Upregulation of VEGF-A and CD24 Gene Expression by the tGLI1 Transcription Factor Contributes to the Aggressive Behavior of Breast Cancer Cells

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Abstract

The Hedgehog signaling pathway is one of the most dysregulated pathways in human cancers. The glioma-associated oncogene homolog 1 (GLI1) transcription factor is the terminal effector of the Hedgehog pathway, frequently activated in human breast cancers and an emerging target of breast cancer therapy. While somatic mutations in the human GLI1 gene have never been reported in any cell or tumor type, we recently uncovered the existence of a novel alternatively spliced, truncated GLI1 (tGLI1) that has an in-frame deletion of 41 codons spanning the entire exon 3 and part of exon 4 of the GLI1 gene. Using glioblastoma models, we showed that tGLI1 has gained the ability to promote glioblastoma migration and invasion via its gain-of-function transcriptional activity. However, the pathological impact of tGLI1 on breast cancer remains undefined. Here, we report that tGLI1 is frequently expressed in human breast cancer cell lines and primary specimens we have examined to date, but is undetectable in normal breast tissues. We found for the first time that tGLI1, but not GLI1, binds to and enhances the human vascular endothelial growth factor-A (VEGF-A) gene promoter, leading to its upregulation. Consequently, tGLI1-expressing MDA-MB-231 breast cancer cells secret higher levels of VEGF-A and contain a higher propensity, than the isogenic cells with control vector and GLI1, to stimulate *in vitro* angiogenesis of human vascular endothelial cells. We further showed that tGLI1 has gained the ability to enhance the motility and invasiveness of breast cancer cells in a proliferation-independent fashion and that this functional gain is associated with increased expression of migration/invasion-associated genes, CD24, MMP-2 and MMP-9. tGLI1 has also acquired the property to facilitate anchorage-independent growth of breast cancer cells. Collectively, our results define tGLI1 as a gain-of-function GLI1 transcription factor and a novel mediator of the behavior of clinically more aggressive breast cancer.

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Keywords

Hedgehog pathway; GLI1; tGLI1; breast cancer; angiogenesis; migration and invasion; VEGF

INTRODUCTION

The glioma-associated oncogene homolog 1 (GLI1) zinc-finger transcription factor is the terminal effector of the Hedgehog pathway playing a central role in many physiological processes (Zhu and Lo 2010). GLI1 has been shown to be constitutively activated in breast cancer (Kasper et al 2009) and associated with poor survival of breast cancer patients (ten Haaf et al 2009). The Hedgehog pathway is also important for differentiation and normal development (Dahmane and Ruiz i Altaba 1999, Dahmane et al 2001, Echelard et al 1993, Roelink et al 1994) and is implicated in tumorigenesis (Clement et al 2007, Dahmane et al 2001, Fiaschi et al 2009, Rao et al 2004), vascular development (Lawson et al 2002, Nagase et al 2008) and stem cell self-renewal (Liu et al 2006). The pathway is highly complex that can be activated following binding of Sonic hedgehog (Shh) to its receptor patched, PTCH (Zhu and Lo 2010). PTCH is a repressor of a 7-transmembrane receptor smoothened, SMO. Shh-binding to PTCH derepresses SMO which, in turn, activates the release of GLI1 transcription factor from SUFU-mediated cytoplasmic sequestration (Kasper et al 2006, Kogerman et al 1999). The released GLI1 translocates into the nucleus and binds to the GLI1-binding element in GLI1-regulated genes, leading to their activation (Kinzler and Vogelstein 1990). Both SMO and GLI1 have emerged as therapeutic targets for breast cancer (Kameda et al 2009, Mukherjee et al 2006, Yang et al 2010). An orally active small molecule SMO inhibitor, GDC-0449, is evaluated in a phase I clinical trial for advanced breast cancer (NCT01071564).

Although the human GLI1 gene was initially identified as an amplified gene from a glioblastoma cell line (Kinzler et al 1987), no GLI1 gene amplification has been reported in breast cancer. Somatic mutations are absent in the human GLI1 gene in any cell or tumor type. Until very recently, the human GLI1 gene transcript was found to be alternatively spliced to yield two shorter isoforms, namely, GLI1 N (Shimokawa et al 2008) and truncated GLI1 (tGLI1) that was identified in our laboratory (Lo et al 2009). GLI1 N contains a large deletion of 128 codons, lacks two functional domains, behaves as a weak GLI1 transcription factor and is expressed in both normal and cancerous cells (Shimokawa et al 2008). The novel tGLI1variant we recently identified contains a small in-frame deletion of 123 bp (41 codons) spanning the entire exon 3 and part of exon 4, and retains functional domains and functionality of GLI1, including, the ability to undergo nuclear import and to activate GLI1-targeted genes, such as, PTCH (Lo et al 2009). Our functional characterization using glioblastoma models showed that tGLI1 is highly expressed in malignant gliomas but is absent in normal brain and other normal tissues, and that tGLI1 gains the ability to regulate expression of genes that are not regulated by GLI1, such as, CD24. We also showed that tGLI1 promotes glioblastoma cell migration and invasion (Lo et al 2009).

However, the expression frequency, functional consequences and pathological role of tGLI1 in breast cancer have not been investigated. To address this knowledge gap, we undertook a series of biochemical and genetic studies aiming to define the pathological significance of tGLI1 in breast cancer. Importantly, our findings indicate that tGLI1 behaves as a gain-of-function GLI1 transcription factor in breast cancer and regulates genes that are not targeted by GLI1. This gained property enables tGLI1 to mediate several important phenotypes of more aggressive breast cancer, such as, angiogenesis, migration and invasion, and anchorage-independent growth that mimics mammary tumorigenesis. The findings of this study establish the first evidence that implicates tGLI1 in the malignant behavior of more aggressive breast cancer.

RESULTS

The novel tGLI1 variant is frequently expressed in human breast cancer cells

We found the tGLI1 transcript to be expressed in some of the human breast cancer cell lines and primary specimens we had examined to date (Fig. 1A). In contrast, tGLI1 was undetectable in normal mammary cells and tissues. All PCR products were excised from the agarose gels and subjected to DNA sequencing to confirm tGLI1 versus GLI1 isoforms. The observed expression pattern of tGLI1 is distinctly different that of GLI1 who is expressed in both cancerous and normal breast. As shown in Fig. 1B, the encoded tGLI1 protein (146 kD) can be detected in the majority of human breast cancer cell lines and distinguished from the GLI1 protein (150 kD) using prolonged 5.5% SDS-PAGE followed by western blotting with an antibody that recognizes both isoforms. The expression status of ER, PR and HER2 (Kao et al 2009) and metastatic potential of each cell line are listed at the bottom of Fig. 1B. An obvious association between the receptors/metastatic potential and tGLI1 was not observed.

tGLI1 is associated with increased VEGF-A gene expression in human breast cancer and medulloblastoma cells

To investigate tGLI1 functionality in breast cancer, we created three isogenic MDA-MB-231 human breast cancer cell lines that stably express control vector (MDA-MB-231-vector), GLI1 (MDA-MB-231-GLI1) and tGLI1 (MDA-MB-231-tGLI1). We chose MDA-MB-231 cells, in part, because of their high degrees of invasiveness and high metastatic potential. As shown in Fig. 2A, the levels of transgenes are comparable to those in two natural breast cancer cells, namely, MCF-7 cells that express only the wild-type GLI1 and SK-BR-3 cells that express both isoforms. Shh ligand has been shown to increase vascularity (Kusano et al 2005, Nagase et al 2008); however, the mechanisms underlying this regulation are still unclear. Since tGLI1/GLI1 are effectors of the shh pathway and VEGF-A stimulates endothelial cell proliferation leading to angiogenesis, we explored if tGLI1 regulates VEGF-A expression. As shown in Fig. 2B, we found that the VEGF-A gene is expressed at a higher level in tGLI1-expressing MDA-MB-231 cells than those with control vector and GLI1. The observed low level of VEGF-A expression is in line with a previous report showing that MDA-MB-231 cells expressed a low level of VEGF-A (Timoshenko et al 2006). The observation of tGLI1-induced VEGF-A expression was further made in human breast cancer HCC38 cells and human medulloblastoma D458 cells (Fig. 2C). We also observed in breast cancer HCC1937 cells that GLI1 and tGLI1 similarly

upregulate VEGF-A expression. These results suggest that VEGF-A gene regulation by tGLI1 and GLI1 is complex and is likely dependent on cellular context. In Fig. 2D, we further showed that human breast cancer cell lines with low tGLI1 levels expressed lower levels of VEGF-A than those with high levels of tGLI1, in which levels of tGLI1 were determined previously in Fig. 1B. Collectively, we show in Fig. 2 that tGLI1 is associated with increased VEGF-A gene expression in human breast cancer and medulloblastoma cells.

tGLI1 has gained the ability to bind to and activate the human VEGF-A gene promoter

Using the quantitative chromatin immunoprecipitation assay (regular ChIP assay in Fig. 3A and quantitative ChIP assay in Fig. 3B), we further observed that tGLI1, but not GLI1, significantly binds to the human VEGF-A gene promoter. To determine whether tGLI1 and GLI1 differ in their ability to transactivate the VEGF-A promoter, we engineered a luciferase reporter construct that is under the transcriptional control of the human VEGF-A gene promoter and a TATAA Box (top panel in Fig. 3C). Using this reporter construct, transfections and luciferase assay, we found that the VEGF-A promoter activity is significantly higher in tGLI1-carrying MDA-MB-231 cells than the isogenic control and GLI1-expressing cells (lower panel in Fig. 3C). Collectively, these results indicate that tGLI1 has gained the ability to upregulate VEGF-A gene expression. Notably, we provide evidence to indicate that the human VEGF-A gene is a novel transcriptional target of the tGLI1 transcription factor.

tGLI1-expressing breast cancer cells secret high levels of VEGF-A and promote *in vitro* angiogenesis of human vascular endothelial cells

To determine whether tGLI1-expressing MDA-MB-231 cells secret more VEGF-A than the control and GLI1-carrying isogenic cells, we conducted enzyme-linked immunosorbent assay (ELISA) to measure VEGF-A concentrations in the culture medium. As shown in Fig. 4A, MDA-MB-231-tGLI1 cells secret higher levels of VEGF-A compared to MDA-MB-231-vector and MDA-MB-231-GLI1 cells. Consistent with this observation, conditioned medium from cultured MDA-MB-231-tGLI1 cells strongly promotes the ability of human umbilical vein endothelial cells (HUVECs) to form capillary-like structure that mimics *in vivo* angiogenesis (Fig. 4B). We then quantified *in vitro* angiogenesis by measuring total tubule length (Fig. 4C, left panel) and the number of branch points (right panel). And the results indicate that the tGLI1- expressing MDA-MB-231 cells had a higher propensity to promote *in vitro* angiogenesis of HUVEC cells compared to their isogenic counterparts. Since VEGF-A primarily binds to VEGFR-1/2, we determined the levels of all VEGFRs in HUVEC cells and found that these cells only express VEGFR-1/2 but not VEGFR-3 (Supplemental Fig. 1). These results show, for the first time, that tGLI1 enhances VEGF-A secretion of breast cancer cells, leading to an enhancement of *in vitro* angiogenesis of human vascular endothelial cells.

tGLI1 significantly enhances breast cancer cell invasion and motility, independent of proliferation

In light of the ability of tGLI1 to promote glioblastoma cells migration and invasion (Lo et al 2009), we investigated whether this gain-of-function property of tGLI1 can be observed in

breast cancer cells. This avenue is important because of the facts that increased motility and invasiveness are the major characteristics of metastatic breast cancer and that metastasis is the leading cause of breast cancer mortality. Using the scratch-wound assay that measures cell motility (Fig. 5A), we found that MDA-MB-231-tGLI1 cells are significantly more migratory than MDA-MB-231-vector and MDA-MB-231-GLI1 cells. Increased migration is indicated by the high Migration Index (Im) derived from MDA-MB-231-tGLI1 cells. Next, we determined whether tGLI1 expression alters the proliferation rate of MDA-MB-231 cells and found that both tGLI1 and GLI1 increase cell proliferation after 48 hrs in culture (Fig. 5B). However, the tGLI1- and GLI1-expressing MDA-MB-231 cells showed similar growth rate. Since the scratch-wound assay was conducted for 48 hrs, the tGLI1-mediated increase in migration occurred independent of cell proliferation. Furthermore, we conducted the invasion transwell assay that measures the ability of tumor cells to invade through basement membrane extracts over 48 hrs (Fig. 5C,D) or 18 hrs (Supplemental Fig. 2). And the staining results indicate that MDA-MB-231-tGLI1 cells are more invasive than the isogenic counterparts. Following normalization against cell proliferation rates, the quantitative invasion assay (Fig. 5D) showed the net invasiveness (invasion:proliferation) of MDA-MB-231-tGLI1 cells to be approximately 4-fold higher than those of MDA-MB-231-vector and MDA-MB-231-GLI1 cells. Together, these results indicate that tGLI1 has acquired the ability to promote the propensity of human breast cancer cells to migrate and to invade, independent of proliferation.

tGLI1 is associated with increased expression of CD24, MMP-2 and MMP-9, but not CD147 or heparanase in breast cancer cells

To determine the molecular mechanisms underlying tGLI1-mediated breast cancer motility and invasion, we examined the effects of tGLI1 (and GLI1) on expression of three genes known to promote tumor invasion, migration and/or metastasis, namely, CD24, CD147 and heparanase. CD24 is a mucin-type glycoprotein and a ligand for a Ca^{2+}-dependent endogenous lectin, P-selectin (Aigner et al 1997) that recruits adhesion molecules to lipid rafts and promotes cell migration, invasion and metastasis (Baumann et al 2005, Kristiansen et al 2003, Runz et al 2008). CD24 is overexpressed in breast cancer and associated with metastasis and shortened patient survival (Kristiansen et al 2003, Lee et al 2009). Accumulation of lactate within tumors has been correlated with poor clinical outcomes. CD147 is a chaperone to some monocarboxylate transporters that influence substrate availability, the metabolic path of lactate and pH balance within the tumor, thereby contributing to tumor progression and metastasis (Kennedy and Dewhirst 2010). Heparanase is a multi-functional protein that (i) cleaves heparan sulfate chains of extracellular matrix proteins, leading to disruption of basement membrane structure and subsequent migration of endothelial cells to tumors, (ii) releases heparan sulfate-bound angiogenic factors bFGF and VEGF, and (iii) generates heparan sulfate that enhances bFGF:FGFR binding (Vlodavsky et al 2002, Vreys and David 2007).

Via gene expression profiling, we previously identified the human CD24 gene to be upregulated in tGLI1-expressing glioblastoma cells (Lo et al 2009). Using isogenic MDA-MB-231 cells, we found that CD24 gene expression is upregulated in MDA-MB-231-tGLI1 cells compared to those in MDA-MB-231-vector and MDA-MB-231-GLI1 cells (Fig. 6A,

left panel). In contrast, levels of CD147 and heparanase are similar in three isogenic cell lines (right panel). The ability of tGLI1 to upregulate CD24 gene expression is also observed in two additional human breast cancer cell lines, namely, MCF-7 and MDA-MB-468 (Fig. 6B). In line with these observations, CD24 gene promoter activity is significantly higher in MDA-MB-231-tGLI1 cells than in MDA-MB-231-vector and MDA-MB-231-GLI1 cells (Fig. 6C). In contrast, both tGLI1 and GLI1 activate the 8×3′Gli-BS luciferase reporter that is under the transcriptional control of eight copies of the consensus GLI1-binding element (Fig. 6D). Next, we asked whether mouse mammary carcinoma cells expressed endogenous tGLI1 and whether transient expression of human tGLI1 activates mouse CD24 gene. The results in Fig. 6E indicate that 4T1 mouse mammary carcinoma cells expressed endogenous mouse tGLI1; however, the human tGLI1 does not elevate mouse CD24 gene expression. Similarly, human tGLI1 does not upregulate mouse VEGF-A gene expression. These results suggest that human tGLI1 may not regulate mouse genes in the same fashion as its human target genes.

Furthermore, we found that MMP-2 is upregulated by tGLI1 in three of the four breast cancer cell lines we had analyzed and that MMP-9 level was increased by tGLI1 in all four cell lines (Fig. 6F). Both MMP-2 and MMP-9 are important factors that promote cancer invasion (Ahmad et al 2009, Gondi et al 2004). Noticeably, MMP-2 expression in MCF-7 cells was not altered by tGLI1 or GLI1 while in HCC38 cells, both tGLI1 and GLI1 upregulate MMP-2 expression. These observations indicate that the regulation of MMP-2 by tGLI1 and GLI1 is complex and likely to be dependent on cellular context. Collectively, results of Fig. 6 indicate that tGLI1 is associated with increased gene expression of CD24, MMP-2 and MMP-9, and has retained the capability to activate a wild-type GLI1-targeted promoter.

tGLI1 enhances anchorage-independent growth of breast cancer cells

In light of the association of the Hedgehog pathway with tumorigenesis, we next investigated whether tGLI1 alters breast cancer anchorage-independent growth, a property that mimics tumorigenesis. Using colony formation assays in the absence (Fig. 7A) and presence (Fig. 7B) of soft agar, we found that subpopulations of the MDA-MB-231-tGLI1 cells (about 3-5%) formed more and bigger colonies in both assays, compared to control cells and those with GLI1. Notably, tGLI1-expressing MDA-MB-231 cells produced colonies in a dose-dependent fashion, 10-14 days after initial seeding (Fig. 7A). In the presence of soft agar (Fig. 7B, left panel), MDA-MB-231-tGLI1 cells produced visible large colonies approximately six weeks after initial seeding. In the right panels of Fig. 7B, we show two representative high-resolution (20X) images from two different microscopic fields, in which MDA-MB-231-tGLI1 cells clearly formed larger colonies than the isogenic counterparts.

DISCUSSION

We reported the discovery of the novel tGLI1 isoform and characterized its functionality in glioblastoma in our 2009 study (Lo et al 2009). In this current study, we further addressed the importance of tGLI1 in breast cancer, in particular, in the more aggressive phenotypes of

breast cancer. The results of this study indicate that tGLI1 has the ability to enhance the expression of four cancer-associated genes, VEGF-A, CD24, MMP-2 and MMP-9 in some breast cancer cell lines, and to promote several pathological processes in breast cancer cells, including *in vitro* angiogenesis, migration and invasion, and anchorage-independent growth. Our results also establish the human VEGF-A gene as a novel transcriptional target of the tGLI1 transcription factor. These observations collectively implicate tGLI1 in breast cancer growth and progression.

Using MDA-MB-231 cells, we found tGLI1 to be a stronger promoter of *in vitro* angiogenesis than GLI1. This findings are in line with previous studies showing that shh ligand increases vascularity (Kusano et al 2005, Nagase et al 2008) and our results demonstrating that shh can activate tGLI1 transcriptional activity (Fig. 6D). However, our results also suggest that tGLI1-mediated transcriptional regulation of VEGF-A may be dependent on cellular context. In this regard, tGLI1 may interact with another transcription factor to co-regulate gene expression. This speculation also applies to the relationship between tGLI1 and MMP-2. Together, these observations call for a need to gain a deeper understanding of the mechanisms underlying tGLI1- versus GLI1-mediated gene regulation.

Several important future tasks are prompted by our novel finding that tGLI1 enables subpopulations of MDA-MB-231 cells to undergo robust anchorage-independent growth. First, an important line of investigations will be necessary to explore if tGLI1 is associated with breast cancer stemness given that anchorage-independent growth is one of the unique features of cancer stem cells (Charafe-Jauffret et al 2009). Second, it will be essential to investigate whether tGLI1 promotes tumorigenesis and metastasis since breast cancer stem cells have a high potential to initiate tumors and to metastasize (Liu and Wicha 2010). Third, it will also be an important task to gain further insights into the molecular mechanisms by which tGLI1 mediates the anchorage-independent growth of MDA-MB-231 cells. In light of our data showing the ability of tGLI1 to enhance VEGF-A expression and previous reports indicating that MDA-MB-231 cells express high levels of VEGFR-1 (Schmidt et al 2008), it is possible that the VEGF-A-VEGFR-1 autocrine effect contributes to their growth under a non-adherent growth condition. The possibility should be addressed by future work.

Our results indicate that CD24 is a transcriptional target of tGLI1, but not GLI1, in breast cancer. This finding is in agreement with our previous study that showed similar results in glioblastoma models using a combination of DNA microarray and subsequent biochemical validations (Lo et al 2009). Using glioblastoma models, we also showed that transcriptional knockdown of CD24 significantly reduced tGLI1-mediated tumor cell migration and invasion in a proliferation-independent fashion. In line with our observations, CD24 has been shown to be involved in cancer cell motility, invasion and metastasis (Baumann et al 2005, Kristiansen et al 2003, Runz et al 2008) and shortened patient survival (Kristiansen et al 2003, Lee et al 2009). Also consistent with these reports are the facts that CD24 recruits adhesion molecules to lipid rafts (Runz et al 2008) and that CD24 indirectly stimulates cell adhesion to fibronectin, collagens I/IV, and laminin through the activation of integrins (Baumann et al 2005). Interestingly, several studies have shown that some breast cancer-initiating stem cells display the characteristic of being low or negative for CD24 expression but high for CD44 (Al-Hajj et al 2003, Kim et al 2007). This notion is not in agreement with

our results showing that tGLI1 expression enables MDA-MB-231 cells to grow in an anchorage-independent fashion, a property commonly displayed by cancer stem cells. However, in support of our results, a recent report showed that CD24 expression does not define breast cancer stem cells (Meyer et al 2010). Also in agreement with our results, another recent study reported that pancreatic cancer stem cells demonstrate the CD44+CD24+ESA+ phenotype (Li et al 2007). It has been suggested that the role of CD24 in cancer stemness may be tissue-specific (Keysar and Jimeno 2010). Clearly, more work is needed to define the role that CD24 plays in both cancer progression and stem cell biology.

MATERIALS AND METHODS

Reagents, Cell Lines and Primary Specimens

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Human breast cancer cell lines and mouse mammary carcinoma cell line 4T1 were obtained from ATCC (Manassas, VA) and were routinely cultured in DMEM supplemented with 10% fetal calf serum and antibiotics. Stable MDA-MB-231 cell lines were established following G418 selection and were maintained in DMEM supplemented with 700 ug/ml G418 and 10% fetal calf serum. HUVEC cells were maintained in EGM-2® Endothelial Cell Growth Medium (LONZA; cc-3162). Human medulloblastoma D458 cells were obtained from the Preston Robert Tisch Brain Tumor Center at Duke University. Frozen primary breast tumor and normal tissues were obtained from the Breast Cancer SPORE Tissue Bank and have been pathologically verified by the pathologist Dr. Geradts.

RT-PCR

Total RNA isolation and RT were conducted using SV Total RNA Isolation System (Promega) and Superscript II First-Strand cDNA synthesis system (Invitrogen), retrospectively. The forward and reverse primers used for the PCR were: 5′-TGTTCAACTCGATGACCC-3′ and 5′-GTCATGGGGACCACAAGG-3′ (exons 1-4 of GLI1 and tGLI1), 5′-GGCGGCACCACCATGTACCC-3′ and 5′-AGGGGCCGGA CTCGTCATACT-3′ (β-actin), 5′-ATGGGCAGAGCAATGGTGGCCA-3′ and 5′-AGAGTGAGACC ACGAAGAGACT-3′ (human CD24), 5′-ATGGGCAGAGCGATGGTGGCCA-3′ and 5′-AGAAGAG AGAGAGAGAGAGCCA-3′ (mouse CD24), 5′-CACCATCGACAGAACAGTCC-3′ and 5′-GAATCC AATTCCAAGAGGGA-3′ (human VEGF-A), 5′-ATGAACTTTCTGCTCTCTTGGGT-3′ and 5′-ACA CAGGACGGCTTGAAGATGTA-3′ (mouse VEGF-A), 5′-ACAGTCTTCACTACCGTAGA-3′ and 5′-TTCTGACGACTTCACAGCCT-3′ (CD147), 5′-GGTCAGCCTCGAAGAAAGAC-3′ and 5′-TAGCAGT CCGTCCATTCAAA-3′ (heparanase), 5′-AGATCTTCTTCTTCAAGGACCGGTT-3′ and 5′-GGCTGG TCAGTGGCTTGGGGTA-3′ (MMP-2), and 5′-CTGCCAGTTTCCATTCATCT-3′ and 5′-GTCAAAGTT CGAGGTGGTAG-3′ (MMP-9).

Western Blotting (WB)

This was performed as described previously (Lo et al 2008a, Lo et al 2008b). Antibodies used included mouse monoclonal antibodies against β-actin (Sigma) and α-tubulin (Sigma), and rabbit polyclonal antibodies for VEGF-A (Santa Cruz; sc-152), GLI1 (Santa Cruz;

H300) and CD24 (Santa Cruz; FL-80), and goat polyclonal GLI1 antibody (Santa Cruz; C-18).

ChIP Assay to Determine Binding of tGLI1 to the VEGF-A Promoter

This was performed using a ChIP Assay Kit (Upstate, Billerica, MA) as we described previously (Lo et al 2005). A rabbit polyclonal GLI1 antibody (Santa Cruz; H300) that recognizes the COOH-terminal region (aa 781-1080) present in both GLI1 and tGLI1 proteins was used in these experiments. DNA sequences for the primers used to amplify the VEGF-A promoter are 5′-GCACATTGTCAGAGGGACAC-3′ (forward) and 5′-CCCTATTTCTGACCTCCCAA-3′ (reverse).

Plasmids, Transfection and Luciferase Assay

Construction of the GLI1- and tGLI1-expressing plasmids was described in our previous study (Lo et al 2009). The GLI1-binding sites-driven luciferase construct, 8×3′Gli-BS Luc, was generously provided by Dr. Hiroshi Sasaki at Osaka University, Japan (Sasaki et al 1997). The reporter construct pCD24-0.25kb-Luc was engineered in our previous study (Lo et al 2009). To generate the pGL3-VEGF-A-Luc reporter, the promoter region of the human VEGF-A (−900 to +100 bp) was cloned into the pGL3-basic Firefly luciferase vector (Promega). All transfections were performed with cells in exponential growth using lipofectamine 2000 (Invitrogen) and FuGENE HD (Roche). A Renilla luciferase expression vector, pRL-CMV was used to control for transfection efficiency. Forty-eight hrs after transfection, the cells were lysed and luciferase activity measured using the Firefly and Renilla Luciferase Assay Kit (Biotium, Hayward, CA), as we previously described (Lo et al 2005, Lo et al 2007, Lo et al 2008a, Lo et al 2008b). Relative promoter activity was computed by normalizing the Firefly luciferase activity against that of the Renilla luciferase.

ELISA that Measures VEGF-A Concentrations in Culture Medium

This was conducted using the VEGF-A ELISA Kit from R&D, according to manufacturer's instructions. Breast cancer cells seeded in 24-well culture plates were incubated in the EBM-2 Basal Medium (LONZA) at 37°C. After 24 hrs, conditioned medium was collected and centrifuged at 1,200 xg for 10 min to remove cell debris. The supernatants were then subjected to ELISA in triplicates. Absorbance at 540 nm (and 450 nm as normalization background) was determined using the Synergy HT Multi-mode Microplate Reader (BioTek). VEGF-A concentrations were computed with reference to standard curves derived from purified VEGF-A supplied in the ELISA Kit.

Tubule Formation Assay that Measures *In Vitro* Angiogenesis of Vascular Endothelial Cells

Tubule formation assay was performed using the *In Vitro* Angiogenesis Kit from Trevigen. Briefly, 96-well culture plates were coated with 50 ul Cultrex® Basement Membrane Extracts per well and incubated at 37°C for 1 hr or until the gel has solidified. A total of 5,000 HUVEC cells were then seeded into each coated well and incubated with EBM-2 Basal Medium (LONZA) with and without supplementation of growth factor (EGM-2 BulletKit; LONZA) or with tumor conditioned medium. Conditioned medium was collected

from stable MDA-MB-231 cells that have been starved in EBM-2 Basal Medium for 24 hrs. After incubation at 37°C for 4-6 hrs, HUVEC cells were examined for capillary-like network formation and photographed under a light microscope. Images were taken from 3-5 different fields in each well. Tubule formation was quantified by measuring total tubule length and total number of branch points in triplicate wells, using the NIH Image J software according to a published protocol (Dasari et al 2010).

Scratch-Wound Assay that Determines Cell Migration

Breast cancer cells seeded in 6-well culture plates were scratched via a pipette tip to create a gap, incubated at 37°C and imaged every 2 hrs using a digital camera attached to a Carl Zeiss microscopy. Gap width was determined from the images taken using AXIO 4.0 software. For each gap, the average width was computed from three measurements taken at the top, middle and bottom end of the microscopic field. The migratory index, I_m, was computed via the equation: $I_m = (g_0-g_t)/g_0$ (g_0 = gap width at time zero; g_t = gap width at time t).

Cell Proliferation Assay

This was performed, as we described previously (Lo et al 2008a, Lo et al 2008b), using the CellTiter Blue Cell Viability Assay kit (Promega), a fluorescent method that is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Briefly, the cells were seeded on 96-well cell culture plates (4×10^3/well) and at 0, 24, 48, and 72 hrs, the degrees of cell viability were determined.

Invasion Assay

This was conducted as we previously described using the InnoCyte™ Quantitative Cell Invasion Assay kit (EMD) (Lo et al 2009). Briefly, 1.75×10^5 cells were placed in the upper chamber of re-hydrated inserts with an 8-μm pore size polycarbonate membrane coated with a uniform layer of basement membrane matrix on the upper surface. Following incubation for 48 hrs, the medium in the upper chamber was discarded and the inserts placed in fresh wells containing fluorescent Calcein-AM cell staining/detachment buffer. Aliquots of the fluorescence-stained dislodged cells were transferred to duplicate wells of a 96-well cell culture plate and the fluorescence measured. The inserts containing the breast cancer cells invaded through the basement membrane matrix were stained in 0.5% crystal violet. Cell proliferation rates were simultaneously determined (Lo et al 2008a, Lo et al 2009, Zhu et al 2010) to derive "invasiveness:proliferation" ratios that indicate net invasiveness.

Determination of Anchorage-Independent Growth by the Colony Formation Assays

Clonogenic growth assays were performed in 6-well cell culture plates with 500, 1,000 and 2,000 cells per well, as we previously described (Lo et al 2005, Lo et al 2010). For the experiments with soft agar, 1,000 cells were seeded in each well and all wells were pre-coated with 0.5% agarose as the bottom layer whereas the top layer is consisted of 0.3% agarose and tumor cells. Following 10-14 days (without soft agar) and 6 weeks (with soft agar), colonies were stained with crystal violet blue solution (Sigma) for 1 hr and counted

under a microscope. Triplicate wells were used for each cell line and three independent experiments were performed.

Statistical Analysis

The student *t*-test was performed using STATISTICA (StatSoft Inc., Tulsa, OK) and Microsoft Excel, as we previously described (Lo et al 2008a, Lo et al 2009, Zhu et al 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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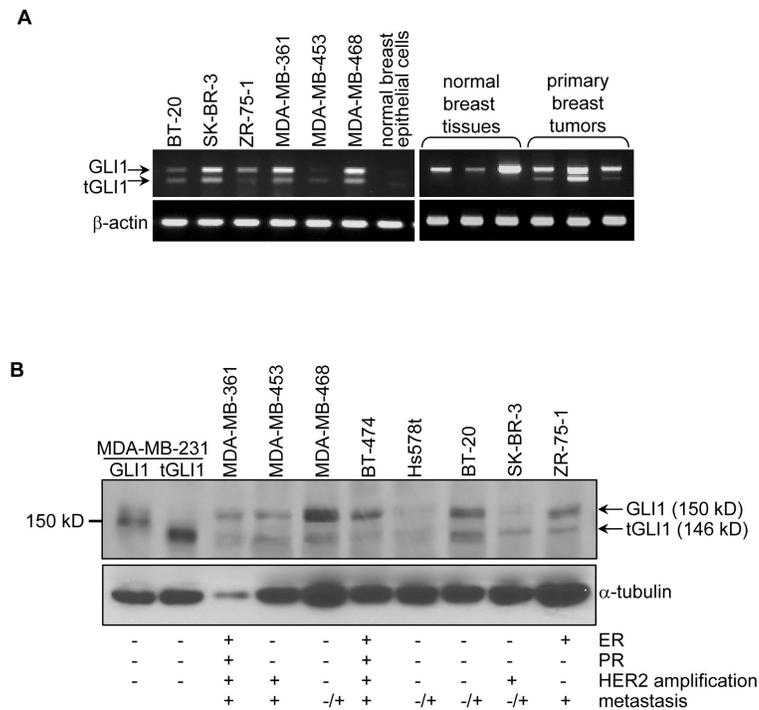
Figure 1. The novel tGLI1 variant is frequently expressed in human breast cancer cells

A. tGLI1 transcript is detected in cell lines and specimens of breast cancer, but not in normal breast cells and tissues. RT-PCR was conducted to amplify exons 1-4 region of the GLI1/tGLI1 transcripts. All PCR products were extracted and DNA sequenced.

B. tGLI1 protein (146 kD) can be detected in the majority of human breast cancer cell lines and distinguished from the GLI1 protein (150 kD) using prolonged 5.5% SDS-PAGE followed by western blotting with an antibody that recognizes both isoforms. The expression status of ER, PR and HER2(Kao et al 2009) and metastatic potential are listed at the bottom of Fig. 1B.
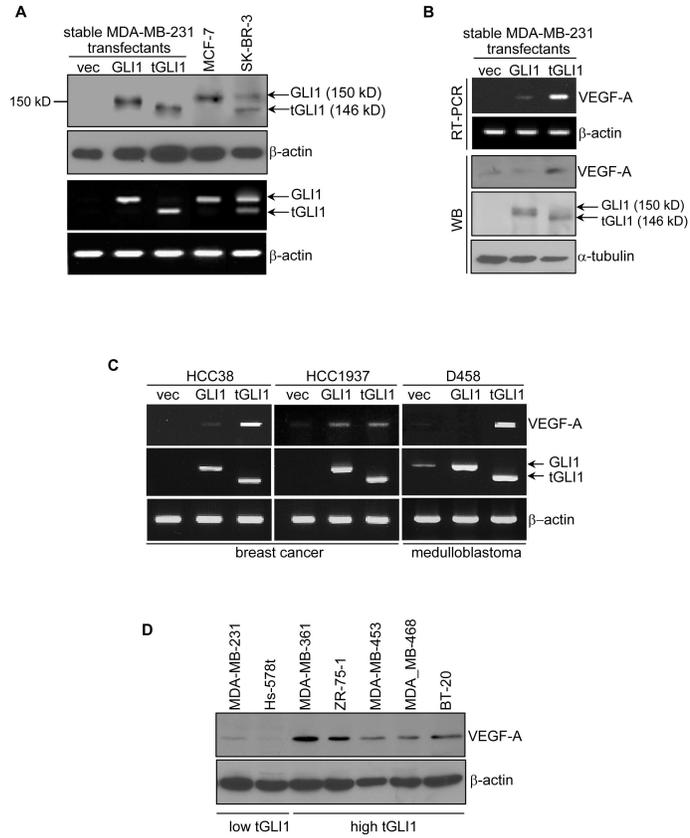
Figure 2. tGLI1 is associated with increased VEGF-A gene expression in human breast cancer and medulloblastoma cells

A: Isogenic MDA-MB-231-GLI1 and MDA-MB-231-tGLI1 cell lines express GLI1 and tGLI1, respectively, at levels similar to those in natural breast cancer cells. The encoded tGLI1 protein (146 kD) was distinguished from the GLI1 protein (150 kD) using prolonged 5.5% SDS-PAGE followed by WB with an antibody that recognizes both GLI1 and tGLI1 isoforms. Top, WB. Bottom, RT-PCR.

B. VEGF-A gene is expressed at a higher level in tGLI1-expressing MDA-MB-231 cells than those with control vector and GLI1. Top, RT-PCR. Bottom, WB.

C: Expression of VEGF-A in breast cancer and medulloblastoma cells transiently transfected to express GLI1 or tGLI1, as shown by RT-PCR.

D: Human breast cancer cell lines with low tGLI1 levels expressed lower levels of VEGF-A than those with high levels of tGLI1, in which levels of tGLI1 were determined previously in Fig. 1B. WB was conducted.
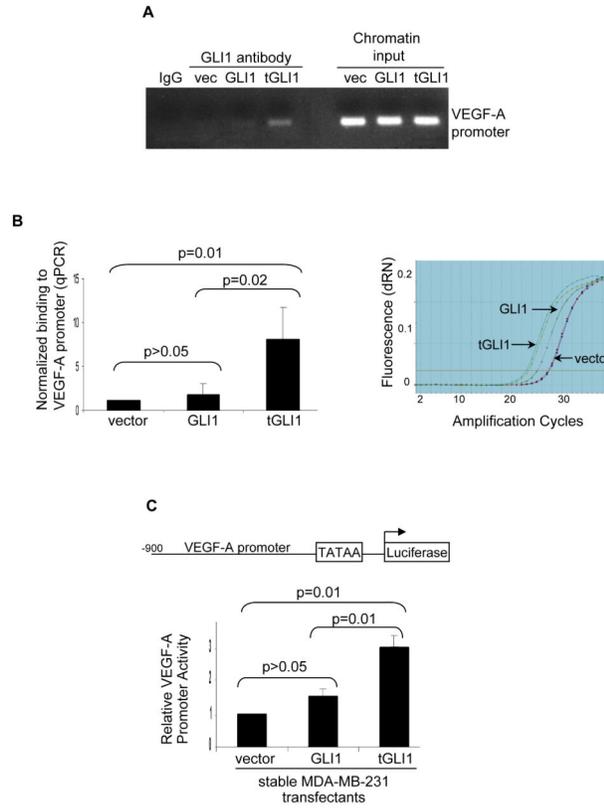
Figure 3. tGLI1 has gained the ability to bind to and activate the human VEGF-A gene promoter
A,B. tGLI1, but not GLI1, binds to the human VEGF-A gene promoter. Regular (A) and quantitative (B) ChIP assay was used to determine the extent to which tGLI1 and GLI1 bind to the human VEGF-A gene promoter. In these assays, IgG was used in immunoprecipitation as the negative control for GLI1/tGLI1 antibody whereas chromatin inputs were used in quantitative PCR as the loading controls. The results were derived from three independent experiments and analyzed via the student *t*-test to determine p-values.
C. tGLI1, but not GLI1, transactivates the human VEGF-A gene promoter. The structure of the human VEGF-A promoter-containing luciferase reporter is shown in the top panel. The VEGF-A gene promoter reporter was co-transfected with the control pRL-CMV Renilla luciferase reporter into the isogenic MDA-MB-231 cells. Luciferase activity was measured 48 hrs after transfections. Relative promoter activity was computed by normalizing the Firefly luciferase activity against that of the Renilla luciferase. The VEGF-A gene promoter activity is significantly higher in tGLI1-carrying MDA-MB-231 cells than the isogenic control and GLI1-expressing cells. The results represent those from three independent experiments and were analyzed via the student *t*-test to derive p-values.
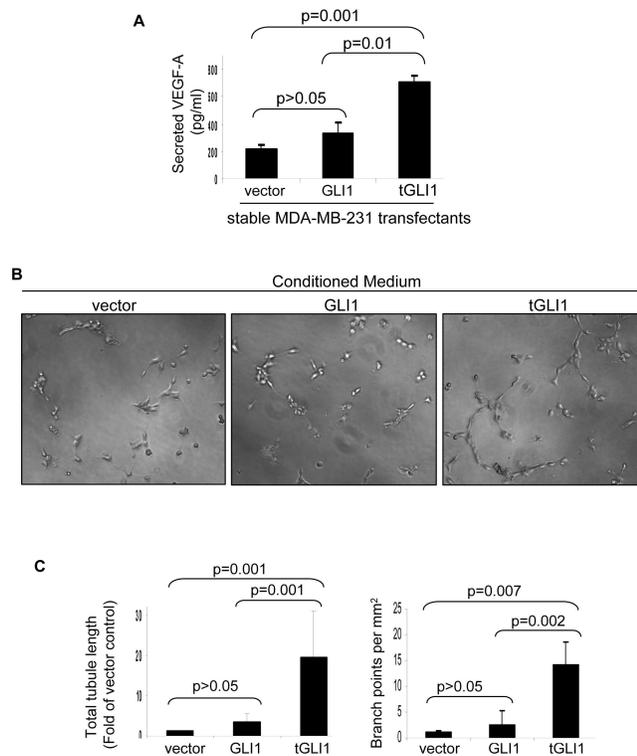
Figure 4. tGLI1-expressing breast cancer cells secret high levels of VEGF-A and promote *in vitro* angiogenesis of human vascular endothelial cells

A. MDA-MB-231-tGLI1 cells secret higher levels of VEGF-A compared to MDA-MB-231-vector and MDA-MB-231-GLI1 cells. ELISA was conducted to measure VEGF-A concentrations in the culture medium. The means and standard deviations were computed from three independent experiments. The student *t*-test was conducted to determine p-values.

B. Conditioned medium from cultured MDA-MB-231-tGLI1 cells strongly promotes the ability of HUVEC cells to form capillary-like structure that mimics *in vivo* angiogenesis. Representative images are shown. **C.** tGLI1 increases the ability of breast cancer cells to promote *in vitro* angiogenesis. *In vitro* angiogenesis was quantified by measuring total tubule length (left panel) and the number of branch points (right panel). The results represent the means and standard deviations of four independent experiments. The student *t*-test was conducted to determine p-values.
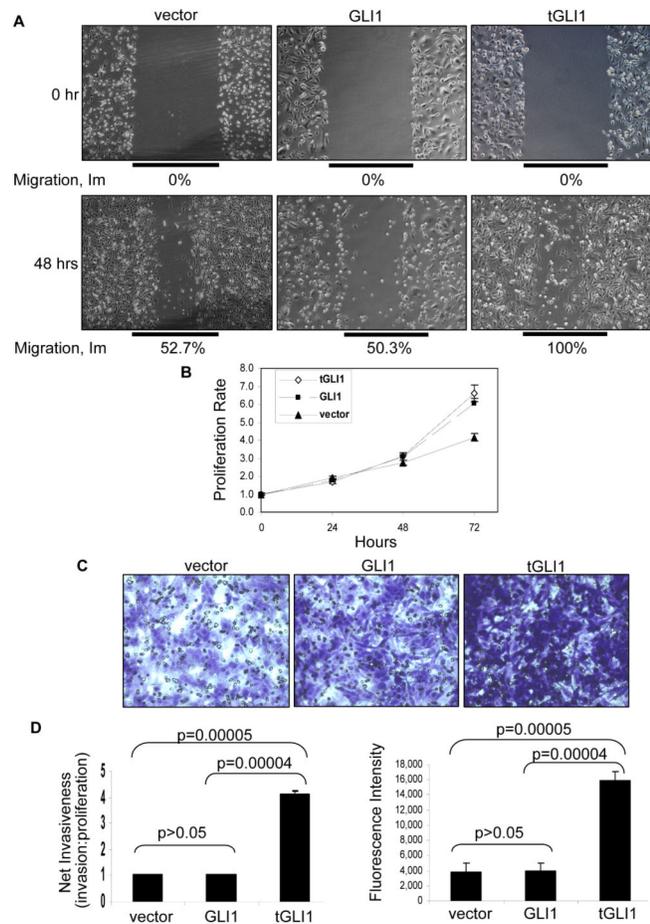
Figure 5. tGLI1, but not GLI1, significantly enhances breast cancer cell invasion and motility, independent of proliferation

A. tGLI1 enhances the motility of MDA-MB-231 cells, independent of proliferation. Isogenic MDA-MB-231 cells were scratched and the Migration Index (Im) was determined 48 hrs after the initial scratch. Im = $(g_0-g_t)/g_0$ (g_0 = gap width at time zero; g_t = gap width at time t). Bars indicate initial gap widths.

B. Proliferation rates of the MDA-MB-231-tGLI1 and MDA-MB-231-GLI1 cells are similar. Using Celltiter Blue Cell Survival assay, we determined the proliferation rates of the isogenic MDA-MB-231 cells over 72 hrs. Both tGLI1 and GLI1 increased MDA-MB-231 cell proliferation after 48 hrs in culture. The tGLI1- and GLI1-expressing MDA-MB-231 cells showed similar growth rate.

C. tGLI1 increases the propensity of MDA-MB-231 cells to invade. Invasion assay was conducted for 48 hrs to measure the ability of tumor cells to invade through basement membrane extracts. Images show invasive cells stained with crystal violet blue.

D. Quantitative invasion assay shows tGLI1 to significantly increase the invasiveness of MDA-MB-231 cells, independent of proliferation. The assay was conducted for 48 hrs. Invasiveness was normalized against cell proliferation rates to derive net invasiveness. The results show that MDA-MB-231-tGLI1 cells were approximately 4-fold more invasive than

MDA-MB-231-vector and MDA-MB-231-GLI1 cells. The results were derived from three independent experiments and were analyzed by the student *t*-test to compute p-values.
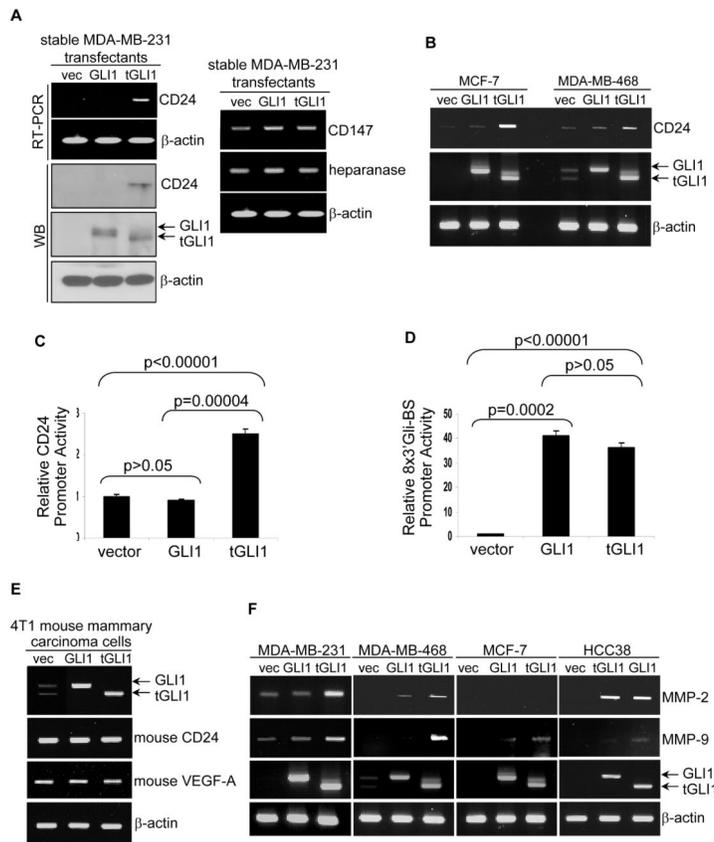
Figure 6. tGLI1 is associated with increased expression of the invasion-, migration-, metastasis-associated genes, CD24, MMP-2 and MMP-9, but not CD147 or heparanase

A. tGLI1 selectively enhances expression of CD24, but not CD147 and heparanase, in breast cancer cells. In WB, GLI1 (150 kD) and tGLI1 (146 kD) proteins were resolved in 5.5% SDS-PAGE.

B. tGLI1, but not GLI1, enhances CD24 expression in transiently transfected MCF-7 and MDA-MB-468 breast cancer cells, as shown by RT-PCR.

C. CD24 gene promoter activity is significantly higher in MDA-MB-231-tGLI1 cells than in MDA-MB-231-vector and MDA-MB-231-GLI1 cells. The pCD24-0.25kb-Luc reporter was co-transfected with the control pRL-CMV Renilla luciferase reporter into the isogenic MDA-MB-231 cells. Luciferase activity was measured 48 hrs after transfections. Relative promoter activity was determined following normalizing the Firefly luciferase activity against that of the Renilla luciferase. The results were derived from three independent experiments and were analyzed by the student *t*-test to compute p-values.

D. Both tGLI1 and GLI1 activate the wild-type GLI1-targeted promoter. The 8×3′Gli-BS luciferase reporter that is under the transcriptional control of eight copies of the consensus GLI1-binding element was co-transfected with the pRL-CMV Renilla luciferase reporter into the three isogenic MDA-MB-231 cell lines. Luciferase activity was determined 48 hrs post transfections. Relative promoter activity was computed by normalizing the Firefly luciferase activity against that of the Renilla luciferase. The results were derived from three independent experiments. The student *t*-test was performed to compute p-values.

E. Endogenous tGLI1 can be detected in 4T1 mouse mammary carcinoma cells. 4T1 cells were transiently transfected with control vector and human GLI1- and tGLI1-carrying expression vectors. After 48 hrs, RT-PCR was conducted to detect both human and mouse tGLI1 transcripts, and those of mouse CD24 and mouse VEGF-A genes. Notably, Endogenous mouse tGLI1 was readily expressed in 4T1 cells. In contrast to the results from human breast cancer cells, human tGLI1 does not affect mouse CD24 and mouse VEGF-A expression in the cells, suggesting that human tGLI1 may not regulate mouse genes in the same fashion as its target genes of the human origin.

F. MMP-2 is upregulated by tGLI1 in three of the four breast cancer cell lines we had analyzed while MMP-9 was increased by tGLI1 in all four cell lines. Noticeably, MMP-2 expression in MCF-7 cells was not altered by tGLI1 or GLI1 while in HCC38 cells, both tGLI1 and GLI1 upregulate MMP-2 expression.
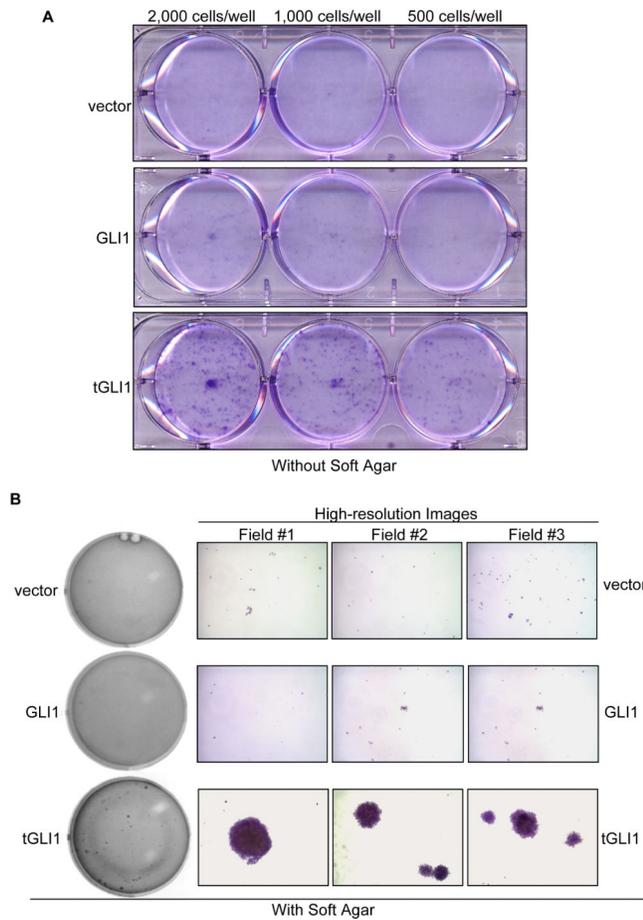
Figure 7. tGLI1 promotes anchorage-independent growth of breast cancer cells
A. Colony formation assay was performed with 500, 1,000 and 2,000 cells/well in the absence of soft agar. Colonies were stained with crystal violet blue 10-14 days after seeding. Notably, MDA-MB-231-tGLI1 cells formed more and bigger colonies than those with GLI1 and vector in a dose-dependent fashion. Experiments were done in triplicates and were repeated twice.

B. Soft agar colony formation assay indicates that MDA-MB-231-tGLI1 cells produced more and bigger colonies than control cells and those with GLI1. 1,000 cells were seeded per well and stained with crystal violet blue approximately six weeks after initial seeding. Left panel shows that tGLI1, but not GLI1, enabled MDA-MB-231 cells to form visible colonies. Right panels show two representative high-resolution (20X) images from two different microscopic fields. Experiments were done in triplicates and were repeated twice.