Co-overexpression of Janus kinase 2 and signal transducer and activator of transcription 5a promotes differentiation of mammary cancer cells through reversal of epithelial–mesenchymal transition

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Signal transducer and activator of transcription (Stat) 5 appears to play a vital role in prolactin (PRL)-induced cell differentiation and normal mammary gland development. We previously showed that PRL-activated Stat5a induced expression of E-cadherin–β-catenin complex *in vitro* and in xenotransplant tumors *in vivo* led to inhibition of breast cancer invasion. In the present study, we show that human breast cancer cells co-overexpressing Stat5a and its tyrosine kinase (Jak) 2 cultured in three-dimensional (3D) Matrigel culture demonstrate changes consistent with induction of mesenchymal–epithelial redifferentiation. Jak2 and Stat5a-co-overexpressing cells treated with cocktail (PRL, dexamethasone, and insulin), effectively reverse epithelial–mesenchymal transition by stimulating 3D organoids more reminiscent of the acinar growth of normal mammary epithelial cells, compared with cells overexpressing only Stat5a or Jak2. In contrast, dominant-negative dominant-negative-Stat5 blocks 3D organoid formation, causing cells to grow in layers instead. Hyperactivation of Jak2 and Stat5a in T-47D cells induces alveolar-like structures, mamospheres, with marked lumen formation through central apoptosis and restores a polarized epithelial phenotype. However, Jak2 and Stat5a overexpression in BT-20 cells induces partially differentiated 3D organoids with no central lumen, but effectively re-expresses estrogen receptor α. Jak2 and Stat5a-induced 3D differentiated organoids are accompanied by increased expression of E-cadherin, zonula occludens-1, and cytokeratins 8 and 18, and decreased levels of vimentin and Snail, indicating a shift from a mesenchymal phenotype toward an epithelial phenotype. Collectively, Jak2 and Stat5a co-overexpression cooperatively reverses epithelial–mesenchymal transition and promotes differentiation in human breast cancer cells, which may provide a mechanism to explain the invasive suppressor role of PRL-activated Stat5a in mammary cancer cells. (*Cancer Sci* 2008; 99: 272–279)

Signal transducer and activator of transcription (Stat) 5 is a hormonally responsive transcription factor of major importance for normal breast epithelial development and differentiation that was originally identified as a prolactin (PRL)-activated mammary gland transcription factor.[1] The PRL pathway is driven through Stat5[1,2] and the protein tyrosine kinase Janus kinase (Jak) 2, a key Stat5 tyrosine kinase in breast epithelial cells both during and outside of pregnancy and lactation.[2–6] The mechanism of activation of cytoplasmic Stat5 involves initial phosphorylation of a positionally conserved tyrosine residue by Jak2, an event followed by translocation of Stat5 to the cell nucleus where it binds as a dimer to specific regulatory DNA.[5] There are two highly (96%) homologous *Stat5a* and *Stat5b* genes that are both activated.[7,8] In mammary epithelial cells, the Stat5a–Stat5b heterodimer and Stat5a homodimers are thought to have essential roles in milk-protein gene regulation.[9,10] Although knockout studies in mice indicate that Stat5a is more critical for lactation at first pregnancy, Stat5b will compensate as a redundant gene product and restores lactation in *Stat5a*$^{-/-}$ mice on subsequent pregnancies.[11,12] Therefore, Stat5a and Stat5b are integral components of a PRL receptor–Jak2–Stat5 signaling pathway that meditates PRL-induced differentiation and lactogenesis in the mammary gland,[6,13] despite the existence of subtle and possibly important differences in their structure and regulation.[14,15]

Initial investigations have indicated a mammary tumor-promoting role of Stat5 in mice, which is consistent with the established mammary tumor-promoting role of PRL in rodents.[16,17] However, the significance of the role of the Jak2–Stat5 pathway for the effects of PRL in human breast carcinoma cells remains unclear. Recent findings suggest that other signal-transduction pathways may be involved in the mediation of PRL-induced biological responsiveness.[18] In addition to the PRL–Stat5 pathway, epidermal growth factor, estrogen, and progesterone are critical factors to the development and proliferation of mammary tissue. However, the progesterone receptor (PR) can synergize with activated Stat5 in the induction of transcription from the β-casein gene promoter.[19] These examples suggest that there may be interplay between these critical molecules and pathways in mammary differentiation.

In human breast cancer, using a specific phospho-Stat5 immunohistochemistry method, constitutive basal activation of Stat5a in healthy, non-pregnant human breast epithelia was detected[11] and inactivation or loss of Stat5a was correlated with metastatic progression of breast cancer.[20] Moreover, a decrease in Stat5a expression also has previously been demonstrated in atypical and malignant breast ductal epithelial cells.[21] Stat5a expression has been shown to be present in approximately 80% of normal breast cells and little or no expression has been seen in atypical ductal hyperplasia or invasive ductal carcinoma.[22] Furthermore, suppression of Jak2–Stat5 signaling in immortalized, near-normal breast epithelial cells led to a hyperproliferative phenotype characterized by increased mitotic rate, reduced apoptosis, and reduced contact inhibition.[23] Additionally, mammary cancer histological differentiation was positively correlated with nuclear levels of Stat5 protein.[24] We have recently uncovered novel evidence that the transcription factor Stat5a acts as a key suppressor of invasive characteristics of mammary cancer cells.[25] In a parallel study, blocking PRL autocrine function in T-47D cells, using pharmacological and genetic approaches, induced mesenchymal-like phenotypic changes and enhanced their invasive propensity.[26]

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The molecular mechanisms involved in epithelial to mesenchymal dedifferentiation are necessary for breast cancer invasion and metastasis, and Jak2 and Stat5a could play a vital role in these processes.

To understand a possible mechanistic regulatory role for Jak2 and Stat5a in epithelial–mesenchymal transition (EMT) and human breast cancer differentiation, we overexpressed Jak2 and Stat5a in BT-20 and T-47D breast cancer cells, using three-dimensional (3D) Matrigel culture *in vitro*. Overexpression of Jak2 plus Stat5a has been demonstrated to be synergic in promotion of mesenchymal to epithelial redifferentiation in human breast cancer that may work directly to suppress breast cancer cell dispersal and metastatic progression.

Materials and Methods

Cell culture. Breast carcinoma cell lines T-47D and BT-20 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 2 mM L-glutamine, and 50 IU/mL penicillin/streptomycin (Sigma, St Louis, MO, USA) at 37°C with 5% CO_2. Unless stated otherwise, subconfluent cells in 75-cm^2 flasks were washed with phosphate-buffered saline (PBS) and maintained in DMEM with 2% FBS for 24 h before culturing on extracellular matrix treated with or without cocktail (1 mM dexamethasone, 20 nM PRL, and 5 mg/mL insulin). Cell numbers were determined using a Beckman Coulter Counter (Fullerton, CA, USA).

Reagents. Human PRL (NIDDK-PRL-SIAFP-B2, AFP-2969 A) was kindly provided by Dr A. F. Parlow under the sponsorship of the National Hormone and Pituitary Program, National Institutes of Health, and the United States Department of Agriculture. Dexamethasone and insulin were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The bicinchoninic acid kit for protein detection was obtained from Pierce (Rockford, IL, USA).

Generation of adenoviral vectors for high-efficiency gene delivery of wild-type and forms of Stat5a, and wild-type Jak2. Details of the generation of adenoviruses carrying wild-type (Wt)-*Stat5a*, dominant-negative (Dn)-*Stat5*, and Wt-*Jak2* were as described previously.[25]

Three-dimensional Matrigel culture of mammary cancer cells. T-47D or BT-20 cells were either mock infected or infected for 90 min with adenovirus carrying Wt-*Jak2* at a multiplicity of infection (MOI) of 15 for T-47D cells or 20 for BT-20 cells, Wt-*Stat5a* or Dn-*Stat5* at a MOI of 25 for T-47D cells, Wt-*Stat5a* at a MOI of 40 for BT-20 cells, or a combination of Wt-*Jak2* and Wt-*Stat5a* at a MOI of 25 for T-47D cells or 40 for BT-20 cells. For the coinfection experiment cells were infected with Wt-*Jak2* and 24 h later, were infected with Wt-*Stat5a*. Subconfluent cultures were transferred into 24-well plates coated with 1.0 mm growth factor-reduced Matrigel (BD Biosciences, Bedford, MA, USA). They were further incubated in DMEM containing 2% FBS and 5% Matrigel and then treated with cocktail (dexamethasone, PRL, and insulin) or control vehicle. The medium was changed every 4 days by gently replacing part of the volume. Mock or adenovirus variants were reinfected on the eighth day of the experiments during the medium change. Fifteen-day culture is necessary to induce morphological changes, especially in undifferentiated BT-20 cells that need a longer incubation period than T-47D cells. After 15-day culture at 37°C, cells were fixed as indicated in 4% paraformaldehyde in PBS for 15 min. Cells were washed twice with PBS and morphological alterations were analyzed using phase-contrast microscopy (Nikon Stereoscope, Chuoku, Japan) at 200× magnification. Cells were collected in Histo-gel according to the recommended protocol (Richard-Allan Scientific, Kalamazoo, MI, USA), fixed in 10% formalin, dehydrated, embedded in paraffin, and sectioned. Non-specific sites were blocked with 5 mg/mL bovine serum albumin plus 1% goat serum in PBS and then subjected to further immunohistochemical analysis using the following antihuman monoclonal antibodies: E-cadherin, MABNCH-38; estrogen receptor (ER)-α, MAB-clone-ID5; or PR, MAB-PgR-6361 (Dako, Carpinteria, CA, USA). Central apoptotic luminal detection in 3D organoids was determined according to the recommended protocol using an Apo-BrdU-IHCTM *In Situ* DNA Fragmentation Assay Kit (catalog no. K403-50; BioVision, Mountain View, CA, USA). Cells were visualized and analyzed under a vanox microscope equipped with a Zeiss 40/0.8 NA objective lens (Thornwood, NY, USA).

Immunoprecipitation and immunoblotting. Immunoprecipitation and western blot analysis of cultured cells were carried out exactly as described previously.[22] The membranes were incubated overnight (4°C) in blocking buffer with specific mouse monoclonal antibodies against the following: 1 μg/mL human E-cadherin (G10; Santa Cruz Biotechnology, Santa Cruz, CA, USA); 0.1 μg/mL human ER-α (clone h-151; Stress General Biotechnologies, Victoria, BC, Canada); 0.4 μg/mL vimentin (Sigma-Aldrich); 10 μg/mL cytokeratin 8 (clone 4.1.18); 1 mg/mL cytokeratin 18 (clone RCK106) (Chemicon, Temecula, CA, USA); or 2 μg/mL anti-zonula occludens (ZO)-1 polyclonal antibody (Zymed Laboratories, San Francisco, CA, USA). Next, the membranes were washed and incubated with secondary antibody (goat antimouse immunoglobulin or goat antirabbit), conjugated to horseradish peroxidase and diluted 1:4000, for 1 h at room temperature and antibody binding was detected using ECL. When needed, blots were stripped in restore plus western blot stripping buffer (Pierce) before being reprobed.

Results

Co-overexpression of Jak2 and Stat5a promotes a phenotypic mesenchymal to epithelial shift and induces 3D differentiated organoids in human breast cancer cells. To explore whether Jak2 and Stat5 have regulatory roles in mesenchymal to epithelial redifferentiation and hence the metastatic potential of mammary cancer cells, BT-20 and T-47D cells were either mock infected or infected with adenovirus carrying Wt-*Jak2*, Wt-*Stat5a*, *Dn-Stat5*, or a combination of Wt-*Jak2* plus Wt-*Stat5a*, grown in 3D Matrigel culture, and treated with cocktail instead of PRL alone or control vehicle for 15 days. We also hypothesized that a 3D environment, a better model of the malignant phenotype that is most closely related to tumorigenicity *in vivo*, may be essential to assess the mechanism of Stat5a-based invasion suppression.[25] It is well established that the hormones PRL, hydrocortisone, and insulin cooperate in the regulation of milk-protein synthesis and differentiation of mammary explants and normal mammary epithelial lines.[27,28] Moreover, glucocorticoids facilitate PRL signaling via Stat5a directly at the level of the β-casein gene promoter, and the glucocorticoid receptor is a ligand-activated coactivator of Stat5 transcription factors.[29]

In BT-20 cells, which showed no active Jak2–Stat5a signaling, mock or Jak2-expressing cells with or without cocktail could not overcome its natural mesenchymal scattering phenotype, as confirmed by phase-contrast stereoscope and hematoxylin–eosin (HE) staining (Fig. 1a–d,a′,b′). Instead, cells grew as layers 1–2-cells thick. In contrast, cells overexpressing either Stat5a alone or Jak2 plus Stat5a grew as spherical organoids more reminiscent of the acinar growth of normal mammary epithelial cells, which was moderately but consistently enhanced by cocktail treatment (Fig. 1e–h,c′,d′). However, the 3D organoids were of heterogeneous size and did not form a lumen, indicating that the overexpression of Stat5a or Jak2 plus Stat5a induces only partial redifferentiation. The immunohistochemistry of E-cadherin showed induced localization of E-cadherin at cell–cell adherens junctions in the differentiated 3D organoids, which was sharply enhanced in Jak2

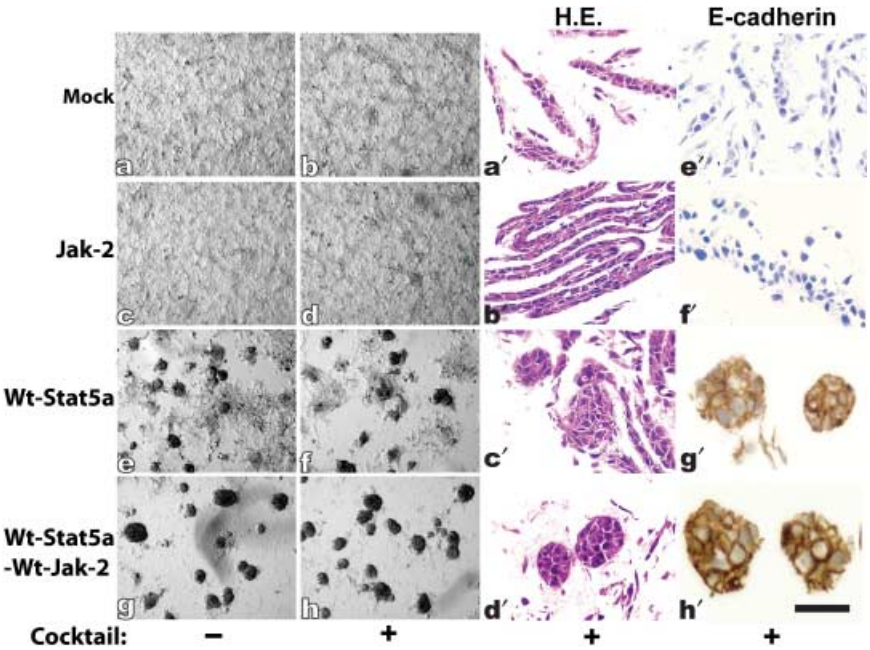

Fig. 1. Co-overexpression of Janus kinase (Jak) 2 and signal transducer and activator of transcription (Stat) 5a signaling reverses the mesenchymal phenotype of BT-20 cells in 3-dimensional (3D) Matrigel culture. BT-20 cells were either mock infected or infected with adenovirus carrying wild-type (Wt)-*Jak2*, Wt-*Stat5a*, or a combination of Wt-*Jak2* and Wt-*Stat5a*. After 24 h, cells were treated with cocktail or control vehicle for 15 days in 3D Matrigel culture, fixed, and then morphological alterations were analyzed using phase-contrast microscopy at 200× magnification (a–h). Matrigel-cultured cells were collected in Histo-gel and paraffin-embedded sections, further subjected to hematoxylin–eosin (HE) staining (a′–d′), or immunohistochemical analysis for E-cadherin localization, using specific E-cadherin monoclonal antibody (e′–h′). Morphological alterations were visualized and photographed (scale bar = 100 μm). Representative data from three independent experiments are shown.

and Stat5a-co-overexpressing cells treated with cocktail compared with Stat5a-expressing cells (Fig. 1g′,h′), indicating that both Jak2 and Stat5a are required to induce partial 3D differentiated organoids in BT-20 cells.

Jak2 plus Stat5a overexpression induces luminal differentiation in T-47D breast cancer cells. Similarly, systematic activation of either Stat5a alone or Jak2 plus Stat5a induces alveolar-like structures, mamospheres, in T-47D cells, but not in mock or Jak2-expressing cells with or without cocktail, as confirmed by HE staining (Fig. 2A,a–i,a′–e′). Mamosphere-like structures were markedly induced in response to overexpression of Jak2 and Stat5a without the effect of cocktail compared with Stat5a alone, and this induction was enhanced by cocktail (Fig. 2A,g,i). Although in BT-20 cells 3D organoids displayed only limited or no lumen formation, marked lumen formation through central apoptosis was observed consistently by HE staining in Jak2 and Stat5a-co-overexpressing T-47D cells (Fig. 2A,d′,e′). Moreover, HE staining of T-47D cells showed polarized columnar cells with basally localized nuclei, and establishment of a single layer of differentiated cells with a secretory phenotype compared with mock or Jak2-expressing cells with or without cocktail (Fig. 2A,a′,c′,B, upper-panel). Establishment of an apoptotic lumen in T-47D cells was indicated by central brown staining and a methyl green-counterstained single layer of cells, using the *in situ* DNA fragmentation method (Fig. 2C). In addition, Stat5a alone with or without cocktail failed to show a clear apoptotic lumen in T-47D cells, which in turn reflects the synergistic effect of Jak2 and Stat5a on central differentiated apoptotic lumen formation (Fig. 2A,d′,e′,b). In contrast, Dn-Stat5 blocked luminal 3D organoid formation even with cocktail treatment; instead, cells grew as layers (Fig. 2A,c,d,b′), indicating that Stat5a is involved in the process of luminal differentiation of T-47D cells. In parallel sections of paraffin-embedded cells of 3D organoids overexpressing Jak2 plus Stat5a, immunohistochemistry of E-cadherin revealed that E-cadherin was enhanced and localized to the cellular junctions in T-47D cells (Fig. 2B, lower panel). In contrast, mock and Jak2-expressing T-47D cells showed a more diffuse pattern of E-cadherin localization, as confirmed previously.[25] Together, overexpression of Jak2 plus Stat5a is necessary to promote a central differentiated apoptotic lumen that was associated with E-cadherin localization to the cellular junctions in breast cancer cells.

Overexpression of Jak2 and Stat5a re-expresses ER-α in ER-negative BT-20 cells. Estrogen receptor-α is a crucial growth-regulatory gene in breast cancer. Its expression is critical for tumor progression and prediction in response to hormonal therapies such as tamoxifen. To investigate whether reconstitution of Jak2–Stat5a signaling in differentiated 3D organoids of BT-20 cells could re-express ER-α or PR, Jak2 and Stat5a co-overexpressing cells were fixed and embedded in paraffin blocks for ER-α and PR immunohistochemical staining. The 3D organoids of Jak2 plus Stat5a-overexpressing cells treated with cocktail showed brown staining of re-expressed ER-α in the nuclei of BT-20 cells (Fig. 3A). No nuclear staining was detected in mock-, PR-, or IgG-stained cells (Fig. 3a). Consistent with the immunohistochemical data, immunoprecipitation and western blot analysis of protein extracts showed a distinct ER-α band at ~65 kDa in cocktail-treated 3D organoids overexpressing Jak2 and Stat5a, with no sign of ER-α induction in mock, Jak2-, or Stat5a-expressing cells (Fig. 3B). Collectively, reconstituted Jak2–Stat5a signaling led to robust ER-α expression in BT-20 cells, which in turn showed a marked positive response to tamoxifen treatment in *in vivo* experiments (data not shown).

Co-overexpression of Jak2 and Stat5a promotes mesenchymal to epithelial redifferentiation. Epithelial–mesenchymal transition has been defined as a three-part process in which cells acquire a fibroblast-like morphology and downregulate epithelial marker proteins such as E-cadherin, ZO-1, and cytokeratins k8 and k18 while simultaneously expressing mesenchymal proteins such as vimentin.[30–32] A key element of EMT is loss of homotypic adhesion and tight-junction components, E-cadherin and ZO-1, respectively. In breast cancer, ZO-1 is usually co-expressed with E-cadherin and is a strong independent marker of more-differentiated phenotypes,[30] suggesting that ZO-1 could act as a tumor suppressor as well.[33]

To further test whether the morphological changes in BT-20 and T-47D cells were associated with induction of epithelial cell markers, we first examined E-cadherin and ZO-1 expression. BT-20 and T-47D cells were exposed to control virus or adenoviral vector carrying Jak2 or Stat5a variants, grown in 3D Matrigel

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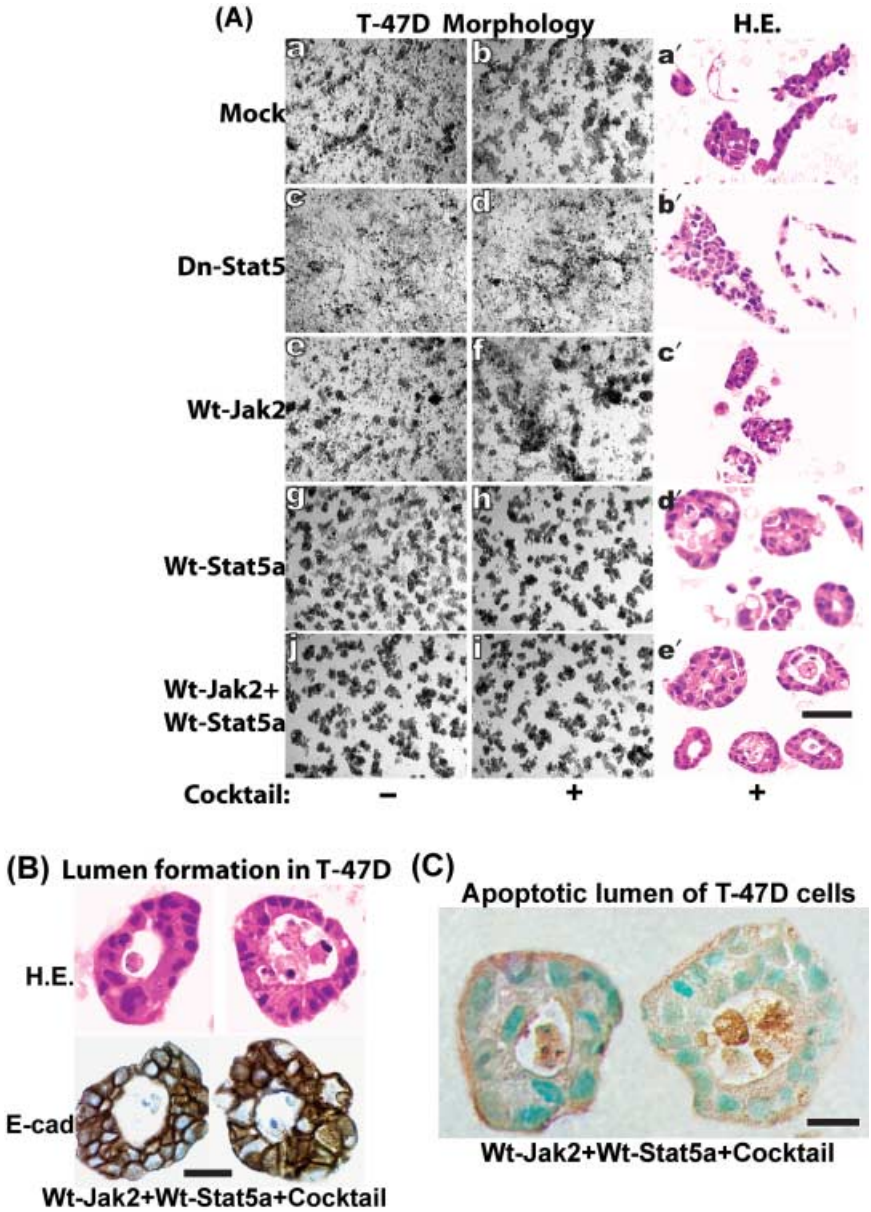

Fig. 2. Hyperactivation of Janus kinase (Jak) 2 and signal transducer and activator of transcription (Stat) 5a promoted luminal differentiated alveolar-like structures of T-47D cells in three-dimensional (3D) Matrigel culture. T-47D cells were either mock infected or infected with adenovirus carrying wild-type (Wt)-*Jak2*, Wt-*Stat5a*, dominant-negative (Dn)-*Stat5*, or a combination of Wt-*Jak2* and Wt-*Stat5a*, treated with cocktail or control vehicle for 15 days in 3D Matrigel culture, and then formalin fixed. (A) Jak2 plus Stat5a induced 3D differentiated organoids in T-47D cells. Morphological alterations of T-47D cells were analyzed using phase-contrast microscopy at 200× magnification (a–i) and hematoxylin–eosin (HE) staining (a′–e′). (B) Jak2 and Stat5a co-overexpression promoted differentiation of T-47D cells and E-cadherin induction at cell–cell contact. T-47D cells were infected with a combination of Wt-*Jak2* and Wt-*Stat5a*, cocktail treated for 15 days in 3D Matrigel culture, and then paraffin-embedded sections were subjected to HE staining and immunohistochemical analysis for E-cadherin localization, using specific E-cadherin antibody. HE staining of T-47D cells showed polarization of columnar cells with basally localized nuclei, and establishment of a single layer of differentiated cells with a secretory phenotype (upper panel). Immunohistochemistry of E-cadherin revealed that E-cadherin was enhanced and localized to the cellular junctions in T-47D cells (lower panel). Morphological alterations were visualized and photographed (scale bar = 200 μm). Representative fields from three independent experiments are shown. (C) Jak2 plus Stat5a overexpression induced a marked differentiated lumen through central apoptosis in T-47D cells. *In situ* DNA fragmentation of T-47D cells using the Apo-Brdu-IHC method. Cocktail-treated T-47D cells overexpressing a combination of Wt-*Jak2* and Wt-*Stat5a* showed a central apoptotic lumen with a single layer of differentiated cells, as indicated by central brown staining and methyl green-counterstained cells. Morphological alterations were visualized, and analyzed (scale bar = 200 μm). Representative fields from three independent experiments are shown.

culture, and treated with cocktail or control vehicle for 15 days. Western blot analysis of whole-cell lysates from BT-20 or T-47D cells showed elevated total levels of ZO-1 under conditions where 3D organoids occurred, with highest levels in cells expressing Stat5a or Jak2 plus Stat5a treated with cocktail, and lowest to no expression in mock, Jak2-, and Dn-Stat5-expressing T-47D cells (Fig. 4A,B), suggesting that Jak2 and Stat5a co-overexpression enhanced ZO-1 levels. The pattern of induction of E-cadherin and ZO-1 was consistent with marked induction in Stat5a- or Jak2 plus Stat5a-overexpressing cells with or without cocktail, but not in mock or Jak2-expressing cells (Fig. 4A,B). Moreover, Dn-Stat5 with or without cocktail decreased E-cadherin expression in T-47D cells compared with mock and transfectants, indicating that Stat5a is involved in E-cadherin upregulation (Fig. 4B). A major mechanism by which E-cadherin is downregulated in EMT is transcriptional repression by Snail.[34] To confirm our finding, Snail expression was examined. Consistent with E-cadherin and ZO-1 upregulation, we found that Snail expression was decreased in Stat5a- and Jak2 plus Stat5a-overexpressing cells, indicating that Snail is involved in the mechanism of Stat5a-regulated E-cadherin induction (Fig. 4A,B). Collectively, we theorized that co-overexpression of Jak2 and Stat5a may play a role in reversing the process of EMT normally required for carcinogenic transformation and inducing epithelial differentiation markers in breast cancer cells.

The intermediate filaments of the cytoskeleton, keratins 8 and 18, characterize the differentiation compartment (the luminal cells), and loss of these keratins is associated with a loss of differentiation.[31] Moreover, the ratio of the intermediate filament proteins keratins 8 and 18 to vimentin is often used to assess the phenotypic properties of cells that have undergone EMT.[31] To examine whether the reversed process of EMT is associated with induction of epithelial markers, western blot analysis was carried out. Western blot data revealed that 3D organoids of Stat5a- and Jak2 plus Stat5a-overexpressing cells showed a significant reduction in vimentin levels compared with mock and Jak2-expressing BT-20 cells, but not of T-47D cells that showed no vimentin expression (Fig. 4C). Conversely, the expression of keratins 8 and 18 was found to be low in mock cells but they were re-expressed in Stat5a- and Jak2 plus Stat5a-overexpressing cells treated with

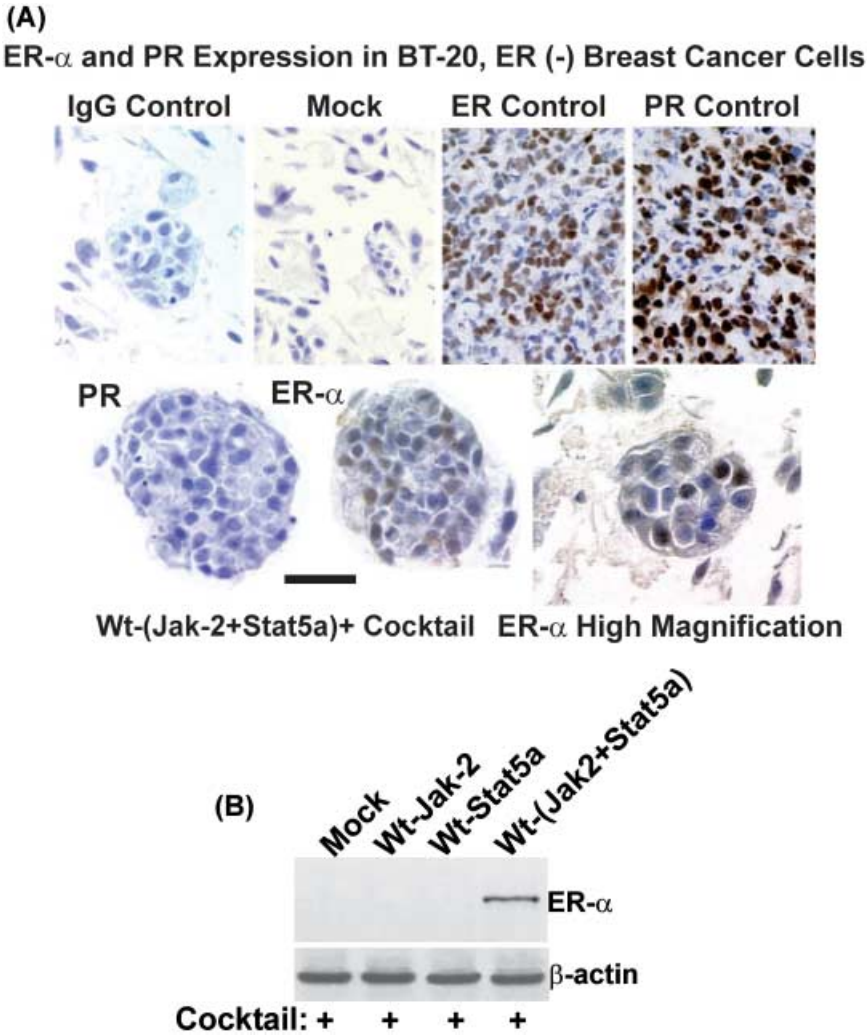

Fig. 3. Reconstitution of Janus kinase (Jak) 2 and signal transducer and activator of transcription (Stat) 5a signaling leads to robust estrogen receptor (ER)-α expression in differentiated three-dimensional (3D) organoids of BT-20 cells. (A) Immunohistochemical staining of ER-α and progesterone receptor (PR) in 3D organoids of BT-20 cells. Brown staining was detected in the nuclei of Stat5a- and Jak2-overexpressing 3D organoids of BT-20 cells treated with cocktail. No nuclear staining was detected in mock-, PR-, or IgG-stained cells. T-47D cells were used as controls for ER-α and PR immunohistochemical staining. (B) Western blot analysis of BT-20 cells in 3D culture. Cells were mock or wild-type (Wt)-*Jak2*-, Wt-*Stat5a*-, or a combination of Wt-*Jak2* and Wt-*Stat5a*-infected, and cocktail-treated for 15 days. Only cocktail-treated cells overexpressing a combination of Jak2 plus Stat5a showed a distinct ER-α band at ~65 kDa. The membrane was reprobed with anti-β-actin antibody to verify equal protein loading. Representative data from three independent experiments are shown.

cocktail (Fig. 4C). The expression of keratins 8 and 18 was markedly enhanced in T-47D cells compared with BT-20 cells as T-47D cells have a luminal phenotype and express comparably high levels of both Jak2 and Stat5a. Collectively, these changes in the protein pattern of keratins 8 and 18 and vimentin were consistent with E-cadherin and ZO-1 induction, indicating that Jak2 and Stat5a co-overexpression reverts the transformed phenotype of breast cancer cells by reversing EMT and induces differentiation in the breast tumor cells.

Discussion

The present study extends previous work where we introduced PRL-activated Stat5a as a coordinate regulator of invasion-related characteristics of mammary cancer cells, including cell-surface association of β-catenin, homotypic adhesion *in vitro* and in xenotransplant tumors *in vivo*, invasion through Matrigel, cell migration, and matrix metalloproteinase activity.[25] Here, we examined the role of the PRL–Jak2–Stat5a pathway on EMT regulation and differentiation in breast cancer cells.

The current study provides novel evidence for the role of Jak2 and Stat5a in reversing EMT normally required for carcinogenic transformation in mammary cancer. We have established conditions in which exogenous co-overexpression of Jak2 and Stat5a effectively reverses the mesenchymal phenotype and induces 3D differentiated organoids in BT-20 and T-47D cells. We also used cocktail instead of PRL alone as we found that dexamethasone can upregulate PRL signaling via Stat5a and stimulate terminal differentiation in T-47D cells, but not in BT-20 cells that have lost Stat5a expression (data not shown).

Although undifferentiated BT-20 cells displayed 3D organoids with limited to no lumen formation, moderately differentiated T-47D cells showed alveolar-like structures, mamospheres, with a marked differentiated lumen through central apoptosis and a polarized epithelial phenotype (Figs 1,2). Notably, these extensive morphological changes induced by Jak2 and Stat5a co-overexpression recapitulate key elements of normal differentiated epithelial cell markers, including expression of E-cadherin, ZO-1, and cytokeratins 8 and 18. Moreover, these findings were associated with a significant reduction in vimentin and Snail levels, indicating a shift from a mesenchymal towards an epithelial phenotype. We further showed that co-overexpression of Jak2 and Stat5a re-expressed ER-α in BT-20 cells, which may improve tumor progression and prediction for response to hormonal therapies. We conclude that Jak2 and Stat5a co-overexpression cooperatively reversed EMT while promoting epithelial differentiation of human cancer cells.

It remains controversial as to whether the central signaling axis used by PRL, the Jak2–Stat5 pathway, contributes positively to the tumor-promoting effect of PRL based on limited studies of breast cancer models in rodents and humans,[17,27,35–38] or acts as an invasive suppressor and differentiation promoter in mammary cancer. However, accumulating data from our laboratory and others suggests that PRL, through the Jak2–Stat5a pathway,

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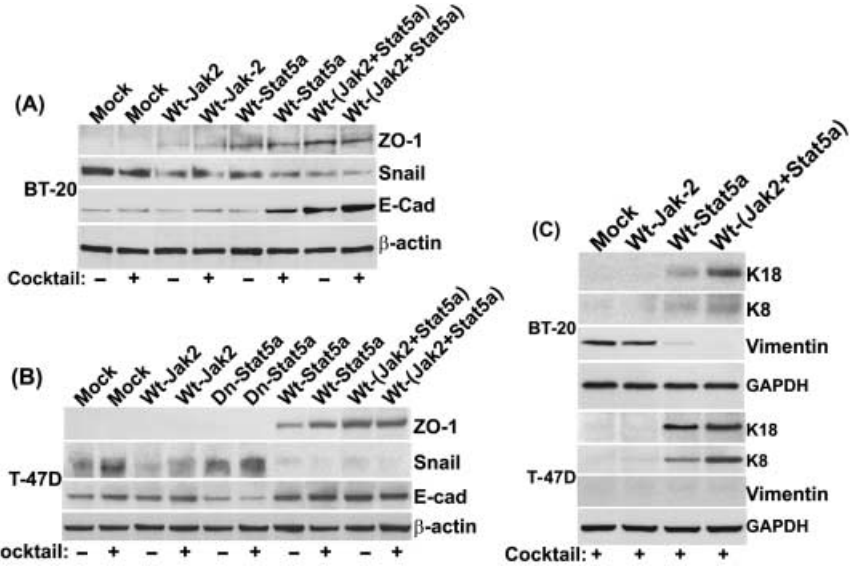

Fig. 4. The synergistic effect of Janus kinase (Jak) 2 plus signal transducer and activator of transcription (Stat) 5a promotes mesenchymal to epithelial redifferentiation. Overexpression of Jak2 plus Stat5a induces E-cadherin and zonula occludens (ZO)-1, and decreases Snail in differentiated three-dimensional (3D) organoids of (A) BT-20, and (B) T-47D. Cells were either mock infected or infected with adenovirus carrying wild-type (Wt)-*Jak2*, Wt-*Stat5a*, dominant-negative (Dn)-*Stat5*, or a combination of Wt-*Jak2* and Wt-*Stat5a*, and treated with cocktail or control vehicle for 15 days in 3D Matrigel culture. Cell extracts were exposed to western blot analysis using specific antibodies for ZO-1, Snail, and E-cadherin. Jak2 plus Stat5a-overexpressing cells induced E-cadherin and ZO-1 expression, but decreased Snail levels in BT-20 and T-47D cells. Membranes were reprobed with anti-β-actin antibody to verify equal protein loading. Representative data from three independent experiments are shown. (C) Jak2 plus Stat5a overexpression induces a mesenchymal to epithelial shift in BT-20 and T-47D cells. Cells were either mock infected or infected with Jak2 and/or Stat5a adenovirus variants, treated with cocktail or control vehicle for 15 days in 3D Matrigel culture, and then subjected to western blot analysis. Jak2 plus Stat5a overexpression significantly reduced the levels of vimentin, whereas they significantly induced the levels of cytokeratins 8 and 18 compared with mock, Jak2-, or Stat5a-expressing cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal gel-loading control. Representative data from three independent experiments are shown.

acts as an invasion suppressor and regulator for EMT through promoting differentiation of mammary breast cancer.[11,20,25,26,37] In addition to the Jak2–Stat5a pathway,[4] PRL receptors may activate several parallel intracellular signaling pathways that could resolve the paradigm associated with the role of the PRL–Stat5a pathway in breast cancer. Whereas the mitogenic effects of PRL have been attributed to the Ras–mitogen activated protein kinase (MAPK) signaling pathway[38–40] as well as additional kinase cascades, such as Src, the phosphatidyl-inositol 3-kinase–Akt pathway,[14,39–41] and Nek3,[42] PRL-induced terminal differentiation of mammary epithelium and milk-protein expression (e.g. β-casein, β-lactoglobulin, and PRL-inducible protein) appears to be mediated by activation of Stat5,[43,44] particularly Stat5a.[9] As loss of active Stat5a is a feature of progressing human breast cancer invasion,[25] the tumor-promoting effects of PRL are probably mediated by other pathways rather than the Jak2–Stat5a pathway, and possibly by Stat5a-incompetent short PRL receptor isoforms. The long receptor form is critical for Stat5a-mediated milk induction, whereas the short isoforms, including the most stable PRLR-S1b isoform, appear to be incapable of activating Jak2–Stat5 signals and act as suppressors of this pathway.[45,46] In light of this new notion of the PRL–Jak2–Stat5a axis as a conditional suppressor and promoter of breast cancer growth and progression, we further postulate that disruption of PRL-receptor signaling in human breast cancer may have distinct effects, depending on whether Stat5a or other pathways are predominant downstream mediators in a given tumor. In addition, Dn-Jak2 plus Dn-Stat5-overexpressing T-47D cells led to a marked increase in basal phosphorylation of the MAPK (extracellular signal regulated kinase ([ERK]1/2) pathway (data not shown) and invasion potential[25] after 24 h, indicating that the MAPK pathway, but not the Jak2–Stat5a pathway, is involved in invasion of mammary cancer.

The new role of Jak2–Stat5a signaling to reverse EMT and restore differentiation is consistent with a series of other experimental observations in normal and malignant breast epithelial cells. First, Stat5 is a key regulator of epithelial differentiation in mouse mammary gland,[7,9,47,48] and Stat5 likewise is hyperactivated during terminal differentiation and lactation in human breast.[11] Second, independent studies in which hyperactive Stat5 or dominant-negative Stat5 were targeted to mouse mammary glands under control of the β-lactoglobulin gene promoter show that tumors arose more frequently and were more aggressive in mice expressing the dominant-negative Stat5.[45,49,50] Third, suppression of Jak2–Stat5 signaling in immortalized, near-normal breast epithelial cells led to a hyperproliferative phenotype characterized by increased mitotic rate, reduced apoptosis, and reduced contact inhibition.[23] In addition, Stat5a is normally active in healthy human breast epithelia outside of pregnancy, and basal activation is lost during metastatic progression of human breast.[11,24] Fourth, and most importantly, our mechanistic data showed that Jak2 and Stat5a can reverse a program of invasive characteristics of mammary cancer cells with induction of cell-surface association of the E-cadherin–β-catenin complex and can restore an epithelial phenotype even in aggressive, ER-negative mammary cancer cells.[25] Fifth, in another study, restoring PRL–Jak2 signaling in MDA-MB-231 suppressed their mesenchymal properties and reduced their invasive behavior, suggesting the negative regulatory role of PRL and Jak2 on EMT.[26] Finally, the present study shows that Jak2 and Stat5a co-overexpression promotes epithelial differentiation and reverses the malignant characteristics of BT-20 and T-47D cells in 3D culture.

To begin to assess the downstream molecules regulated by Jak2 and Stat5a expression in BT-20 and T-47D cells that may be related to the Stat5a-based mechanisms of invasive suppression and E-cadherin induction in mammary cancer, we examined the process of tumorigenesis (EMT). We showed that overexpression of Jak2 and Stat5a mediated a shift toward a more luminal epithelial-cell phenotype, which was confirmed by increased

cytokeratin 8 and 18 levels in BT-20 and T-47D cells, whereas vimentin levels decreased and the cells were less invasive.[25] Both of these cytoskeletal proteins are indicators of EMT, and epithelial cells are known to have a less-invasive potential than mesenchymal cells.[51] Full reversion to a luminal differentiated epithelial phenotype, however, would include restoration of cell polarization, which was confirmed using HE staining in differentiated 3D organoids of T-47D cells, but not BT-20 cells (Figs 1,2). Moreover EMT is a multistep process that is hypothesized to be highly influenced by the tissue microenvironment.[52] It is therefore not surprising that Jak2 and Stat5a co-overexpression alone does not allow the complete reversion of mammary tumor cells to an epithelial phenotype but suggests that Jak2 and Stat5a may be essential components in this process.

The redifferentiation and invasive-suppression role of the Jak2–Stat5a pathway in mammary cancer cells is best explained by the role of Stat5a in induction of E-cadherin, ZO-1 levels, and decreased Snail expression in BT-20 and T-47D cells. Snail is known to repress E-cadherin, cytokeratin 18, and other epithelial marker genes such as occludin,[53] either directly or indirectly, whereas its expression is associated with upregulation of mesenchymal markers, including vimentin and fibronectin,[54] leading to full EMT induction. Here, cells co-overexpressing Jak2 and Stat5a induced E-cadherin and ZO-1 expression with a concomitant reduction in Snail levels, indicating the shift toward epithelial redifferentiation. The linkage of the effect of Jak2 and Stat5a overexpression on E-cadherin induction and Snail reduction could provide one mechanism by which Stat5a may regulate E-cadherin and thus reverse EMT. Furthermore, E-cadherin is required for proper ZO-1 localization at the tight junction and the interaction of ZO-1 with the catenin proteins raises the possibility that ZO-1 could function downstream of E-cadherin in an adhesion-dependent signaling pathway.[30,31,55]

In summary, we present novel evidence that co-overexpression of Jak2 and Stat5a can be synergic in reversion of epithelial to mesenchymal dedifferentiation while promoting epithelial-cell differentiation, and hence control the invasion potential of human mammary cancer cells. Jak2 and Stat5a restoration in undifferentiated, ER-negative BT-20 cells, which lost Jak2–Stat5a signaling, induced partial 3D differentiated organoids with no lumen formation, but effectively re-expressed ER-α, whereas moderately differentiated T-47D cells expressing comparably high levels of both Jak2 and Stat5a showed Jak2- and Stat5a-induced alveolar-like structures, mamospheres, with marked lumen formation through central apoptosis and restored a polarized epithelial phenotype. Taken together, the findings implicate Jak2–Stat5a signaling as a critical dedifferentiation and invasion suppressor of potential importance for marking effective individualized therapeutic approachs to suppress human breast cancer invasion.

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