

# Blockade of Autocrine TGF- $\beta$ Signaling Inhibits Stem Cell Phenotype, Survival, and Metastasis of Murine Breast Cancer Cells

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## Abstract

Transforming growth factor beta (TGF- $\beta$ ) signaling has been implicated in driving tumor progression and metastasis by inducing stem cell-like features in some human cancer cell lines. In this study, we have utilized a novel murine cell line NMuMG-ST, which acquired cancer stem cell (CSC) phenotypes during spontaneous transformation of the untransformed murine mammary cell line NMuMG, to investigate the role of autocrine TGF- $\beta$  signaling in regulating their survival, metastatic ability, and the maintenance of cancer stem cell characteristics. We have retrovirally transduced a dominant-negative TGF- $\beta$  type II receptor (DNRII) into the NMuMG-ST cell to abrogate autocrine TGF- $\beta$  signaling. The expression of DNRII reduced TGF- $\beta$  sensitivity of the NMuMG-ST cells in various cell-based assays. The blockade of autocrine TGF- $\beta$  signaling reduced the ability of the cell to grow anchorage-independently and to resist serum deprivation-induced apoptosis. These phenotypes were associated with reduced levels of active and phosphorylated AKT and ERK, and Gli1 expression suggesting that these pathways contribute to the growth and survival of this model system. More interestingly, the abrogation of autocrine TGF- $\beta$  signaling also led to the attenuation of several features associated with mammary stem cells including epithelial-mesenchymal transition, mammosphere formation, and expression of stem cell markers. When xenografted in athymic nude mice, the DNRII cells were also found to undergo apoptosis and induced significantly lower lung metastasis burden than the control cells even though they formed similar size of xenograft tumors. Thus, our results indicate that autocrine TGF- $\beta$  signaling is involved in the maintenance and survival of stem-like cell population resulting in the enhanced metastatic ability of the murine breast cancer cells.

**Keywords:** Autocrine TGF-beta; Breast cancer; Apoptosis; Lung metastasis, Cancer stem cell

## Introduction

Transforming growth factor beta (TGF- $\beta$ ) is a multifunctional polypeptide growth factor that has a variety of biological effects that range from regulating cellular proliferation, differentiation, migration, extracellular matrix remodeling, wound healing and repair, and estrogen signaling [1,2]. TGF- $\beta$  signaling is initiated by ligand binding to two cell surface receptors, T $\beta$ RI and T $\beta$ RII. Upon binding of TGF- $\beta$ 1 or  $\beta$ 3, T $\beta$ RII forms a homodimer and auto-phosphorylates. The activated T $\beta$ RII complex binds to and transphosphorylates T $\beta$ RI. Activated T $\beta$ RI subsequently phosphorylates Smad2 and Smad3. The complex of phosphorylated Smad2 and phosphorylated Smad3 together with Smad4 translocate into nucleus to regulate the transcription of TGF- $\beta$ -responsive gene. TGF- $\beta$  is also known to mediate its signaling through a Smad-independent pathway by activating the mitogen-activated protein kinase (MAPK) pathways of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs), and p38 [3-5].

It is generally accepted that TGF- $\beta$  pathway acts as a tumor suppressor in the early stage of cancer progression by its induction of senescence [6] and apoptosis [7,8], and its inhibition of cell cycle progression and genomic instability [9]. On the other hand, it is also well known to act as a tumor promoter in later stages of carcinogenesis [10,11]. Loss of TGF- $\beta$  signaling has been reported in variety of cancers. For example, T $\beta$ RII has been found mutated in gastric and colon carcinoma cells due to microsatellite instability [12,13]. Frameshift mutation in T $\beta$ RI has been identified in one-third of ovarian cancers [14]. Mutations in Smad2 and Smad3 have been identified in colorectal

cancers, and Smad4 mutations have been identified in pancreatic cancers and juvenile polyposis [15-18]. These mutations are thought to drive the progression of these carcinomas [19-21]. However, studies have indicated that inactivation mutations in T $\beta$ RII and Smad proteins are restricted to certain types of cancers and are rare in others including breast cancers [20,22,23], which implies that the autocrine TGF- $\beta$  pathway mediated by T $\beta$ RII might be necessary for the progression of carcinogenesis in certain types of cancers. TGF- $\beta$  pathway was reported to support the survival of some types of cancer cells [24-26]. Our previous studies also showed that inhibiting autocrine TGF- $\beta$  signaling in MDA-MB-231 and MCF-7 human breast carcinoma cells induced apoptosis [20,27]. A recent study demonstrated that epithelial cell plasticity is controlled by an autocrine TGF- $\beta$  signaling network through epigenetic modification using the Madin Darby canine kidney cell line (MDCK) model [28]. But the cellular and molecular mechanism(s) by which autocrine TGF- $\beta$  signaling maintain the growth and survival of breast tumor cells is under-explored. Recent

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evidences indicated that a subpopulation of tumor initiating cells, referred as cancer stem cells (CSC), exhibit phenotypes resembling normal stem cell properties such as expression of stem cell markers, self-renewal, migration during tissue regeneration and repair, and resistance to cytotoxic agents [29-31]. These features of CSC and the pathways that regulate them, which includes epithelial to mesenchymal transition (EMT) [29] and self-renewal pathways such as hedgehog and Notch [32,33], have been implicated not only in the breast cancer initiation but also in malignant progression which includes invasion and establishment of distant metastasis.

In this study, we have evaluated the regulation of autocrine TGF- $\beta$ -induced tumor cell growth and survival by abrogating endogenous TGF- $\beta$  signaling in a spontaneously transformed murine mammary epithelial cell, NMuMG-ST [34] expressing markers of EMT and stem cell self-renewal pathways. Our results indicated that blockade of autocrine TGF- $\beta$  signaling, in this murine cell model of late stage tumor progression, have the potential to inhibit both cancer stem cell maintenance pathways and metastasis-promoting activity.

## Materials and Methods

### Cell culture

NMuMG-ST, a spontaneously transformed malignant murine mammary epithelial cell line was developed from the untransformed mammary epithelial cell line NMuMG in our laboratory [34]. These transformed highly malignant cells displays higher level of expression of several stem cell markers including Gli1, CD44 (HCAM), CD49f (integrin  $\alpha 6$ ), CD29 (integrin  $\beta 1$ ), Oct 3/4 and P-Glycoproteins (MDR) compared to the parental non-transformed cells (data not shown). The cell line was maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), pyruvate, vitamins, amino acids and antibiotics as described earlier [34]. Cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

The NMuMG-ST cells infected with pLPCX-DNR II retrovirus which expresses dominant negative RII without intracellular kinase domain was referred as DNR II cells. While, the NMuMG-ST cells infected with pLPCX retrovirus vector was referred as control cells.

### Immunoblotting analysis

Exponentially growing cells were rinsed twice with ice-cold PBS and lysed in a cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40) containing protease inhibitor (Boehringer Mannheim) and phosphatase inhibitors, 1 mM NaVO<sub>3</sub> and 1 mM NaF. Equal amounts of total protein (80  $\mu$ g) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with TBST (100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat powder milk and incubated with primary antibody overnight at 4°C. Primary antibodies were purchased from the following sources: T $\beta$ RII and E-cadherin from R&D Systems and T $\beta$ RII antibody was also used to detect DNR II expression Phospho-Smad3 (Ser 465/467) (provided by Dr. Edward Leof); pAkt and pErk (Thr202/Tyr204) from cell signaling; ALDH1/2, CD29, NOTCH1, GLI1, GLI2 and Tubulin from Santa Cruz Biotechnology; SMO and Snail from Abcam; Vimentin from Sigma; GAPDH from Ambion. After three washes with TBST, the membranes were incubated with HRP-linked secondary antibodies (1:3000 dilution, Santa Cruz) for 1 hour at room temperature and washed again. Bound complexes were detected

using chemiluminescence procedures according to the manufacturer's instructions (NEN Life Science Products). Relative expression levels of indicated gene were quantified with Image J software.

### Transient transfection and luciferase Assay

To determine autocrine TGF- $\beta$  activity, we measured a TGF- $\beta$ -responsive promoter activity using a plasmid called pSBE4-Luc [35] and was analyzed as described previously [20]. Briefly, control and DNR II cells were plated into 12-well plates and cultured for one day. The pSBE4-Luc plasmid (0.5  $\mu$ g) and a  $\beta$ -galactosidase expression plasmid (0.5  $\mu$ g) were transiently co-transfected into the cells using Eugene6 (Roche Molecular Biochemicals) according to manufacturer's instruction. After 4 hours incubation, the cells were treated with or without TGF- $\beta 3$  (0.5 ng/ml). After an additional 20 hours of incubation, the cells were lysed and the activity of luciferase and  $\beta$ -galactosidase in the cell lysates were measured. Luciferase activity was normalized to  $\beta$ -galactosidase activity before being plotted.

### Cell proliferation assay

Cells were seeded in a 96-well plate at 2,000 cells/well with or without TGF- $\beta 3$  (0.5 ng/ml) treatment at various time points and MTT assay was performed as described previously [36]. Two hours before each time point, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (2mg/ml in PBS) was added at 50  $\mu$ l per well and cells were incubated at 37°C for 2 hours. DMSO (100  $\mu$ l) was added into each well after the medium was removed to dissolve the purple product by gently shaken on a shaker for 10 minutes. The absorbance was measured at 595 nm with a microplate reader (Bio Tek Instrument, VT).

### Soft-agar colony formation assay

Cells in 1 ml 0.4% soft agarose (Life Technologies, Carlsbad) mixed with 10% FBS containing medium were plated at 6,000/well on top of a 1 ml solidified underlayer of 0.8% agarose in the same medium in a 6-well tissue culture plate. After 2 weeks of incubation, cell colonies were visualized by overnight staining with 1 ml *p*-iodonitrotetrazolium violet (sigma).

### Cell Cycle analysis

NMuMG-ST Control and DNR II cells were collected by trypsinization at day 0, 2, and 4 following serum starvation. The cells were fixed with 100% ethanol, stained with 10  $\mu$ g/mL propidium iodide in the presence of 5  $\mu$ g/ml RNase A and analyzed for fluorescence using a FACScan flowcytometer (Becton Dickinson, San Diego, CA).

### Immuno-cytochemical staining

Control and DNR II cells were harvested and plated at 3,000 cells per coverslip, which was placed inside the well of a 24-well plate. When the cells grown to 80% confluence, the cells were fixed in 2% paraformaldehyde, permeabilized in 0.1% Triton X-100 and blocked with 1.0% bovine serum albumin. Subsequently, the cells were incubated with anti-E-cadherin and anti-vimentin antibody at 40°C overnight and in Alexafluor 568 fluochrome conjugated secondary antibody for 1 hr at room temperature. The slides were mounted with Vectashield mounting medium (vector) and examined under a fluorescence microscope.

### Cell migration assay

Cell migration assays were performed in 24-well Boyden Chambers with 8- $\mu$ m pore polycarbonate membranes (BD Biosciences) as

described earlier [37]. Cells in serum free medium were seeded in the upper chamber at 25,000 cells per chamber. The lower chamber contained the medium with 10% of FBS as chemoattractant. After incubation with 5% CO<sub>2</sub> at 37°C for 16 hours, cells were removed from the upper surface of the chamber membrane with a cotton swab and the membrane were then stained for the migratory cells at the lower surface of the chamber with HEMA3 staining solution (Fisher Scientific, Houston, TX) according to the manufacturer's protocol. The stained cells were counted under a microscope.

### Mammosphere culture

To study the effect of autocrine TGF- $\beta$  signaling on mammosphere formation, control and DNRII cells were harvested and filtered through a 40- $\mu$ m strainer after trypsinization to obtain a single-cell suspension. Cells were seeded (1000 cells/100  $\mu$ l/well) in 96 well ultra-low attachment plate (Costar) with Mammosphere medium (StemCell Technologies) in triplicate. The number of spheres in each well was counted after 6 days of incubation.

### Tumorigenicity and lung metastasis studies

Tumorigenicity studies were performed as described previously [38]. Briefly, cells from exponential cultures of Control and DNRII cells were labeled with GFP by lentivirus and inoculated subcutaneously in the flanks of 5-week-old female athymic nude mice. The growth of tumors was monitored every alternate day and tumor sizes were measured with a caliper in two dimensions. Tumor volumes were calculated with the equation  $V = (L \times W^2) \times 0.5$ , where L is length and W is width of a tumor. To study lung metastasis, lungs were removed during autopsy and GFP expressing metastases colonies were counted (at x40 magnification) under a TE-200 Nikon fluorescence microscope.

### TUNEL assay

Tumors formed by Control and DNRII cells in nude mice were fixed in 10% buffered formalin (Fisher Scientific) and embedded in paraffin. Sections of 5  $\mu$ m were cut from the embedded tumors and used for TUNEL staining as described earlier [20]. Briefly, DNA fragmentation associated with apoptosis in tumor cells was detected *in situ* by the addition of digoxigenin-labeled nucleotides to label free 3'-end of DNA fragments using the ApopTag *in situ* Apoptosis detection kit (Intergen) according to the manufacturer's instruction.

### Statistical analysis

Two-tailed Student t-tests were used to determine a significant difference between control and experimental data. All the statistical analysis was performed with GraphPad Prism 3.03 software.

## Results

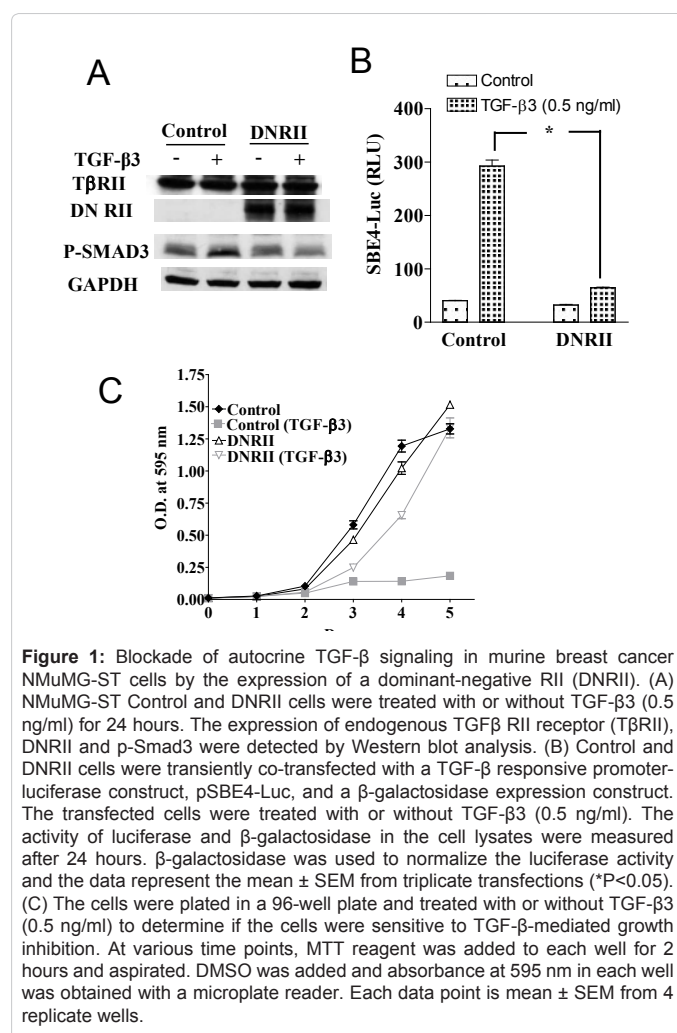
### Blockade of autocrine TGF- $\beta$ signaling by the expression of DNRII

The expression of DNRII and its inhibitory effect on the TGF- $\beta$  signaling pathway was confirmed by Western blot analysis after NMuMG-ST cells were retrovirally transduced with a DNRII retroviral expression vector (Figure 1A). TGF- $\beta$  treatment stimulated phosphorylation of Smad3 in the control cells, but not in DNRII cells (Figure 1A). The inhibition of Smad3 phosphorylation also blocked the transcriptional activity of Smad proteins as indicated by the TGF- $\beta$ -responsive promoter-luciferase reporter assay (Figure 1B). These data

show that the expression of DNRII in NMuMG-ST cells significantly antagonized TGF- $\beta$  signaling.

### Autocrine TGF- $\beta$ signaling supports cell growth and survival

To determine the role of autocrine TGF- $\beta$  signaling in cell growth and survival, we first compared the anchorage-dependent growth property on plastic of the control and DNRII cells. While the growth of DNRII cells were much less inhibited by TGF- $\beta$  treatment than the control cells confirming the blockade of TGF- $\beta$  signaling by DNRII expression, their growth rate appeared a little lower than that of the control cells (Figure 1C). Because TGF- $\beta$  has been shown to promote anchorage-independent growth in some model systems, we also studied the effect of the abrogation of autocrine TGF- $\beta$  signaling on anchorage-independent colony formation in soft-agarose. Significant reduction in colony formation by the DNRII cells was observed when compared to the control cells (Figure 2A,2B). Our results indicated that autocrine TGF- $\beta$  signaling supports both the anchorage dependent and independent growth of NMuMG-ST cells. To further determine if autocrine TGF- $\beta$  signaling was necessary for cell survival in NMuMG-ST cells, we studied the effect of serum deprivation in the culture medium as a stress on cell apoptosis. Cell cycle analysis revealed that there was a remarkable increase of sub-G1 fraction in DNRII cells after four days of serum deprivation indicating the presence of apoptotic

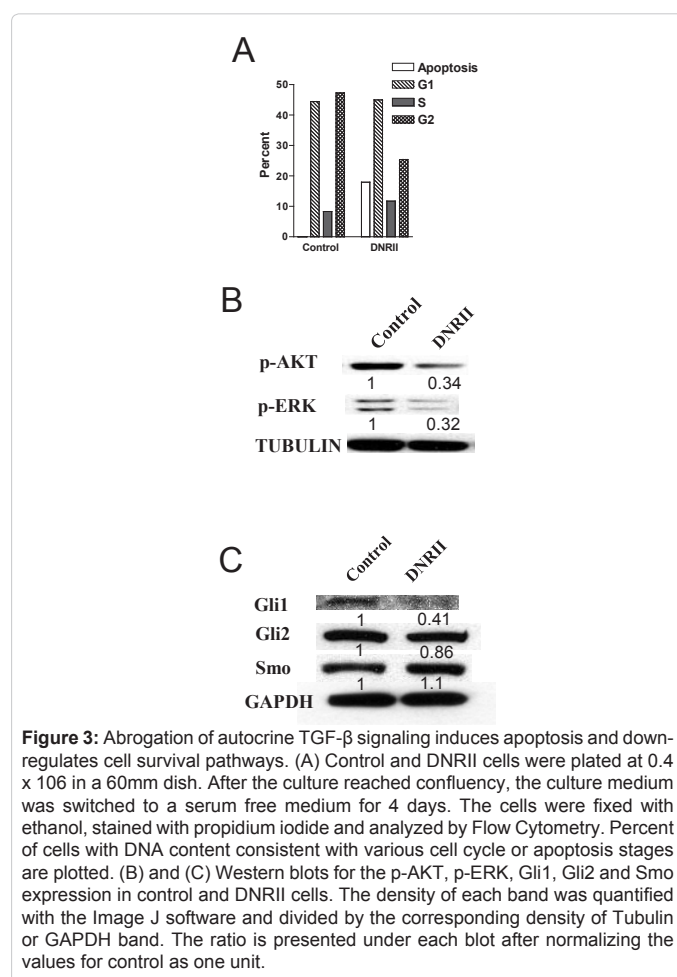
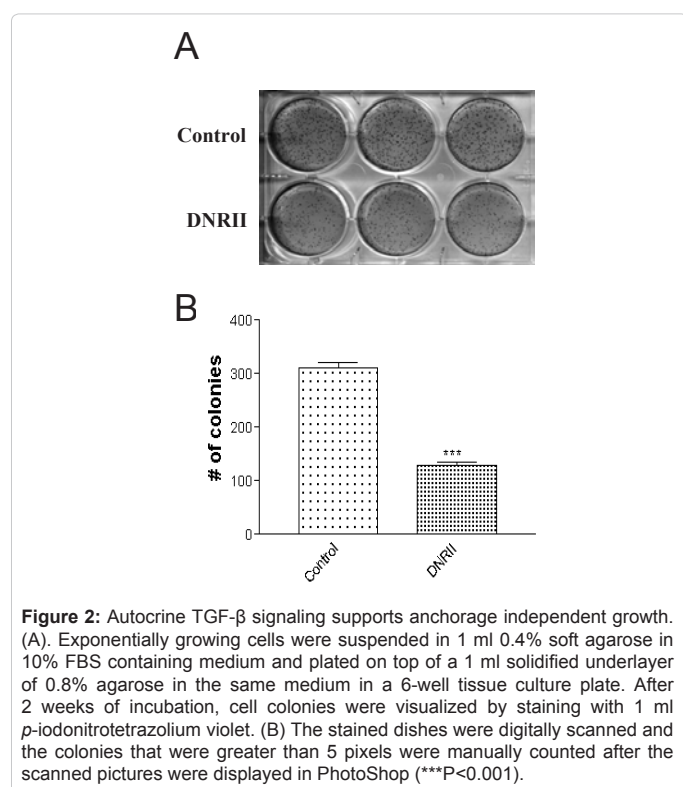




cells (Figure 3A). In contrast, the control cells did not have the sub-G1 fraction indicating little apoptosis after four days of serum deprivation (Figure 3A). The mitogen-activated protein kinase (MAPK) pathway and phosphoinositide 3-kinase (PI3K)/Akt pathway are known to be activated by TGF- $\beta$  receptors [3,39] and have been shown previously by us to mediate TGF- $\beta$ -induced cell survival [20,38]. Interestingly, both phosphorylated Erk and Akt levels were noticeably lower in the DNR11 cells than in the control cells (Figure 3B) suggesting that the reduced MAPK and PI3K/Akt signaling activity might have contributed to the reduced resistance to apoptotic stimulation in the DNR11 cells. In addition to MAPK and PI3K/Akt pathways, TGF- $\beta$  has been shown to stimulate expression of Gli1 and Gli2, the mediators of sonic hedgehog signaling [40,41]. It has been shown that signaling through Gli proteins promotes cell survival [42]. Our data showed that the expression of Gli1 and, to a lesser extent, Gli2 was decreased in DNR11 cells compared to the control cells. In contrast, the blockade of autocrine TGF- $\beta$  pathway did not affect the expression of Smoothened protein (SMO) (Figure 3C), suggesting autocrine TGF- $\beta$  regulation of Gli proteins may also mediate the survival of NMuMG-ST cells.

### Autocrine TGF- $\beta$ signaling is necessary for EMT and migration of NMuMG-ST cells

TGF- $\beta$  is a well-known stimulator of EMT [43,44]. Decreased expression of epithelial marker E-cadherin and enhanced expression of mesenchymal marker vimentin was observed during the process of EMT [45]. The transcription factor Snail is also known as a repressor of E-cadherin gene expression in epithelial tumor cells [46] and Snail silencing was shown to suppress tumor growth and invasiveness [47]. To investigate the role of autocrine TGF- $\beta$  regulation of EMT in NMuMG-ST cells, we analyzed the expression of E-cadherin, Vimentin and Snail by immuno-blotting. We observed that DNR11



cells expressed enhanced epithelial marker E-cadherin and decreased mesenchymal marker Vimentin and Snail when compared to the control cells (Figure 4A). Furthermore, similar results were obtained by immuno-cytochemical staining (Figure 4B). Our results indicated that autocrine TGF- $\beta$  signaling contributes to EMT. The link between EMT and the tumor cell migration has been shown by several studies [48,49]. Therefore, we have examined whether abrogation of autocrine TGF- $\beta$  signaling could attenuate the motility of NMuMG-ST cells. We found that DNR11 cells showed a significantly decreased migration rate in comparison of the control cells (Figure 4C,4D).

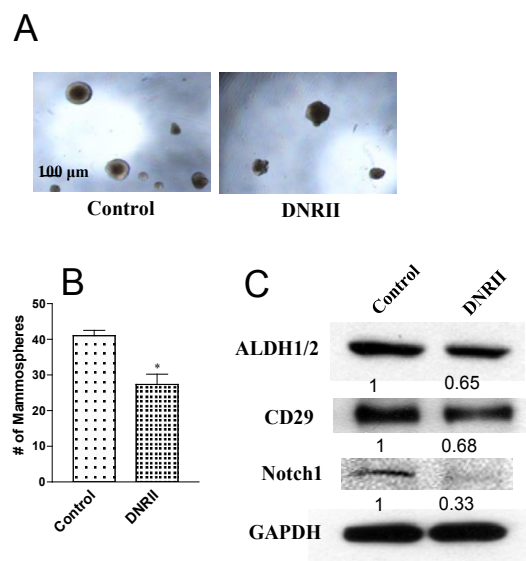
### Autocrine TGF- $\beta$ signaling is essential for the maintenance of a stem cell-like subpopulation

The major characteristic of the stemness of a cell is its self-renewal capacity to generate differentiated progeny. Recently, a non-adherent culture system has been developed [50] in which a small population of stem-like mammary epithelial cells, capable of surviving anoikis, can divide and form discrete spheroid structures termed 'mammospheres'. Each spheroid represents a mammary stem/progenitor cell with limited self-renewal and capable of multi-lineage differentiation. We found that the number of mammospheres formed by DNR11 cells are significantly lower than that formed by the control cells (Figure 5A,5B) indicating functional regulation of the stem like characteristics of tumor cells by autocrine TGF- $\beta$  signaling. We have also analyzed the expression of cell surface stem cell markers CD29 (Integrin  $\beta$ 1) and Notch1, and also

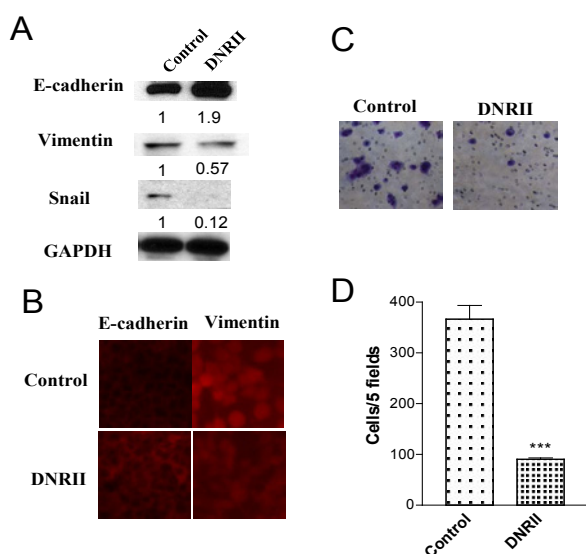
metabolic stem cell marker aldehyde dehydrogenase (ALDH), which are considered as markers for both normal and cancer stem cells [33,51-53] by immunoblotting. We detected reduced expression of ALDH1/2, CD29 and Notch1 in the DNR11 cells compared to the controls (Figure 5C). Expression of Gli1, a component of hedgehog signaling pathway, which is involved in stem cell self-renewal process was also reduced by the abrogation of TGF- $\beta$  signaling (Figure 3C). Recent studies showed that acquisition of stem cell phenotype is linked to the EMT process [29]. Our studies in the previous section also showed that blockade of autocrine TGF- $\beta$  signaling could inhibit the expression of EMT-related markers. All these evidences indicate that autocrine TGF- $\beta$  signaling might be an essential component to maintain cancer stem cell population and their functional pathways.

### Abrogation of autocrine TGF- $\beta$ signaling induces apoptosis and decreases lung metastasis *in vivo*

TGF- $\beta$  has been shown to promote tumor growth and metastasis at the late stage progression of breast cancer. Therefore, we have examined the effect of the abrogation of TGF- $\beta$  signaling on tumor growth *in vivo*. Expression of DNR11 had no significant effect initially on tumor growth (Figure 6A). However, just before the termination of the experiment at day 18, the DNR11 cell-derived tumors showed diminished growth rate in comparison to the control cell-derived tumors. We also determined whether the blockade of autocrine TGF



**Figure 5:** Autocrine TGF- $\beta$  signaling is essential for the maintenance of a stem cell-like subpopulation. (A) Cells from exponential cultures of control and DNR11 were plated in a 96-well low attachment plate at 1,000 cells/well with Mammosphere medium. Representative images of the mammospheres formed by the two cell lines are presented. (B) The number of mammospheres in each well was counted after six days of culture. The data are presented as mean  $\pm$  SEM of 3 replicate wells (\* $p$ <0.05). (C) Cells from exponential cultures of the control and DNR11 cells were harvested and cell extracts were used to detect the expression of ALDH1/2, CD29 and Notch1 by Western blotting. The density of each band was quantified with the Image J software and divided by the density of the corresponding GAPDH band. The ratio is presented under each blot after normalizing the values for the control as one unit.

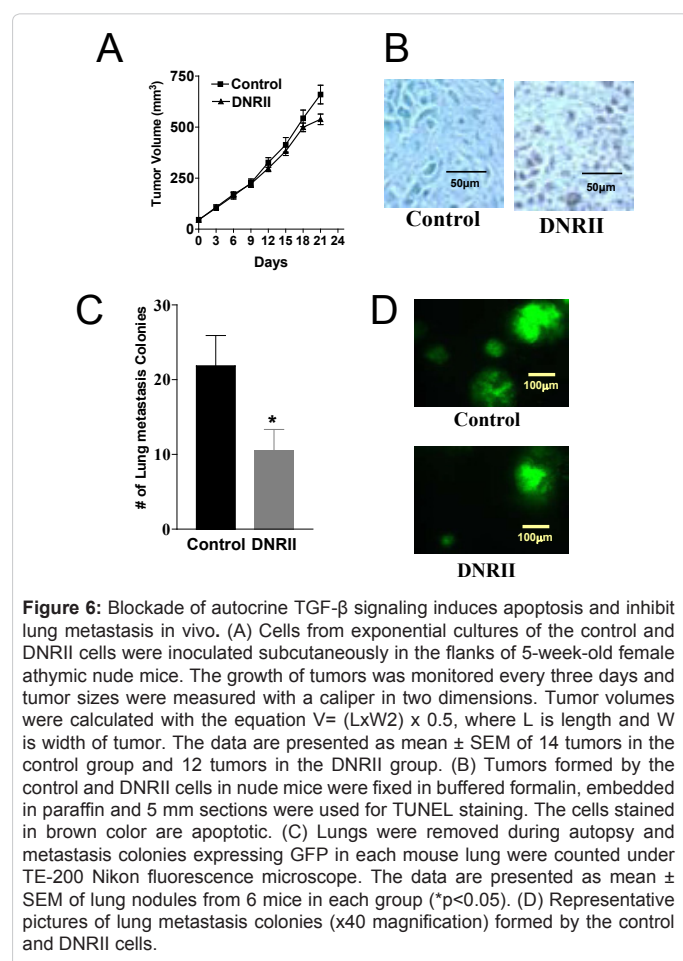


**Figure 4:** Autocrine TGF- $\beta$  signaling is necessary for the progression of EMT in NMuMG-ST cells. (A) Cells from exponential cultures of the control and DNR11 cells were plated in T-25 flasks. After culturing for 4 days, cell extracts were used for Western blot analysis to detect the expression of E-cadherin, Vimentin and Snail. The density of each band was quantified with The Image J software and divided by the density of the corresponding GAPDH band. The ratio is presented under each blot after normalizing the values for control as one unit. (B) Control and DNR11 cells were grown on cover slips in a 24-well plate till 80% confluence. Cells were fixed, permeabilized, blocked and incubated with an anti-E-cadherin or anti-vimentin antibody followed by the incubation with fluorescent dye-tagged secondary antibody. (C) and (D). In a Boyden chamber, 25,000 cells in serum-free medium were seeded in the upper chamber. The lower chamber contained the medium with 10% of FBS as chemoattractant. After incubation for 16 hours, cells were removed from the upper surface of the chamber membrane and stained for the migratory cells at the lower surface of the chamber with HEMA3 staining solution. Cells were counted under a microscope (\*\*\* $p$ <0.005).

beta signaling induced apoptosis *in vivo* by TUNEL assay. As shown in Figure 6B. There were more apoptotic cells in the DNR11 cell-formed tumors than in the control cell-formed tumors. The decreased ability of migration, the inhibition of EMT, and the down-regulation of stem cell-like phenotypes associated with the abrogation of autocrine TGF- $\beta$  signaling *in vitro* indicate that the DNR11 cells might have lower metastasis potential *in vivo* than the control cells. In order to investigate the effect of autocrine TGF- $\beta$  signaling on metastasis, we examined the lungs from the mice after the termination of the xenograft study and counted the GFP expressing micro-metastatic colonies in the lungs. Our results indicated that number of lung metastasis colonies was significantly reduced in the DNR11 cell derived tumor bearing mice when compared to the control cell derived tumor bearing mice (Figure 6C,6D).

### Discussion

In this study, we have investigated the role of autocrine TGF- $\beta$  signaling on the survival, metastasis ability and the maintenance of stem cell-like features in a spontaneously transformed murine breast cancer cell line in which endogenous TGF- $\beta$  signaling was abrogated by the introduction of a dominant negative RII receptor. Our results indicated that autocrine TGF- $\beta$  signaling was necessary for the survival of murine breast cancer NMuMG-ST cells. The DNR11 cells showed more apoptosis than the control cells during growth factor starvation. Blockade of autocrine TGF- $\beta$  signaling in NMuMG-ST cells reduces the tumorigenic potential of the cells *in vitro*, indicated by the formation of fewer anchorage independent colonies in soft agar. Besides the Smad pathway, MAPK/ERK pathway and PI3K/AKT pathway are



also known to be activated by TGF- $\beta$  pathway. It was reported that autocrine TGF- $\beta$  modulate the survival and apoptosis process of cancer cell through MAPK/ERK and PI3K/AKT pathways [20,25-27]. In consistent with the previous studies, we also found that decreased phosphorylation of ERK and AKT in the DNR11 cells was associated with decreased resistance to serum-deprivation-induced apoptosis. We have also analyzed the expression level of PTEN (Phosphatase and tensin homolog), a phosphatase catalyzing dephosphorylation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) involved in the inhibition of the AKT signaling pathway. However, according to our results, the expression level of PTEN was not affected by the expression of DNR11 in the NMuMG-ST cells (data not shown), which suggested that attenuation of PI3K/AKT pathway induced by the abrogation of autocrine TGF- $\beta$  signaling was not mediated by PTEN. Our results also showed that Gli1, an oncogene and a component of hedgehog signaling, which has been shown to promote cell survival [54], might also be involved in the autocrine TGF- $\beta$  regulated survival of tumor cells.

Loss of cell-to-cell contacts and acquisition of the mesenchymal characteristics defined as epithelial-mesenchymal transition (EMT) are important for embryonic development and the progression of cancer metastasis [55]. Although various growth factors are implicated in the regulation of EMT, TGF- $\beta$  is believed to be a major inducer of EMT and metastasis of tumor cells [56-58]. Clinical studies also showed enhanced TGF- $\beta$  signaling in 75% of breast cancer bone metastasis specimens

indicated by the immunohistochemical detection of phosphorylated-Smad2 [59]. Consistent with the reports described above, our study showed that the abrogation of autocrine TGF- $\beta$  pathway in NMuMG-ST cells inhibited the expression of the markers of EMT and decreased their rate of migration. TGF- $\beta$  pathway is believed to regulate the EMT through both Smad and non-Smad pathways. Among the non-Smad signaling responses, activation of ERK/MAPK pathway and PI3K/Akt pathway was considered to contribute to TGF- $\beta$  induced EMT [3]. Our results also confirmed that decreased phosphorylation of ERK and Akt are associated with the inhibition of EMT in the DNR11 cells.

Emerging studies have shown that many tumors, including breast cancers, contain a sub-population of cells identified by their ability to self renew and multipotency. These cells are known as cancer stem cells (CSCs), which exhibit tumorigenic and drug resistance phenotypes both in vitro and in vivo [60]. The metastatic process of cancer cells is very similar to the processes of tissue repair and regeneration by stem cells [61], which might imply a relationship between metastatic ability and stem cell function of cancer cells. A recent study suggests that cells, which have undergone an EMT, behave in many respects similar to stem cells isolated from normal or neoplastic cell populations [29]. Our results also indicate that the EMT is associated with the property of cancer stem cells in NMuMG-ST cells. For example, the attenuation of EMT induced by the abrogation of autocrine TGF- $\beta$  pathway, evidenced by the downregulation of vimentin and upregulation of E-cadherin, was associated with the reduced cancer stem cell population as indicated in the mammosphere assay and decreased expression of Notch1 and Gli1 expressions which are involved in the self-renewal pathways of cancer stem cell maintenance.

Metastases are believed to be the major cause of death in breast cancer patients. It was reported that 60%-74% of the patients who die of breast cancer were diagnosed with lung metastasis [62]. TGF- $\beta$  pathway has been shown to promoter breast cancer metastases [63,64]. Consistent with these studies, our results showed a reduction of lung metastasis burden in the mice inoculated with the DNR11 cells in comparison to those inoculated with the control cells indicating that the autocrine TGF- $\beta$  pathway is necessary for the cells to maintain their metastatic capacity. Our results indicate that the observed enhanced lung metastasis of the control cells is likely due to the induction of EMT and the increase of metastasis promoting mammary stem cell subpopulation modulated by the autocrine TGF- $\beta$  pathway.

In conclusion, our study shows that autocrine TGF- $\beta$  signaling is involved in the maintenance and survival of a stem-like and metastasis-capable subpopulation in the NMuMG-ST cell line. The results of our study suggest therapeutic utility of targeting autocrine TGF- $\beta$  signaling for the attenuation of cancer stem cell activity and prevention of metastasis of breast cancer.

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