup> subpopulations (Fig. 5D) and mammosphere formation (data not shown). These results indicated that Has2 overexpression expanded the CSC subpopulation via TGF-β and p38 MAPK signaling pathways.

Coordinated Roles of Snail and Twist in Has2-induced CSC Expansion—The transcriptional induction of two major EMT regulators, Snail and Twist, was observed in Has2-overexpressing cancer cells. These factors may coordinately stabilize the
transition of epithelial cells to a mesenchymal state. To explore this hypothesis, we first investigated whether Snail was necessary for EMT maintenance and CSC expansion. Treatment of Has2\(^{-Neo}\) cells with GN25, a p53-Snail binding inhibitor, reduced the CD44\(^{high}/\)CD24\(^{low}\) subpopulation and inhibited mammosphere formation (Fig. 6, A and B). As demonstrated by the expression profile of E-cadherin, Has2\(^{-Neo}\) cells reverted to an epithelial state by this treatment (Fig. 6C). qRT-PCR demonstrated that Snail expression was up-regulated by GN25 treatment (Fig. 6D), suggesting that it was controlled by feedback regulation.

To verify the role of Twist in EMT and CSC expansion, we silenced Twist gene expression in Has2\(^{-Neo}\) cells using RNA interference. Short hairpin RNA (shRNA) against murine Twist mRNA was introduced into Has2\(^{-Neo}\) cells using a lentiviral...
vector, and three stably transduced cell lines were established. As a negative control for the experiments, Has2^ΔNeo^ cells were infected with control lentiviral shRNA encoding a scrambled sequence that would not lead to the specific degradation of any cellular message. Parental and transduced cells were analyzed for levels of endogenous Twist mRNA by qRT-PCR. Twist

**Figure A**

SB431542 (TGF-βRI inhibitor) and SB202190 (p38 inhibitor)

| (μM) | 0 | 1 | 2.5 | 5 | 10 | 20 |
|------|---|---|-----|---|----|----|
| p-Smad2 | | | | | | |
| Smad2 | | | | | | |
| p-p38 | | | | | | |
| p38 | | | | | | |

**Figure B**

Relative Snai mRNA and Twist mRNA

**Figure C**

Hyaluronan Overproduction Promotes Cancer Stemness

**Figure D**

Control, TGF-βRI inhibitor, p38 inhibitor

**Figure E**

CD44^high^CD24^low^ cells (%)
knockdown decreased its expression by ∼70% as compared with Has2<sup>ΔNeo</sup> cells with control shRNA (Fig. 6G), which resulted in decreased expression of Snail and increased E-cadherin staining at cell-cell boundaries (Fig. 6, F and G). As evidenced by flow cytometric analysis, Twist knockdown markedly reduced the CD44<sup>high</sup>/CD24<sup>low</sup> subpopulation (Fig. 6E). Together with the above results, these findings supported our hypothesis that Snail and Twist potentiated EMT and CSC expansion in a coordinated fashion.

**Excess HA Production Induces EMT and Expands CSCs—**To ascertain the role of Has2 in the induction of EMT and CSC expansion, we established several stable cell lines constitutively expressing the Has2 transgene from primary Neu carcinoma cells that were derived from primary mammary tumors developed in the MMTV-Neu cancer model. Ectopic Has2 expression induced HA production in stable transfectants. Has2 #3 and #4 transfectants exhibiting moderate and high levels of HA production, respectively, were chosen for further experiments (Fig. 7A). Transfectants expressing the same plasmid vector alone or the Has2 mutant W354Y were also established as negative controls. The mutant Has2 gene has a single nucleotide substitution that causes the replacement of tryptophan at the 354th amino acid in the catalytic site and thereby lacks HA synthase activity of the gene product (Fig. 7A). Control mock and Has2 mutant transfectants displayed similar characteristics to parental cancer cells. The moderate HA production in Has2 #3 transfectants did not increase Snail and Twist expression (Fig. 7B). In agreement with the results obtained from Has2<sup>ΔNeo</sup> cells, however, HA overproduction in Has2 #4 transfectants increased Snail and Twist expression (Fig. 7B) and significantly reduced E-cadherin accumulation at cell-cell boundaries (Fig. 7C). The stable Has2 #4 transfectants additionally displayed CSC characteristics as defined by the generation of CD44<sup>high</sup>/CD24<sup>low</sup> subpopulations (Fig. 7D) and mammosphere formation (data not shown). It was therefore strongly implicated that HA overproduction induced EMT and potentiated cancer cells to enter a stem cell-like state.

To further validate that HA overproduction drives CSC expansion, Has2<sup>ΔNeo</sup> cells were infected with a retrovirus carrying the tamoxifen-dependent Cre recombinase (CreER<sup>T2</sup>). We selected several stable clones in which the Neo cassette of the Has2 transgene was deleted by CreER<sup>T2</sup>-mediated recombination in response to 4-hydroxytamoxifen (4-OHT) (Fig. 8A). We named these clones as delta Neo-like cells (DLCs). Neo cassette deletion elevated HA production to varying degrees in all DLC clones (Fig. 8B). In some but not all clones, Has2 overexpression increased the proportion of CD44<sup>high</sup>/CD24<sup>low</sup> subpopulations to a higher degree than in parental Has2<sup>ΔNeo</sup> and the 4-OHT-unadjusted control cells (CreER<sup>T2</sup> (4-OHT)) (Fig. 8C). Interestingly, the levels of HA production in the DLCs were nearly correlated with the number of CSCs (Fig. 8, B and C).

The above results suggested that HA overproduction aided in the CSC conversion of only pre-existing specialized cell populations that could be readily switched to a stem cell state. To test this idea, we examined whether forced Has2 expression in Has2<sup>ΔNeo</sup> SP cells easily shifted cancer cells from an epithelial to a mesenchymal phenotype and led to a substantial expansion of CSCs as compared with Has2<sup>ΔNeo</sup> parental cells. Has2<sup>ΔNeo</sup> SP cells were infected with a retrovirus carrying the CreER<sup>T2</sup> and then selected with the CreER<sup>T2</sup>-mediated deletion of the Neo cassette as an index (Fig. 9A). Neo cassette deletion elevated HA production in all clones (Fig. 9B). High HA production diminished E-cadherin expression and up-regulated EMT-related marker genes (Fig. 9, C and D). We next classified the Has2<sup>ΔNeo</sup> SP cell clones expressing CreER<sup>T2</sup> into CSC-enriched and poor groups based on their proportion of CSCs. After treatment with 4-OHT, the proportion of the CD44<sup>high</sup>/CD24<sup>low</sup> subpopulation was increased in the CSC-enriched clones (Cre #1, #2, #6, and #12) as well as in the poor clones (Cre #4 and #7) (Fig. 9E). Taken together, our findings clearly indicated that excess HA production promoted the CSC conversion of pre-existing specialized cell populations that readily switch to a stem cell state.

**Exogenous HA Does Not Affect EMT Phenotype or CSC Expansion—**To elucidate whether exogenous HA could induce EMT and CSCs, control Has2<sup>ΔNeo</sup> cells were cultured in the presence of HMW-HA and examined for morphology and EMT marker expression after 7 and 30 days. HMW-HA treatment failed to evoke transition from epithelial to mesenchymal cell morphology (data not shown). Exogenous HMW-HA also had no effect on the expression of Snail or Twist or on that of TGF-β or TNF-α (Fig. 10A). Flow cytometric analysis of CD44 and CD24 further demonstrated that HMW-HA treatment could not expand CD44<sup>high</sup>/CD24<sup>low</sup> cells, although prolonged treatment of 30 days increased the number of CD44<sup>low</sup>/CD24<sup>high</sup> cells (Fig. 10B). Similar results were obtained when Has2<sup>ΔNeo</sup> cells were treated with HA oligosaccharides of a molecular size of 8 kDa or with tetrasaccharides (data not shown). Thus, exogenous HA appeared to have no effect on EMT or CSC expansion.

**Inhibition of HA Production Suppressed CSC Expansion—**We tested the effect of 4-MU, an inhibitor of HA biosynthesis, to confirm that HA production is required for the CSC expansion in Has2<sup>ΔNeo</sup> cells. Because high doses of 4-MU

**FIGURE 5. Inhibition of TGF-β and p38 signaling pathways abrogated Has2-induced EMT and CSC expansion.** A, phosphorylation status of Smad2 and p38 after treatment with signaling inhibitors. Has2<sup>ΔNeo</sup> SP cells were treated with 0–20 μM SB431542, TGF-βR inhibitor, for 6 h or 0–20 μM SB202190, p38 inhibitor, for 30 min. Cell lysates were prepared and subjected to Western blot analysis for phospho-Smad2 and -p38. Total forms of Smad2 and p38 were used as internal controls. B, Snail and Twist expression after TGF-β/p38 signaling inhibition. Has2<sup>ΔNeo</sup> parental (open bar) and SP (solid bar) cells were treated with 10 μM SB431542 or SB202190 for 2 days, and qRT-PCR was performed to analyze Snail and Twist expression. Data represent the mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01 compared with untreated control cells. C, cellular localization of E-cadherin in Has2<sup>ΔNeo</sup> parental and SP cells after TGF-β/p38 signaling inhibition. Has2<sup>ΔNeo</sup> parental and SP cells were treated with 10 μM SB431542 or SB202190 for 2 days and then stained with DAPI (blue) and anti-E-cadherin antibody (green). Fluorescent images were captured under a confocal microscope. Scale bar, 25 μm. D, flow cytometric analysis of CD44<sup>high</sup>/CD24<sup>low</sup> subpopulation after TGF-β/p38 signaling inhibition. Has2<sup>ΔNeo</sup> parental and SP cells were treated with 10 μM SB431542 or SB202190 for 7 days and then analyzed for CD24 and CD44 expression by flow cytometry. Data represent the mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01 compared with untreated control cells.
markedly affected cell viability (data not shown), we used a moderate doses of 4-MU in our trials. 4-MU significantly reduced HA synthesis without reduction of Has2 gene expression (Fig. 11A). Notably, this inhibitor reduced the proportion of CD44high/CD24low and mammosphere formation in a dose-dependent manner (Fig. 11, B and C). These observations suggested that HA production is important for CSC induction.

FIGURE 6. Coordinated roles of Snail and Twist in Has2-induced CSC expansion. A, flow cytometric analysis of CD44high/CD24low subpopulations after Snail inhibition. Has2Neo cells were treated with 20 μM GN25, a p53-Snail binding inhibitor, for 7 days and then analyzed for CD44 and CD24 expression by flow cytometry. B, mammosphere formation of Has2Neo cells after Snail inhibition. Has2Neo cells were treated with 20 μM GN25 for 2 days, cultured for an additional 7 days, and then examined for mammosphere formation. Scale bar, 100 μm. Data represent the mean ± S.D. of three independent experiments. **, p < 0.01 compared with untreated control cells. C, cellular localization of E-cadherin (green) in the Has2 transfectants. Nuclei were stained with DAPI (blue). fluorescent images were captured under a confocal microscope. Scale bar, 25 μm. D, flow cytometric analysis of CD44high/CD24low subpopulation in Twist-silenced Has2Neo cells. Has2Neo cells were infected with a lentivirus carrying either Twist or control shRNA, and three stable clones (Si-Twist #1, #7, and #12) were then analyzed for CD44 and CD24 expression by flow cytometry. E and G, cellular localization of E-cadherin (F) and expression of Snail and Twist (G) were analyzed in Twist knockdown cell lines. Data represent the mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01 compared with control shRNA (Si-control) cells.

FIGURE 7. Forced Has2 expression in Neu breast carcinoma cells induces EMT and expands CSCs. A, Has2 expression and HA production in the Has2 transfectants. Neu breast carcinoma cells were transfected with expression plasmids carrying murine Has2 and its mutant (W354Y) cDNAs. Total RNAs from mock, Has2 (W354Y), Has2 #3, and Has2 #4 transfectants were subjected to qRT-PCR analysis. HA concentration was measured using conditioned media collected from transfected cultures by a competitive ELISA-like assay. Data represent the mean ± S.D. of three independent experiments. **, p < 0.01 compared with mock transfectants. B, expression of Snail and Twist in the Has2 transfectants. Data represent the mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01 compared with mock transfectants. C, cellular localization of E-cadherin in the Has2 transfectants. Scale bar, 25 μm.

Hyaluronan Overproduction Promotes Cancer Stemness
Hyaluronan Overproduction Promotes Cancer Stemness

![Figure 8](image)

**DISCUSSION**

This study shows that HA overproduction allows breast cancer cells to revert to a stem cell state through EMT. CD44\(^{high}\)/CD24\(^{low}\) CSC-like cells were enriched in HA-overproducing cells with an EMT phenotype, and inhibition of the TGF-\(\beta\)-Snail signaling axis as well as silencing of Twist impaired the enrichment of the CD44\(^{high}\)/CD24\(^{low}\) subpopulation.

The phenotypic plasticity of CSCs is dynamically regulated by intrinsic and extrinsic signals originating from the cancer microenvironment. TGF-\(\beta\), a major inducer of EMT, has an important role in the inter-conversion and equilibrium of CSCs and non-CSCs by regulating the EMT process (33). Indeed, we found that TGF-\(\beta\) was up-regulated in HA-overproducing cancer cells and that the inhibition of its signaling pathway significantly reduced EMT and CSC conversion. This was consistent with an earlier report showing that inhibition of the TGF-\(\beta\) signaling pathway reversed EMT and induced mesenchymal-to-epithelial transition differentiation in CD44-positive breast cancer cells (34). Prolonged exposure of breast cancer cells to a combination of TGF-\(\beta\) and TNF-\(\alpha\) has also been demonstrated to generate CSCs via EMT acceleration (30). Although TGF-\(\beta\) treatment alone was sufficient to up-regulate Snail transcription factor and induce EMT, it could not increase the number of CD44\(^{high}\)/CD24\(^{low}\) cells even in combination with TNF-\(\alpha\). This implies that full CSC conversion of plastic cancer cells requires additional stimuli that may be triggered by HA overproduction.

Excess HA production provoked potent up-regulation of Twist as well in our study. However, just as activation of the TGF-\(\beta\)-Snail axis could not drive conversion to a CSC phenotype, neither could Twist overexpression expand the CSC subpopulations (data not shown). Therefore, both Snail and Twist might induce whole processes in progressive stages of EMT and thereby CSC conversion, in a coordinated fashion; either the silencing of endogenous Twist or the inhibition of Snail function reversed mesenchymal Has2\(^{\Delta}\)Neo cells to a more epithelial phenotype and reduced CSC numbers. This was in agreement with evidence that Snail and Twist coordinated in promoting the mesenchymal transition of mammary epithelial cells (32).

Forced Has2 expression significantly enhanced TGF-\(\beta\) expression in both SP and non-SP cells. However, Snail was up-regulated in the Has2\(^{\Delta}\)Neo SP cells only. Thus, there was a discrepancy between TGF-\(\beta\) and Snail expressions in non-SP cells. It is likely that SP cells are more responsive to TGF-\(\beta\), based on an earlier report that the TGF-\(\beta\) response is greater in SP than non-SP cells (35). TGF-\(\beta\) is secreted by most cells as a biologically inactive complex, termed a large latent TGF-\(\beta\) complex and stored in the ECM. Proteinases such as plasmin catalyze the release of active TGF-\(\beta\) from the complex to initiate the signaling cascades required for many physiological functions. Non-SP cells may therefore have a lesser ability to activate the latent form of TGF-\(\beta\) complexes. Other possible mechanism to account for this difference is that antagonists secreted from non-SP cells suppress TGF-\(\beta\) signaling. For instance, retinoic acid has been reported to inhibit endogenous TGF-\(\beta\) signaling in the mesenchyme of primary lung buds in mice (36). Further investigation to address the details of TGF-\(\beta\) signaling regulation in non-SP cells is warranted.

Like normal stem cells, recent lines of evidence have indicated the existence of significant phenotypic plasticity that allows non-CSCs to acquire CSC traits in cancers (37, 38). **In vitro** induction of Has2 expression in control Has2\(^{\Delta}\)Neo cells resulted in the frequent occurrence of CD44\(^{high}\)/CD24\(^{low}\) subpopulations. Because some but not all of the Has2\(^{\Delta}\)Neo cells acquired CSC signatures when Has2 was overexpressed, it was of particular interest to us to examine whether forced Has2...
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A. 4-OHT

B. HA (ng/10^6 cells)

C. Relative Snail mRNA

D. Control 4-OHT

E. CD44 CD24

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expression in Has2\(^{+/Neo}\) SP cells easily shifted cancer cells from an epithelial to a mesenchymal phenotype and led to substantial expansion of CSCs as compared with Has2\(^{+/Neo}\) Neo parental cells. This study suggests that Has2 overexpression and HA overproduction may aid in the CSC conversion of pre-existing specialized cell populations that readily switch to a stem cell state. An alternative mechanism for CSC expansion is one of interconversion between different CSC compartments. Biddle et al. (39) demonstrated that self-renewing CSCs in squamous cell carcinoma contained two biologically distinct phenotypes as follows: a population of CSCs that had undergone EMT (EMT CSCs), and a separate population of CSCs that had retained their epithelial characteristics (non-EMT CSCs). Their experiments revealed that the EMT and non-EMT CSCs are switchable (39). We observed in this study that SP fractions of both Has2\(^{+/Neo}\) and Has2\(^{2\Delta Neo}\) Neo cells contained a significant number of CD44\(^{high}/CD24^{low}\) cells. However, these SP cells differed in their characteristics, whereas Has2\(^{+/Neo}\) SP cells restored epithelial phenotypes, Has2\(^{2\Delta Neo}\) Neo SP cells tended to display mesenchymal properties. Because EMT was induced by the forced expression of Has2 in epithelial Has2\(^{+/Neo}\) cells, it is plausible that HA acts as one of the key regulators for switching between the two phenotypic states.

Our cumulative observations suggest a central role of the HA and TGF-\(\beta\) network in controlling EMT-mediated CSC conversion. A previous study demonstrated that elevated HA production increased the invasive potential of tumor cells (40). The induction of HAS2 expression in HAS2\(^{+/Neo}\) Neo cells produced a cytoplasmic accumulation of CD44 and a loss of E-cadherin, which is characteristic of EMT. Furthermore, treatment with HA significantly increased the expression of EMT markers such as Snail and Twist, and the cellular localization of E-cadherin was altered.

**FIGURE 9.** Induction of Has2 expression in Has2\(^{+/Neo}\) SP cells promotes EMT and CSC conversion. A, PCR analysis of CreER\(^{2\Delta}\)-mediated genomic DNA recombination. Has2\(^{+/Neo}\) SP cells were infected with a retrovirus carrying the tamoxifen-dependent CreER\(^{2\Delta}\). Genomic DNA samples isolated from Has2\(^{+/Neo}\) SP cell clones expressing CreER\(^{2\Delta}\) were collected before (open bar) and after (solid bar) treatment with 4-OHT, and HA concentrations were measured by a competitive ELISA-like assay. Data represent the mean \(\pm\) S.D. of three independent experiments. **, \(p < 0.01\) compared with untreated control cells. B, cellular localization of E-cadherin after the induction of Has2 expression. Has2\(^{+/Neo}\) SP cell clones expressing CreER\(^{2\Delta}\) were immunostained with an anti-E-cadherin antibody (green) before and after treatment with 4-OHT. Nuclei were stained with DAPI (blue). Scale bar, 25 \(\mu\)m. C, flow cytometric analysis of CD24\(^{high}/CD44^{low}\) subpopulations after the induction of Has2 expression. Has2\(^{+/Neo}\) SP cell clones expressing CreER\(^{2\Delta}\) were analyzed before (open bar) and after (solid bar) treatment with 4-OHT for CD24 and CD44 expression by flow cytometry. Data represent the mean \(\pm\) S.D. of three independent experiments. **, \(p < 0.01\) compared with untreated control cells.

**FIGURE 10.** Exogenous HA does not induce EMT or CSC conversion. A, qRT-PCR analyses of EMT-related gene expression. Has2\(^{+/Neo}\) cells were cultured for 7 and 30 days in the presence (open bars) or absence (solid bars) of 100 \(\mu\)g/ml HMW-HA. Total RNA was isolated and analyzed for the expression of TGF-\(\beta\), TNF-\(\alpha\), Snail, and Twist by qRT-PCR. Data represent the mean \(\pm\) S.D. of three independent experiments. B, flow cytometric analysis of the CD24\(^{high}/CD24^{low}\) subpopulation. Has2\(^{+/Neo}\) cells were treated with (+ HA) or without (− HA) 100 \(\mu\)g/ml HMW-HA for 7 and 30 days and then analyzed for CD24 and CD44 expression by flow cytometry.
FIGURE 11. Inhibition of HA biosynthesis suppressed CSC expansion. A, HA production and Has2 expression in Has2<sup>Neo</sup> cells after treatment with 4-MU. Has2<sup>Neo</sup> cells were cultured in the presence of the indicated concentrations of 4-MU for 2 days, and the conditioned media were analyzed for HA concentration by a competitive ELISA-like assay. qRT-PCR was performed to evaluate Has2 gene expression. Data represent the mean ± S.D. of three independent experiments. **, p < 0.01 compared with untreated control cells.

B, flow cytometric analysis of CD44<sup>high</sup>/CD24<sup>low</sup> subpopulation after treatment with 4-MU. Has2<sup>Neo</sup> cells were treated with 4-MU (300 or 600 μM) for 7 days and then analyzed for CD24 and CD44 expression by flow cytometry. Data represent the mean ± S.D. of three independent experiments. **, p < 0.01 compared with untreated control cells.

C, mammosphere formation of Has2<sup>Neo</sup> cells after treatment with 4-MU. Has2<sup>Neo</sup> cells were treated with 4-MU (300 or 600 μM) for 7 days and then examined the mammosphere formation. Scale bar, 100 μm. Data represent the mean ± S.D. of three independent experiments. **, p < 0.01 compared with untreated control cells.
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As a major HA receptor, CD44 has been reported to play critical roles in EMT and stemness regulation by modulating signal transduction in both normal and cancer stem cells. Qu et al. (41) found that bone marrow-derived mesenchymal stem cells maintained their stemness by retaining HA on the cell surface by CD44. Ju et al. (42) also revealed that CD44 is required to maintain human colon cancer stemness; knockdown of CD44 expression reduced the colony forming ability and decreased the expression of Snail and Twist. Mima et al. (43) demonstrated that CD44s regulate TGF-β-mediated mesenchymal phenotype in hepatocellular carcinoma cells. Furthermore, the loss of CD44 expression abrogated TGF-β-induced vimentin expression and mesenchymal spindle-like morphology (43). In this study, however, the treatment of HA-overproducing Has2ΔNeo cells with neutralizing CD44 antibody did not abrogate EMT or CSC conversion (data not shown), suggesting the involvement of a CD44-independent pathway in the control of HA-induced EMT and CSC conversion.

Finally, because our Has2-overexpressing Has2ΔNeo cells were established from primary mammary tumors, we could not eliminate the possibility that cells that displayed EMT- and CSC-like phenotypes were predominantly enriched during in vivo tumor development. We have previously reported that the HA-rich tumor microenvironment enhanced the recruitment of tumor-associated macrophages and stromal cells to accelerate tumor progression (17, 21). Okuda et al. (44) have also recently found that highly metastatic breast CSCs up-regulated HAS2 expression to maintain self-renewal capability by interacting with tumor-associated macrophages. Thus, the recruitment of such cells in the HA-rich microenvironment may also play important roles in the promotion and maintenance of CSC properties.

In summary, our current observations strongly support the notion that excess HA production expands CSC subpopulations through the coordinated regulation of Twist and the TGF-β-Snail signaling axis (Fig. 12).

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