HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2020 February 28.

Published in final edited form as:

Oncogene. 2020 January ; 39(1): 64–78. doi:10.1038/s41388-019-0959-3.

TGLI1 transcription factor mediates breast cancer brain metastasis via activating metastasis-initiating cancer stem cells and astrocytes in the tumor microenvironment

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Abstract

Mechanisms for breast cancer metastasis remain unclear. Whether truncated glioma-associated oncogene homolog 1 (TGLI1), a transcription factor known to promote angiogenesis, migration and invasion, plays any role in metastasis of any tumor type has never been investigated. In this study, results of two mouse models of breast cancer metastasis showed that ectopic expression of TGLI1, but not GLI1, promoted preferential metastasis to the brain. Conversely, selective TGLI1 knockdown using antisense oligonucleotides led to decreased breast cancer brain metastasis (BCBM) *in vivo*. Immunohistochemical staining showed that TGLI1, but not GLI1, was increased in lymph node metastases compared to matched primary tumors, and that TGLI1 was expressed at higher levels in BCBM specimens compared to primary tumors. TGLI1 activation is associated with a shortened time to develop BCBM and enriched in HER2-enriched and triple-negative breast cancers. Radioresistant BCBM cell lines and specimens expressed higher levels of TGLI1, but not GLI1, than radiosensitive counterparts. Since cancer stem cells (CSCs) are radioresistant and

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Competing Interest: None.

metastasis-initiating cells, we examined TGLI1 for its involvement in breast CSCs and found TGLI1 to transcriptionally activate stemness genes CD44, Nanog, Sox2, and OCT4 leading to CSC renewal, and TGLI1 outcompetes with GLI1 for binding to target promoters. We next examined whether astrocyte-priming underlies TGLI1-mediated brain tropism and found that TGLI1-positive CSCs strongly activated and interacted with astrocytes *in vitro* and *in vivo*. These findings demonstrate, for the first time, that TGLI1 mediates breast cancer metastasis to the brain, in part, through promoting metastasis-initiating CSCs and activating astrocytes in BCBM microenvironment.

Keywords

TGLI1; breast cancer; cancer stem cells; brain metastasis; astrocytes

INTRODUCTION

Breast cancer is the second leading cause of cancer-related mortality in women and metastasis to distant organs result in 90% of breast cancer deaths[1]. Metastatic breast cancers occur in 20–30% of cases with a 5-year survival rate of 22%[2]. The most common sites of breast cancer metastases are bone, lung, brain, and liver. HER2-enriched and triple-negative breast cancer (TNBC) subtypes have the highest propensity to metastasize to the brain, ranging from 20–30% in HER2 and 45–60% in TNBC[2]. Breast cancer brain metastasis (BCBM) constitutes ~10–30% of metastatic breast cancer cases[3]. Current therapeutic options for women with BCBM include whole brain radiotherapy, surgical resection, and stereotactic radiosurgery[4]. Despite our current therapies and understanding of BCBM, breast cancer patients with brain metastases have a dismal prognosis of only 6–18 months of survival following diagnosis[3]. Mechanisms underlying BCBM remain poorly understood.

Our laboratory discovered a truncated form of glioma-associated oncogene homolog 1 (TGLI1 or tGLI1) and we have demonstrated that it is a gain-of-function GLI1 zinc-finger transcription factor[5–10]. TGLI1 is an alternatively spliced variant of GLI1, as a result of a 41 amino acid in-frame deletion, which retains all of the functional domains as GLI1, and is activated by the Shh-PTCH1-SMO signaling axis[8]. We have shown that TGLI1 is expressed in GBM and breast cancer, but is not expressed in normal brain or breast tissue[5, 8, 9, 11]. Consistent with our findings, TGLI1 was reported to be expressed in highly invasive hepatocellular carcinoma but is not detected in normal hepatocytes[12]. TGLI1 has the ability to regulate GLI1 target genes but gains the ability to upregulate seven target genes which are not regulated by GLI1[5–10], namely, VEGF-A, VEGF-C, VEGFR2, TEM7, HPSE, CD24, and CD44 resulting in increased growth, angiogenesis, migration and invasion of glioma cells *in vivo* and breast cancer cells *in vitro*. In line with our results, VEGFR2 was reported to be transcriptionally activated by TGLI1 in TNBC cells leading to increased angiogenesis[13]. Whether TGLI1 plays any role in metastasis for any tumor type has never been investigated.

In this study, we aimed to determine whether TGLI1 plays a role in breast cancer metastasis. Through the use of three *in vivo* mouse models of metastasis, we observed that TGLI1

promotes BCBM and facilitates the intracranial growth of breast cancer cells. We further observed that TGLI1 activation correlates with shortened metastasis-free survival and that TGLI1 expression is enriched in BCBM patient samples. Moreover, we found TGLI1 to play an important role in radioresistance and cancer stem cells (CSCs), through upregulating stemness gene expression and that TGLI1 outcompetes GLI1 for binding to target gene promoters. In agreement with an important role for astrocytes in intracranial tumor growth, TGLI1-positive CSCs strongly activate and interact with astrocytes and TGLI1-positive BCBM tumors strongly activate astrocytes in mouse brain. Lastly, knockdown of TGLI1 led to decreased cancer stemness *in vitro* and reduced BCBM *in vivo*. Collectively, our study defined TGLI1 as a novel mediator of BCBM, breast CSCs, activation of astrocytes in BCBM microenvironment, and resistance to radiation therapy.

MATERIALS AND METHODS

Cell lines, tumor specimens, and expression plasmids

Human breast cancer cell lines MCF10A, MCF7, and BT20 were purchased from ATCC (Manassas, VA) and cultured as specified by ATCC. HMLE, MDA-MB-231, and brain metastatic MDA-MB-231-BRM cell lines are from the Weinberg and Massagué laboratories, respectively. The brain metastatic cell line (SKBRM), radio-resistant cell lines (SKBRM-RR and 231BRM-RR), and E6/E7/hTERT immortalized human astrocytes were kind gifts from Drs. Fei Xing and Kounosuke Watabe. BC tissue microarrays (BR10010, BR952, BC01116) were purchased from US Biomax (Rockville, MD). BCBM patient specimens are from the Wake Forest Tumor Tissue Core and Dr. Kounosuke Watabe. The expression plasmids for GLI1 and TGLI1 were previously developed in our laboratory[8].

Animal studies

All animal experiments were conducted as approved by the Wake Forest Institutional Animal Care and Use Committee. Isogenic MDA231 lines with vector, GLI1, or TGLI1, and SKBRM with negative control oligos or TGLI1-AS-ON (1×10^5 cells) were injected into the left cardiac ventricle of female *nu/nu* mice and monitored bi-weekly using an *In Vivo* Imaging System (IVIS). Intracranial implantations were performed as we described previously[9]. Briefly, female *nu/nu* mice were anesthetized with ketamine/xylazine and isogenic cells (2×10^4 cells/5 uL PBS) were stereotactically implanted into the right frontal lobe of the brain. Mice were monitored weekly using IVIS.

Selective knockdowns of GLI1 and TGLI1

Control or TGLI1 specific locked nucleic acid (LNA) antisense oligonucleotides (AS-ON) were custom designed and purchased from Qiagen. Knockdown of TGLI1 was conducted as described previously[9]. Briefly, BT20 or SKBRM cells were transfected for 48 hours with control or TGLI1 AS-ON using Lipofectamine 2000 (Invitrogen) and then cells were harvested for qRT-PCR, seeded for mammosphere assay, or intracardially injected into female *nu/nu* mice described above.

Statistical analyses

Results are represented as ± SE. One-way ANOVA, Fisher's Exact Test, Log-Rank test, and student's *t*-test were performed using GraphPad Prism 5.

RESULTS

TGLI1 promotes brain metastasis of TNBC in an intracardiac injection metastasis mouse model

A role of TGLI1 in metastasis in any tumor type has not been investigated. To determine the effects of TGLI1 on metastasis, we generated isogenic TNBC MDA-MB-231 cell lines to overexpress TGLI1, GLI1 or control vector using lentiviral constructs[9] and the firefly luciferase reporter. Stable expression of TGLI1 or GLI1 was confirmed using western blotting (Fig. 1A). Exogenous GLI1 and TGLI1 were expressed at physiologically relevant levels as indicated by comparable levels of GLI1 and TGLI1 in mammary fat pad (MFP) xenografts and primary breast carcinoma samples (Fig. 1B). Immunohistochemistry was conducted using TGLI1- and GLI1-specific antibodies we previously developed and validated[6, 7, 9, 10]. Of note, commercially available GLI1 antibodies detect both GLI1 and TGLI1 isoforms. Expression of TGLI1 in MDA-231-TGLI1 MFP tumor was equivalent to that detected in breast cancer PDXs as indicated by western blots (Fig. 1C); BCM-2174 (TNBC) and BCM-3963 (HER2-enriched) PDXs were shown to undergo brain metastasis[14]. We next used an experimental metastasis model to mimic several stages of metastasis including evasion of anoikis, intravasation, and colonization to distant organs, mainly to the brain and bone. For this, the isogenic lines were injected into the left ventricle of athymic female mice and the mice were imaged biweekly using an In Vivo Imaging System (IVIS). Representative bioluminescent images showed TGLI1 preferentially increased breast cancer metastasis to the brain compared to GLI1 and vector groups (Fig. 1D). To confirm bioluminescent signals were strictly from the brain or bone, but not surrounding tissues, we imaged the brain and bones *ex vivo* and we observed TGLI1 mice to have increased brain metastasis (Fig. 1E–G), but reduced bone metastasis compared to the vector group (Fig. 1F–G). We further found the TGLI1 group to have the greater number of tumor foci among the three groups (Fig. 1H–I). These results suggest that TGLI1 promotes brain metastasis of circulating breast cancer cells.

TGLI1 promotes the growth of TNBC in mouse brain in an intracranial injection mouse model

We next evaluated the effects of TGLI1 on breast cancer intracranial growth using an intracranial implantation model. The isogenic MDA-MB-231 cell lines were implanted into the brain of female athymic mice and imaged weekly. The results showed TGLI1-expressing breast cancer cells were more tumorigenic than those with vector or GLI1 (Fig. 2A–B). TGLI1-positive brain metastases were significantly larger than GLI1 and vector tumors (Fig. 2C). Mouse brains carrying TGLI1-expressing BCBM were visibly larger and highly vascularized (Fig. 2D). We next examined tumors for expression of GLI1 and TGLI1 via immunohistochemistry (IHC), and confirmed their expression (Fig. 2E). TGLI1-expressing BCBM were more invasive as indicated by the hematoxylin and eosin (H&E) staining, more vascularized (CD31 IHC; Fig. 2F), and more proliferative (Ki-67 IHC; Fig. 2G) compared to

vector and GLI1 xenografts. These results indicate that TGLI1 promotes intracranial growth, invasion and angiogenesis of TNBC cells.

Activation of TGLI1 is enriched in HER2-enriched and triple-negative breast cancers and associated with poor metastasis-free survival

Whether TGLI1 target genes are associated with breast cancer patient outcomes remains unknown. DNA microarray probes do not distinguish TGLI1 from GLI1 transcripts. Therefore, we generated a TGLI1 activation signature (tGAS-6) containing six validated TGLI1 target genes (VEGFA, VEGFC, VEGFR2, TEM7, HPSE, and CD24) known to be regulated by TGLI1 but not GLI1[5–8, 14] and determined whether tGAS-6 is correlated with patient survival using two large datasets retrieved from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO). The cDNA microarray probes used for GLI1 expression in both platforms detect both GLI1 and TGLI1 together. We found that TGLI1 is activated to a higher extent in HER2 and TNBC subtypes compared to luminal A/B subtypes in both TCGA and GEO datasets, whereas GLI1/TGLI1 expression was decreased in both datasets (Fig. 3A–B). Kaplan-Meier analysis of the GEO dataset indicated that patients with high tGAS-6, but not high GLI1/TGLI1 expression, had shortened overall metastasis-free survival (MFS; Fig. 3C), shortened brain MFS (Fig. 3D), and shortened lung and bone MFS (Supplementary Fig. 1A–B). These results indicated that TGLI1 activation is common in HER2-enriched breast cancer and TNBC, and associated with poor MFS, including brain MFS.

Expression of TGLI1 is increased in lymph node metastases and BCBM samples compared to primary breast carcinomas

A systematic analysis for TGLI1 expression, in comparison to GLI1, of breast cancer specimens has not been carried out. Analysis of primary breast carcinomas for TGLI1 and GLI1 protein expression by IHC indicated that TGLI1 expression was significantly higher in lymph node-positive, LN(+), primary breast tumors compared to LN(−) tumors, and that in LN(+) tumors, TGLI1 levels were higher than GLI1 expression (Fig. 3E). Furthermore, we immunostained 47 pairs of primary breast carcinomas and their matched LN metastases and found that TGLI1 expression was higher in LN metastases than in matched primary tumors and that TGLI1 levels were higher than GLI1 expression in both primary tumors and LN metastases (Fig. 3F–G). As shown in Supplementary Fig. 1C, GLI1 expression is higher in the paired lymph node metastases compared to the primary tumor but this trend was seen in 66% of the matched pairs and approximately 32% of the matched pairs have decreased GLI1 expression. TGLI1 expression was higher than GLI1 in LN(+) breast cancer in a pairwise analysis (N=57; Fig. 3H). Next, we analyzed 37 BCBM specimens via IHC and compared their TGLI1/GLI1 expression levels to those in primary breast tumors; the results showed that TGLI1 levels were higher in BCBM than primary tumors (Fig. 3I). Pairwise analysis showed that in BCBM, TGLI1 was expressed at higher levels than GLI1 (Fig. 3J–K). These results showed that TGLI1 expression is increased in LN metastases and BCBM samples compared to primary breast carcinomas, suggesting its important role in BCBM.

TGLI1 contributes to BCBM radioresistance

Standard treatment modalities for patients with BCBM include surgical resection, whole brain radiotherapy, and stereotactic radiosurgery[4]. Whether TGLI1 has any effect on radioresistance for any tumor type has never been investigated. Here, we evaluated the expression levels of TGLI1 and GLI1 in HER2-enriched brain-trophic (SKBRM) cells treated with 0, 2, or 5 Gray (Gy) of radiation. As shown in Fig. 4A, TGLI1 expression was significantly induced by radiation in a dose-dependent fashion whereas GLI1 expression was not significantly altered. We further observed that TGLI1 was expressed at higher levels in radioresistant SKBRM (SKBRM-RR) and MDA-MB-231-BrM (231BRM-RR) cells than the radiosensitive parental lines (Fig. 4B), whereas GLI1 levels were reduced in the radioresistant lines (Fig. 4C). Since radioresistance is an intrinsic hallmark of CSCs and the Hh pathway is an important mediator of tumorigenesis and stemness[15], we determined whether TGLI1 contributes to the radioresistant phenotype of breast CSCs. We grew SKBRM-RR and SKBRM cells as mammospheres and evaluated levels of TGLI1; results showed that TGLI1 was expressed at higher levels in SKBRM-RR mammospheres than SKBRM mammospheres, whereas GLI1 expression was significantly decreased (Fig. 4D). Further shown in Fig. 4E–F, we observed that TGLI1 overexpression rendered radiosensitive stem cells (mammosphere) resistant to irradiation, and radioresistant stem cells were more resistant to irradiation. Furthermore, we confirmed these findings using 20 radiosensitive and 16 radioresistant BCBM samples by IHC. Radiosensitive samples were from patients that did not have recurrence one year after gamma-knife radiosurgery whereas radioresistant BCBMs were from patients with recurrence within one year of gamma-knife treatment. As shown in Fig. 4G–I, TGLI1 expression was significantly higher in radioresistant BCBMs than in radiosensitive samples whereas GLI1 was not differentially expressed between the two groups. Collectively, these data suggest a novel role that TGLI1 plays in radioresistance of BCBM.

TGLI1 promotes breast CSCs; TGLI1-positive stem cells activate human astrocytes

To elucidate the mechanism by which TGLI1 mediates BCBM and radioresistance, we examined the effects of TGLI1 on breast CSCs as they are resistant to radiation and are considered the metastasis-initiating cells[16]. Here, we determined TGLI1 expression levels in monolayer versus mammosphere (stem cell population) culture, and found TGLI1 to be induced by mammosphere culture in three different breast cancer cell lines, including luminal A (MCF-7), TNBC basal (BT-20), and brain-trophic HER2-enriched (SKBRM; Fig. 5A–B). As shown in Supplementary Fig. 2A, TGLI1 expression in monolayer was not induced by mammosphere media, indicating TGLI1 induction was due to CSCs and not growth factors present in the media. Furthermore, TGLI1 overexpression in MDA-MB-231 cells led to increased stem cell population defined by $CD44^{high}/CD24^{low}$ (Fig. 5C). Ectopic expression of TGLI1 enhanced the mammosphere-forming ability of BT-20 cells (Fig. 5D–F), SKBRM cells (Fig. 5G–I), and promoted mammosphere-forming ability of two immortalized normal mammary epithelial cell lines, MCF10A and HMLE (Supplementary Fig. 2B–C), and enhanced the anchorage-independent growth of HMLE cells (Supplementary Fig. 2D).

Reactive astrocytes have been shown to support the arrest, extravasation, and growth of breast cancer cells in the brain[17]. To explore whether TGLI1-expressing breast CSCs have any effect on astrocyte activation, we stimulated human astrocytes with conditioned medium from SKBRM cells grown in monolayer or mammosphere culture. As shown in Fig. 5J–K, TGLI1-positive mammospheres significantly activated astrocytes as indicated by increased glial fibrillary acidic protein (GFAP) expression. Further shown in Fig. 5L, astrocytes only survived and interacted with TGLI1-positive breast CSCs under the sphere-forming stem cell culturing condition. Immunostaining of mouse BCBM tumors (described in Fig. 2) revealed TGLI1-positive tumors significantly activated astrocytes *in vivo* (Fig. 5M–N). In contrast, no difference was observed between vector and GLI1 groups. Together, these results indicated that TGLI1 promotes breast CSCs and that TGLI1-positive stem cells and tumors can activate astrocytes *in vitro* and *in vivo* respectively, a mechanism underlying TGLI1-mediated brain metastasis.

TGLI1 upregulates stemness genes in breast cancer cells

Whether TGLI1 transcriptionally regulates stemness genes in breast cancer has never been investigated. Here, we determined the extent to which TGLI1 mediates breast CSCs by examining the ability of TGLI1 to regulate expression of four genes that are important for pluripotency and stem cell self-renewal, namely, Nanog, SOX2, OCT4, and CD44[18, 19]. GLI1 has been previously shown to directly regulate expression of SOX2 and Nanog[20, 21]. We recently reported that TGLI1, but not GLI1, transcriptionally upregulated CD44 gene expression in glioma stem cells[9]. Whether OCT4 can be regulated by TGLI1 has not been reported. To identify whether these genes are associated with BCBM, we used Kaplan-Meier analysis and the same GEO datasets used in Fig. 3C–D, and found that SOX2 and OCT4, but not Nanog and CD44, were associated with shortened brain MFS (Supplementary Fig. 3A). We found that TGLI1 upregulates expression of all four stemness genes (Fig. 6A–C & Supplementary Fig. 3B–D). Of note, Nanog, SOX2, and OCT4 were significantly upregulated by TGLI1 in comparison to both vector and GLI1 in three cell lines. Consistent with the mRNA results, protein levels of all four stemness genes were enhanced by TGLI1 in SKBRM cells (Fig. 6D). We next examined whether TGLI1 regulates expression of GLI2 target genes[22–24] and found TGLI1 to upregulate GLI2 target genes to a higher extent than GLI1 (Supplementary Fig. 3E). Promoter luciferase reporter assay showed that TGLI1 possessed the ability to transactivate the promoters of Nanog and SOX2 (two known GLI1 target genes; Fig. 6E–F). Using chromatin immunoprecipitation (ChIP) and promoter luciferase reporter assays, we further found that TGLI1 significantly bound to and transactivated CD44 (Fig. 6G–H) and OCT4 (Fig. 6I–J) promoters. Interestingly, we found that TGLI1 bound to the OCT4 promoter to a similar degree as GLI1; however, TGLI1 was able to transactivate the OCT4 promoter more than GLI1 to increase OCT4 mRNA and protein expression. Although GLI family of proteins do not dimerize with each other, we determined whether TGLI1 and GLI1 compete to bind to target gene promoters. We used isogenic MDA-MB-231 cells overexpressing both GLI1 and TGLI1, and performed ChIP assay using TGLI1- and GLI1-specific antibodies. As shown in Fig. 6K, TGLI1 preferentially binds to the OCT4 promoter in the presence of GLI1, indicating that TGLI1 outcompetes GLI1 for binding to target gene promoters. Collectively, these results demonstrate that TGLI1 is a gain-of-function GLI1 transcription factor with the ability to

upregulate stemness genes and that TGLI1 is a novel transcriptional activator of Nanog, SOX2, and OCT4.

Eight-gene TGLI1 activation signature (tGAS-8) is predictive of breast cancer metastasis and radioresistance

We recently identified two new TGLI1 target genes, CD44 and OCT4, which prompted us to create the 8-gene TGLI1 activation signature (tGAS-8) and determine its utility as a prognostic marker for breast cancer metastasis. Using tGAS-8 and the GEO datasets used earlier in Fig. 3, we found that tGAS-8 was more enriched in the HER2 and TNBC subtypes than the luminal A/B subtypes (Supplementary Fig. 3F). Metastatic breast tumors had higher tGAS-8 than non-metastatic counterparts (Fig. 6L & Supplementary Fig. 3G). Patients with brain or lung metastases had higher tGAS-8 in their primary tumors compared to those with bone metastases and no metastases (Supplementary Fig. 3H). In contrast, GLI1/TGLI1 mRNA was decreased in breast cancer with metastases (Supplementary Fig. 3G–H). Furthermore, Kaplan-Meier analyses showed that patients with high tGAS-8 have a shortened overall MFS, as well as, shortened brain, lung, and bone MFS, compared to those with low tGAS-8 (Fig. 6M & Supplementary Fig. 3I). Next, we investigated whether tGAS-8 is associated with metastasis and radioresistance using Gene Set Enrichment Analysis (GSEA) and the same GEO datasets. For this, we retrieved previously established signatures for cancer metastasis[25], BCBM[26, 27], EMT[28], breast cancer stemness[29], and breast cancer radioresistance[30] (Fig. 6N & Supplemental Fig. 3J–M). The results showed that breast tumors with high tGAS-8, but not GLI1/TGLI1 mRNA, were enriched for all five signatures. Taken together, these results indicated that tGAS-8 can be used as a prognostic indicator for metastatic breast cancer and radioresistance.

TGLI1 Knockdown suppresses breast CSCs *in vitro* and reduces BCBM *in vivo*

To further validate the effects of TGLI1 on breast CSCs, we used validated antisense oligonucleotides (AS-ON) to selectively knockdown TGLI1 in BT20 and SKRBM cells then determined the effect on mammosphere-forming ability. Consistent with our recent report[9] the AS-ON led to selective knockdown of TGLI1 in both cell lines and significantly decreased the number of mammospheres compared to control (Fig. 7A–B). TGLI1 knockdown decreased expression of the recently identified TGLI1 target genes, OCT4 and CD44 (Fig. 7C). To determine the functional role of TGLI1 on breast cancer metastasis, we knocked down TGLI1 in brain trophic SKBRM cells and performed intracardiac injections to study brain and bone metastasis (Fig. 7D). *Ex vivo* analysis of the brain and bone revealed a significant decrease in brain metastases in the TGLI1 knockdown group (Fig. 7E–F) but no difference in bone metastases (Fig. 7E–G). These results further support the novel role of TGLI1 in mediating breast cancer stem cells and BCBM.

DISCUSSION

In this study we made the following novel and impactful observations: (1) TGLI1 promotes breast cancer metastasis to brain, and intracranial growth of breast cancer cells in two mouse models of breast cancer metastasis; (2) TGLI1 activation is increased in HER2-enriched and TNBC subtypes and is associated with shortened brain MFS; (3) TGLI1 expression is

enriched in LN metastases and BCBM specimens compared to primary breast carcinomas; (4) TGLI1 is increased in radioresistant BCBM cell lines and specimens compared to radiosensitive counterparts, and contributes to resistance of BCBM cells; (5) TGLI1 promotes mammosphere formation in breast cancer cells and normal mammary epithelial cells; (6) TGLI1 mediates breast CSCs by transcriptionally activating cancer stemness gene expression; (7) TGLI1 upregulates expression of GLI2 target genes; (8) TGLI1 outcompetes with GLI1 to bind to OCT4 gene promoter; (9) TGLI1-postive breast CSCs strongly activate and interact with astrocytes *in vitro* and *in vivo*; and (10) TGLI1 knockdown decreases mammosphere formation, decreases stemness gene expression, and reduces breast cancer metastasis to the brain. Through making these significant observations, our study defines TGLI1 as a novel mediator of brain metastasis, astrocyte activation, and resistance to radiation therapy in breast cancer, thereby providing novel insights into the molecular mechanisms for BCBM.

We observed in this study that TGLI1 promotes breast CSCs. This is consistent with previous studies reporting that a subpopulation of breast CSCs can be characterized by cell-surface expression of $CD44^{high}/CD24^{low}$ [31, 32]. Interestingly, we have previously reported that both CD44 and CD24 can be transcriptionally activated by TGLI1[5] while this study shows TGLI1 to increase the $CD44^{high}/CD24^{low}$ stem cell subpopulation. We speculate that TGLI1 may have increased the expression of nuclear CD24 but not cell-surface CD24 expression. It has been recently discovered that biologically active CD24 is localized in the nucleus of cancer cells with low cell-surface CD24 expression. Importantly, they found that nuclear CD24 correlated with metastasis and that a nuclear CD24 gene signature was predictive of shortened survival for breast cancer patients[33]. In agreement with these findings, and the role of the TGLI1-CD24 signaling axis in promoting cancer migration and invasion[5, 8] and metastasis (Figs. 1–2), other groups have shown that CD24-positive breast cancers are associated with tumor progression and poor survival[34, 35]. These results suggest that TGLI1 may increase nuclear CD24 expression but reduce cell-surface CD24 expression, and thereby contributes to breast CSCs and cancer metastasis. This possibility should be addressed in future studies.

Our results from three mouse models of breast cancer metastasis indicate that TGLI1 directs breast cancer metastasis to the brain and promotes breast cancer growth once in the brain. We also demonstrate TGLI1-positive CSCs strongly activate and interact with astrocytes *in vitro* and TGLI1-positive BCBM tumors activate astrocytes *in vivo*. The ability of TGLI1 to activate astrocytes should be investigated further. Apart from mediating breast CSCs to drive BCBM and activating astrocytes to alter the tumor microenvironment, another potential mechanism by which TGLI1 may mediate BCBM is through regulating exosomal microRNAs. Through secretion of exosomes, small extracellular vesicles containing growth factors, DNA, and microRNAs, primary tumors can prime distant organs for colonization of tumor cells[36]. Whether TGLI1-positive tumors secrete exosomal microRNAs to prime the brain for colonization remains to be investigated. Furthermore, results from our bioinformatic analysis implicate TGLI1 in facilitating lung and bone metastases (Supplementary Figs. 1 and 3). Using one cell line in our *in vivo* model, to overexpress TGLI1, we did not observe significant increase in lung (not shown) or bone metastases between vector, GLI1 or TGLI1 groups suggesting that TGLI1-positive breast cancers

preferentially metastasize to the brain. Similarly, TGLI1 knockdown in a brain trophic HER2-enriched cell line (SKBRM) did not lead to significant differences in lung and bone metastases. Future experiments using additional breast cancer cell lines, or genetically engineered mouse models, are needed to further define the impact of TGLI1 on breast cancer metastasis in the context of organ preference.

We found that TGLI1 overexpression transformed normal mammary epithelial cells resulting in increased mammosphere formation and anchorage-independent growth, key features of CSCs. This finding suggests TGLI1 may play an oncogenic role for mammary tumorigenesis. Future studies examining the role of TGLI1 in malignant transformation of normal mammary cells are needed. To meet this need, conditional transgenic mouse models may be established to allow for GLI1 gene knocked out and human TGLI1 gene knocked in. These floxed mice can then be crossed with mammary-specific Cre mice such as MMTV-cre mice. This transgenic mouse model will shed new light on the effects of TGLI1 on malignant transformation of mammary cells, breast tumor growth, and progression.

We identified OCT4 as a novel transcriptional target of TGLI1 in breast cancer cells (Fig. 6). OCT4 is an established stemness gene[18]. This novel TGLI1-OCT4 signaling axis represents a previously undescribed molecular mechanism underlying breast CSCs. Aberrant expression of OCT4 has been shown to promote breast cancer progression, chemoresistance, and metastasis, and to be associated with TNBCs[37]. In contrast, OCT4 overexpression resulted in decreased migration and invasion *in vitro*, as well as, decreased lung metastases *in vivo*[38]. Interestingly, a study demonstrated that OCT4 is expressed in adult stem cells and has been shown to mediate adult neural stem cell differentiation into dopaminergic neurons[39]. The important role of OCT4 in the adult brain may contribute to the observed preferential brain metastasis of TGLI1-positive breast cancers. Future studies are needed to address this possibility.

To fully understand the mechanism by which TGLI1 promotes breast CSCs, RNA-seq will be carried out in future to identify additional TGLI1-specific stemness genes. The current study identified OCT4 as a stemness gene that underlies TGLI1-mediated breast cancer stemness. This study also confirmed the existence of the TGLI1-CD44 signaling axis in breast cancer which was first discovered in GBM stem cells[9]. Based on these findings, TGLI1 may mediate breast CSCs through upregulating both OCT4 and CD44 plus additional genes. We demonstrate that TGLI1 outcompetes GLI1 for binding to the OCT4 gene promoter which may contribute to the gain-of-function ability of TGLI1. We further uncovered that TGLI1, but not GLI1, upregulates GLI2 target genes including PDGFRβ, FGF19, and ARHGEF16. PDGFRβ is an important neuroprotective factor during brain injury and is associated with breast cancer brain metastasis and gastric cancer stem cells[24, 40]. FGF19, the human ortholog of FGF15, is essential for embryonic brain development[41] and overexpression of FGF19 is implicated in breast cancer progression and metastasis[42]. ARHGEF16 was recently shown to promote glioma cell migration and proliferation[22]. Upregulation of these GLI2 target genes by TGLI1 further contributes to the aggressiveness of TGLI1. Future studies are needed to address how TGLI1 regulates GLI2 target genes. One possibility for TGLI1's gain-of-function ability is that TGLI1 may interact with other transcription factors to co-regulate gene expression. Another possibility is that

TGLI1 may bind to different consensus sites that GLI1 cannot bind. However, these possibilities will need to be thoroughly investigated in future studies.

In summary, our current study reports important findings on the novel functions of TGLI1 and mechanisms contributing to BCBM, stem cells, reprogramming of tumor microenvironment via astrocyte activation, and radioresistance. The results from this study, together with previous studies, have further strengthened the rationale to further investigate the roles of TGLI1 in regulating exosomal microRNAs in the context of BCBM and mammary tumorigenesis, as well as, identify the mechanism that underlie the gain-of-function transcriptional ability of TGLI1.

Supplementary Material

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ACKNOWLEDGEMENTS

We thank Dr. Shadi Qasem for providing pathology support. We acknowledge the funding support from NIH grants R01NS087169 (to H-WL), F31CA224805-01A1 (to SRS, H-WL), T32CA079448 (to SK, RLC), R01NS087169-3S1 (to H-WL, SRS), P30CA012197 (to BP; core facility), and DoD grants W81XWH-17-1-0044 (to H-WL) and W81XWH-19-1-0072 (to H-WL).

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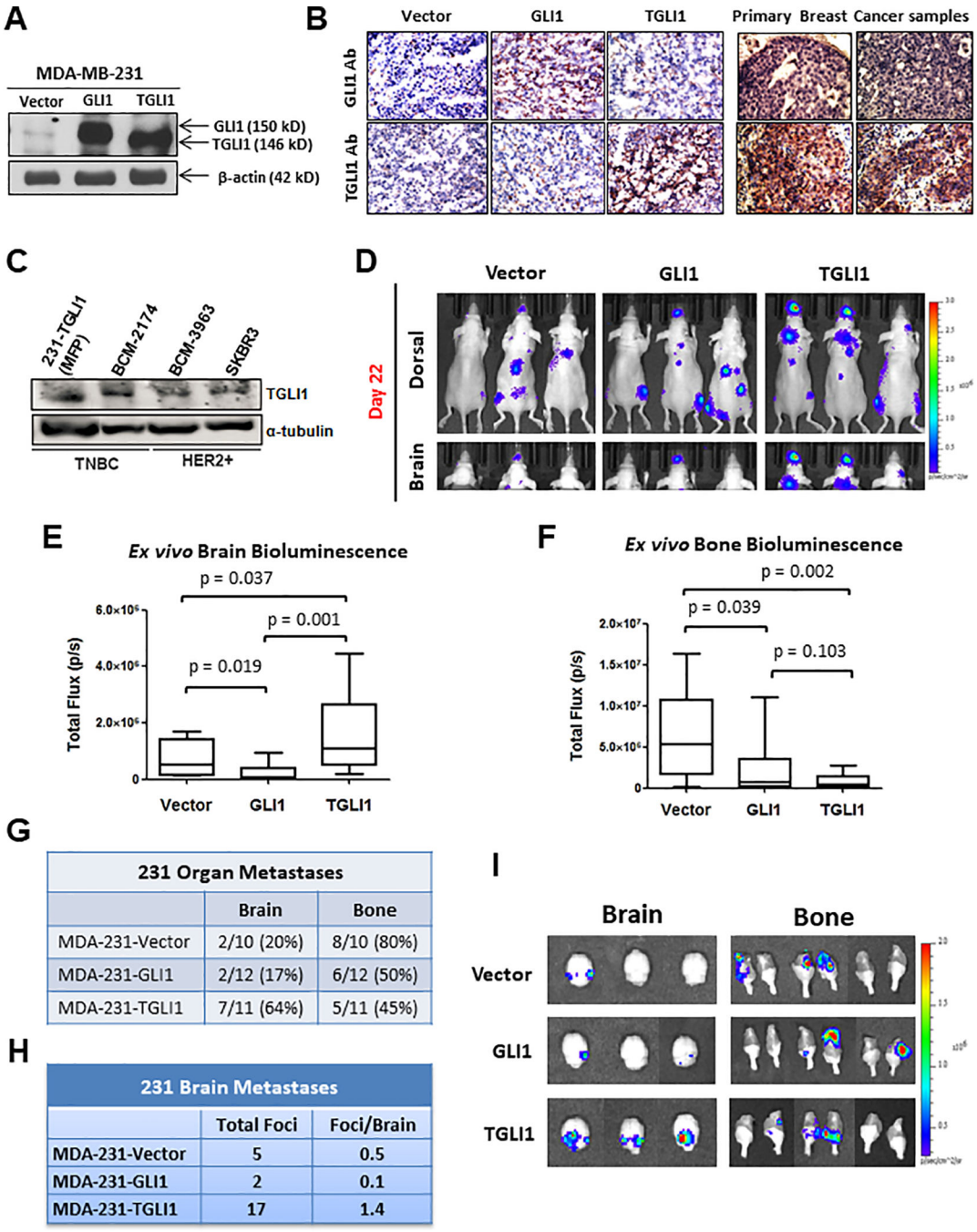

231 Organ Metastases		
	Brain	Bone
MDA-231-Vector	2/10 (20%)	8/10 (80%)
MDA-231-GLI1	2/12 (17%)	6/12 (50%)
MDA-231-TGLI1	7/11 (64%)	5/11 (45%)

231 Brain Metastases		
	Total Foci	Foci/Brain
MDA-231-Vector	5	0.5
MDA-231-GLI1	2	0.1
MDA-231-TGLI1	17	1.4

Figure 1: TGLI1 promotes brain metastasis of TNBC in an intracardiac injection metastasis mouse model.

A) Western blot analysis of isogenic MDA-MB-231 cell lines. **B)** IHC analysis of mammary fat pad MDA-MB-231 xenografts from female athymic mice and primary breast cancer patient samples using GLI1- and TGLI1-selective antibodies. **C)** Expression of TGLI1 in MDA-MB-231-TGLI1 MFP xenograft was similar to that found in two breast cancer PDXs. Western blots using the TGLI1-specific antibody were conducted. **D)** Bioluminescent images of female athymic mice 22 days post intracardiac injection of isogenic MDA-

MB-231 cell lines. Dorsal and brain views are shown. Twelve mice per group were used. Two mice in vector and one mouse in TGLI1 groups died during quantified. **G)** Tumor foci within mouse brains were determined via *ex vivo* brain imaging. **H)** Representative *ex vivo* IVIS images of brain and bone are shown. Student's *t*-test was used to compute p-values for panels E and F.

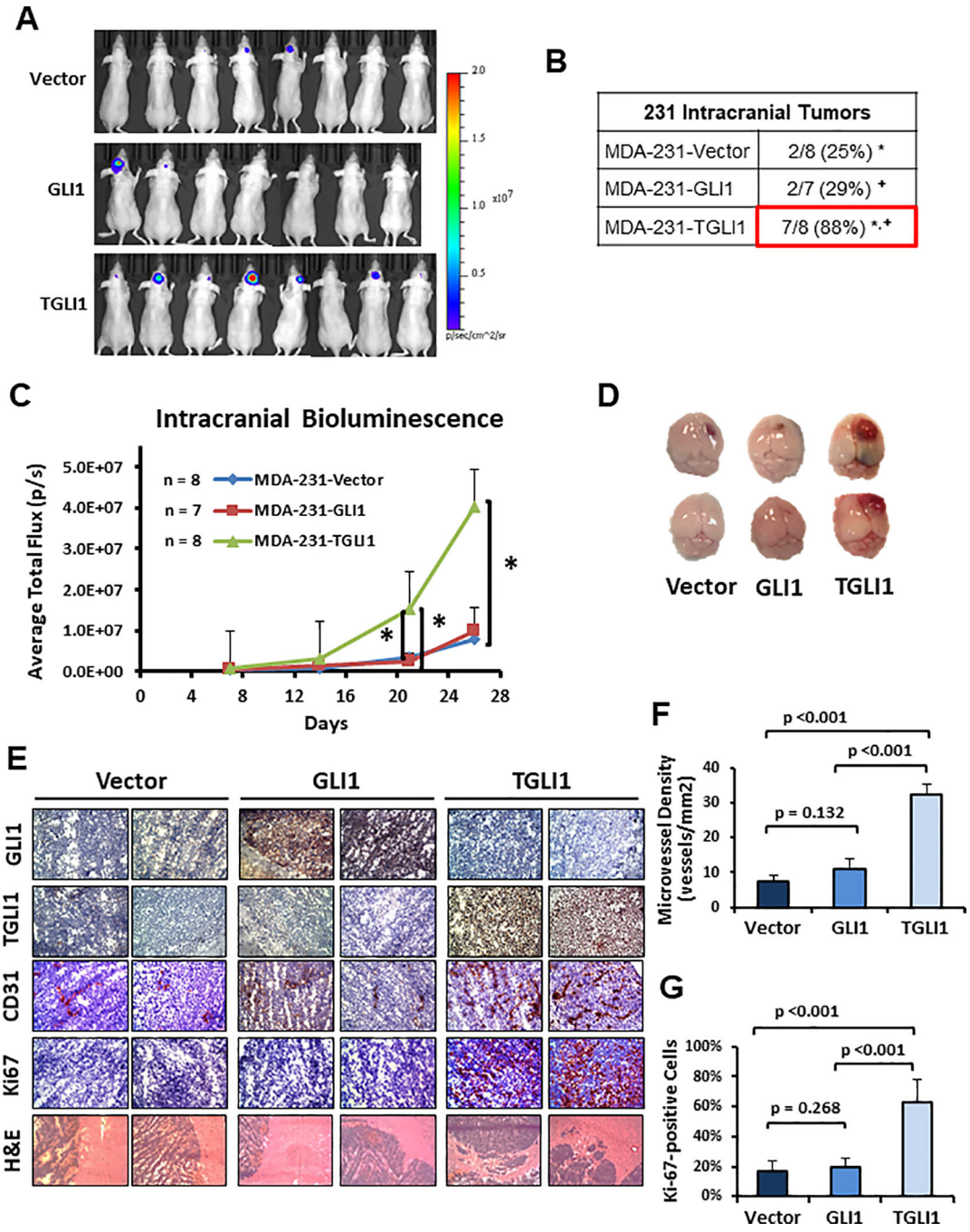

Figure 2: TGLI1 promotes TNBC growth in mouse brain in an intracranial injection mouse model.

Isogenic MDA-MB-231 lines were implanted into mouse brain and imaged weekly via an IVIS imager. Eight mice per group were used in which one mouse in the GLI1 group died of ketamine overdose. **A)** IVIS images of female athymic mice 3 weeks post intracranial implantation. **B)** Incidence of BCBM formation in each group. *, $^{+}$ indicates $p < 0.05$ between vector and TGLI1 or GLI1 and TGLI1, respectively. Fisher's Exact Test was used. **C)** Growth curve for intracranial tumors. * indicates $p < 0.05$. **D)** Representative mouse

brains showing TGLI1 BCBM being more vascularized**. E)** Analysis of BCBM for GLI1/TGLI1 expression (IHC), invasiveness (H&E), microvessel density (CD31 IHC) and proliferation index (Ki-67 IHC). **F)** Microvessel density. **G)** Proliferation Index. Student's *t*-test was used to compute p-values for panels F and G.

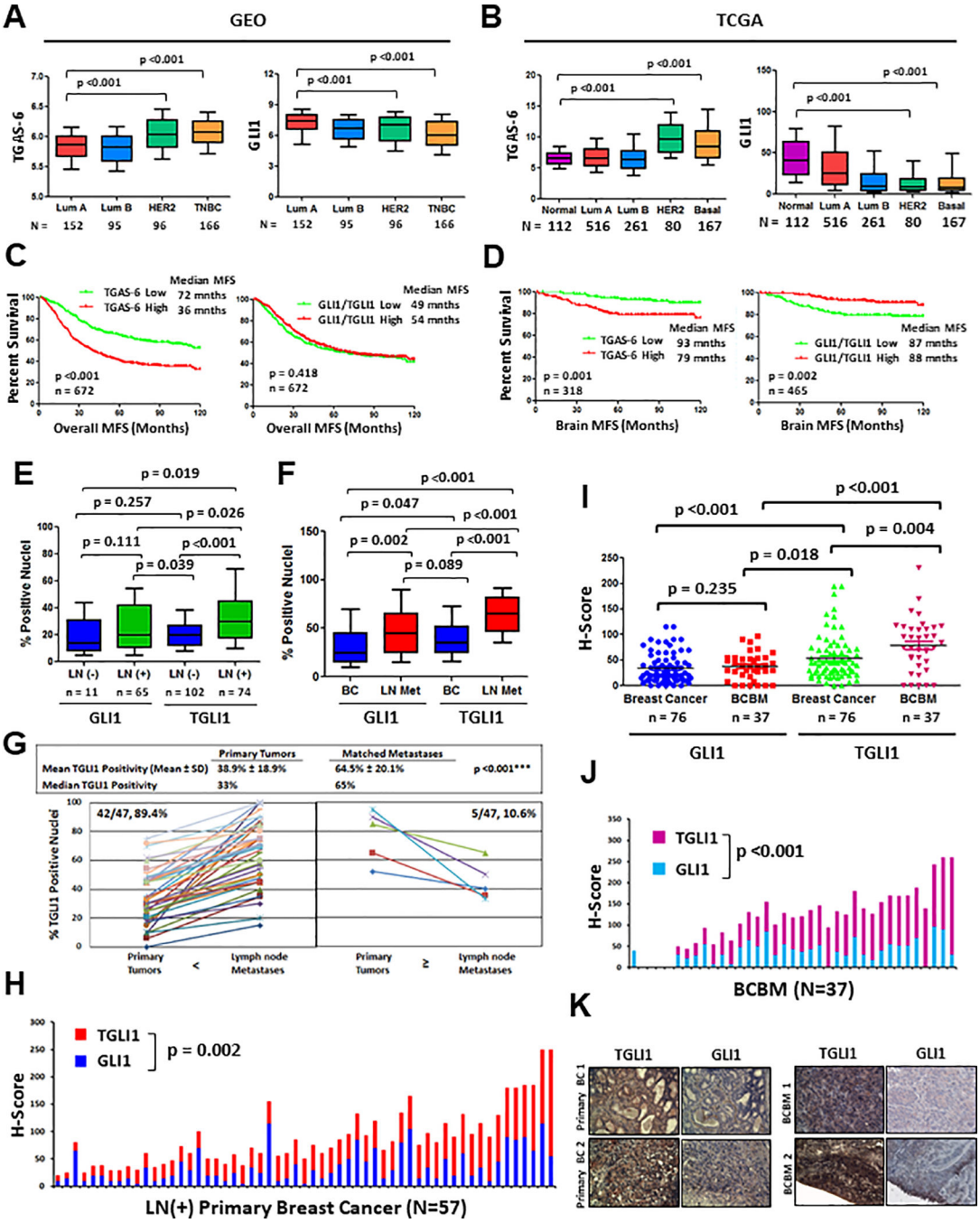

Figure 3: TGLI1 activation is enriched in HER2-enriched breast cancer and TNBC, and associated with poor MFS; TGLI1 expression is increased in lymph node metastases and BCBM specimens.

A-B) Bioinformatic analysis of GEO datasets (GSE12276/2034/2603/5327/14020; Panel A) comprised of 710 breast tumors and TCGA dataset (Panel B) comprised of 1,136 breast tumors for tGAS-6 enrichment and GLI1/TGLI1 mRNA expression. **C-D)** Kaplan-Meier curves showing the correlations between tGAS-6 or GLI1/TGLI1 mRNA expression with overall MFS (Panel C) and brain MFS (Panel D). Only 672 out of 710 patients in the GEO cohort had survival data. Log rank test was used to compute p-values. **E-K)** IHC analysis of

patient specimens using TGLI1 and GLI1 specific antibodies developed in our laboratory. **Panel E** shows the expression of GLI1 and TGLI1 in primary breast tumors that are either lymph node-negative, LN(−), or lymph node-positive, LN(+). **Panels F-G**, expression levels of GLI1 and TGLI1 in matched primary breast cancers and lymph node metastases (N=47). **Panel H** shows that TGLI1 expression is higher than GLI1 in LN(+) breast cancer in a pair-wised analysis (N=57). **Panel I** shows GLI1 and TGLI1 expression in 56 primary breast tumors and 37 BCBM specimens. **Panel J** contains a pairwise analysis of 37 BCBM for GLI1 and TGLI1 expression; **Panel K** shows representative primary breast cancer and BCBM samples immunostained for GLI1 and TGLI1. Student's *t*-test was used to compute p-values.

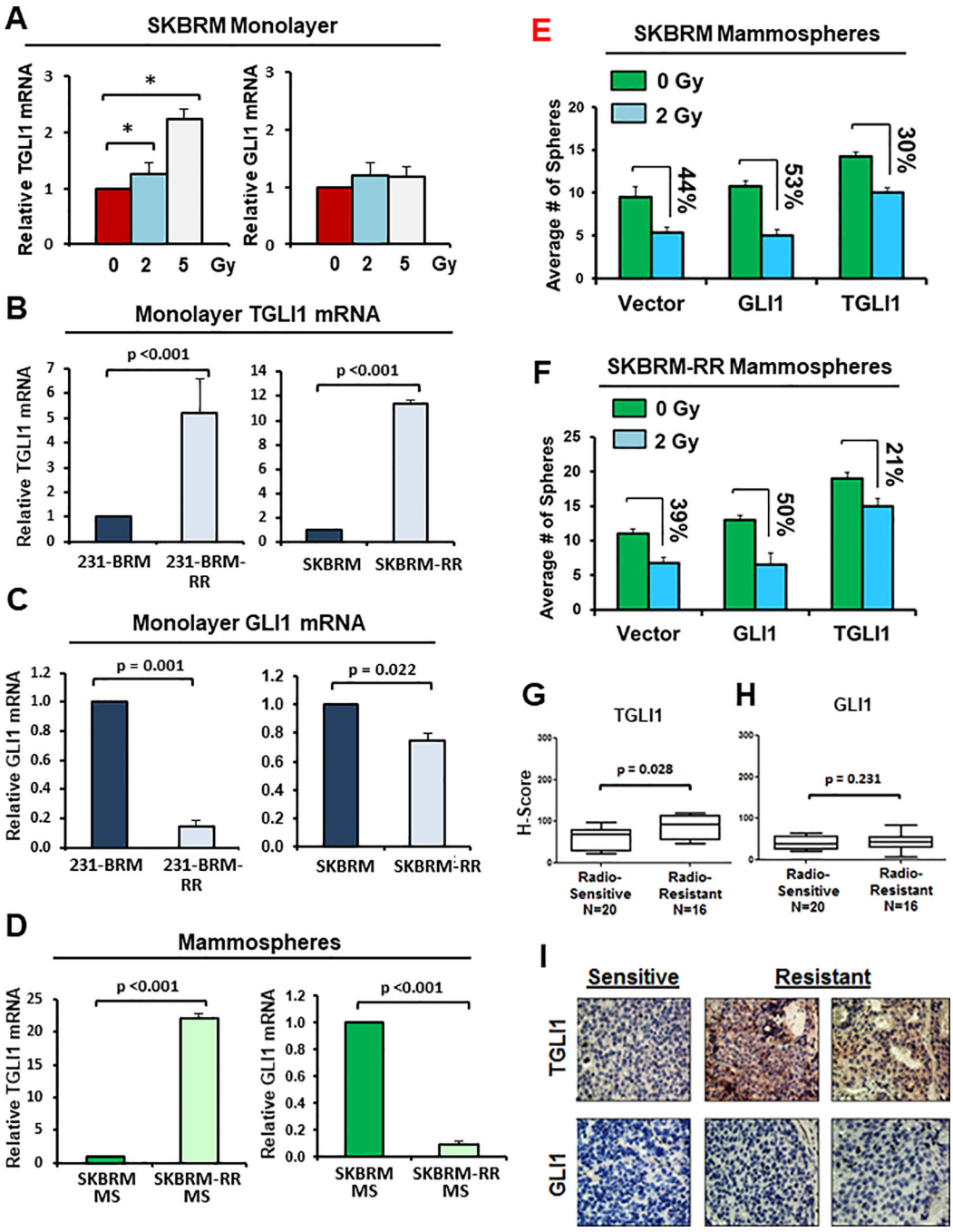

Figure 4: TGLI1 contributes to BCBM radioresistance.

A) Expression levels of TGLI1 and GLI1 in SKBRM cells treated with 0, 2, or 5 Gy of radiation. TGLI1 and GLI1 mRNA expression was determined 30 minutes after irradiation. **B-C)** Relative TGLI1 or GLI1 mRNA expression in radiosensitive and radioresistant brain-tropic breast cancer cell lines. **D)** TGLI1 mRNA was expressed at higher levels in SKBRM-RR mammospheres than SKBRM mammospheres. GLI1 expression was significantly decreased in the radioresistant mammospheres. **E-F)** SKBRM and SKBRM-RR cells transfected with control, GLI1-, or TGLI1-vector were irradiated (2 Gy) and subjected to

mammosphere formation assay. **G-H)** IHC staining of 20 radiosensitive BCBM tumors and 16 radioresistant BCBM tumors using GLI1 and TGLI1 specific antibodies developed in our laboratory. **I)** Representative immunostained BCBM specimens. *Indicates significant difference ($p<0.05$). Student's *t*-test was used to compute p-values.

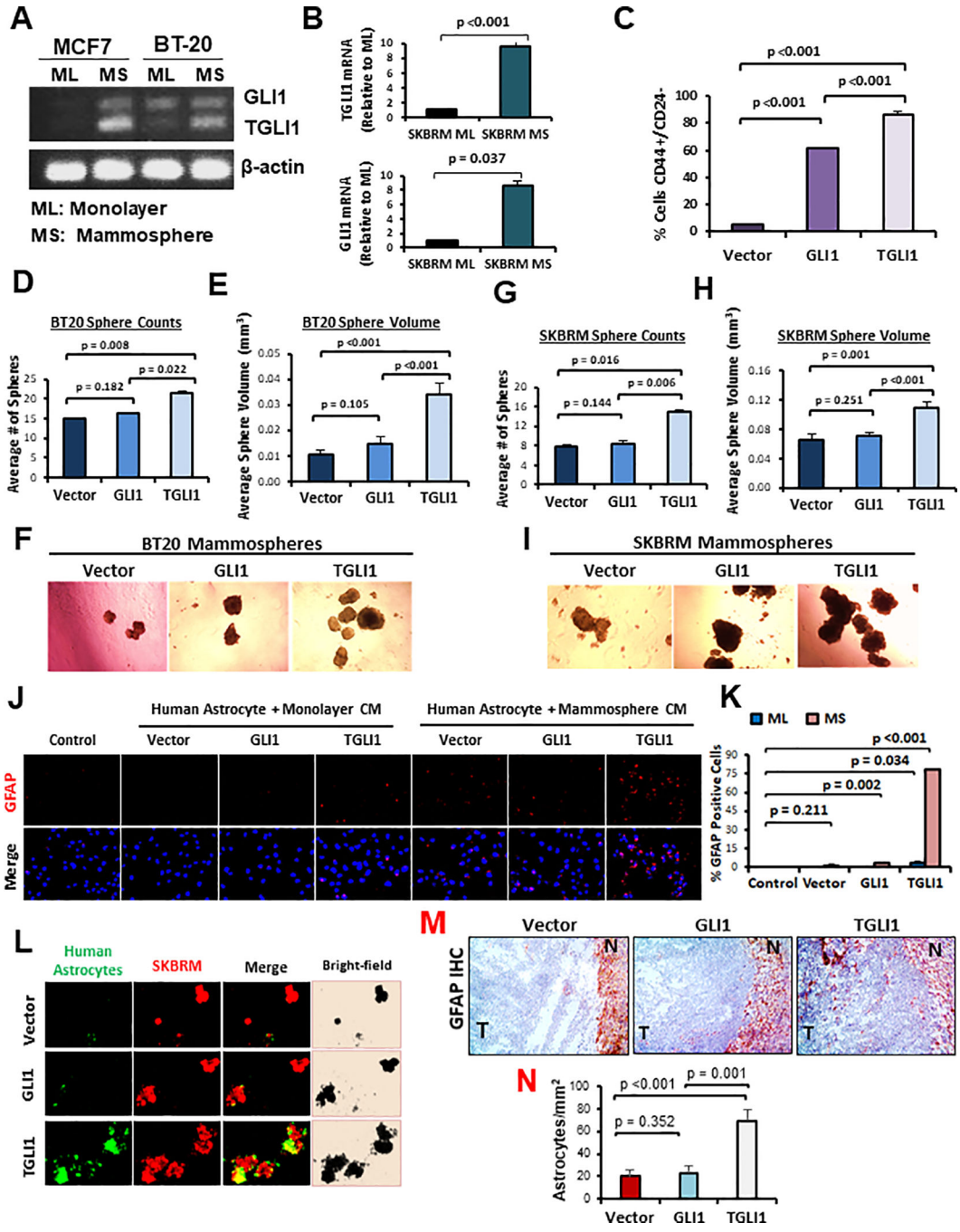

Figure 5: TGLI1 promotes breast cancer stem cells and TGLI1-positive stem cells activate human astrocytes.

A) Expression of GLI1 and TGLI1 mRNA in a two breast cancer cell lines grown in monolayer or mammospheres. Regular RT-PCR was conducted. MS, mammosphere. ML, monolayer. **B)** GLI1 and TGLI1 mRNA expression in SKBRM MS relative to ML, as determined by qRT-PCR. **C)** TGLI1 overexpression in MDA-MB-231 cells led to increased stem cell population defined by $CD44^{high}/CD24^{low}$ as determined by FACS. **D-F)** TGLI1 ectopic expression enhanced the mammosphere-forming ability of BT20 cells. Panel D,

average number of mammospheres. Panel E, average size of mammospheres. Panel F, representative images of mammospheres. **G-I)** TGLI1 ectopic expression enhanced the mammosphere-forming ability of SKBRM cells. Panel G, average number of mammospheres. Panel H, average size of mammospheres. Panel I, representative images of mammospheres. **J-K)** GFAP expression is increased in human astrocytes upon stimulation with conditioned medium (CM) from TGLI1-positive mammospheres. Isogenic SKBRM cells grown in monolayer or mammospheres were used. Panel J, representative images. Panel K, percent GFAP positive cells. **L)** Co-culture of human astrocytes (GFP-tagged) with isogenic SKBRM cells (RFP-tagged) under the sphere-forming condition. **M-N)** Analysis of isogenic MDA-MB-231 intracranial tumors for activated astrocytes (GFAP IHC). **M)** Representative images. **N)** Quantified number of astrocytes per area of tumor. Student's *t*-test was used to determine p-values.

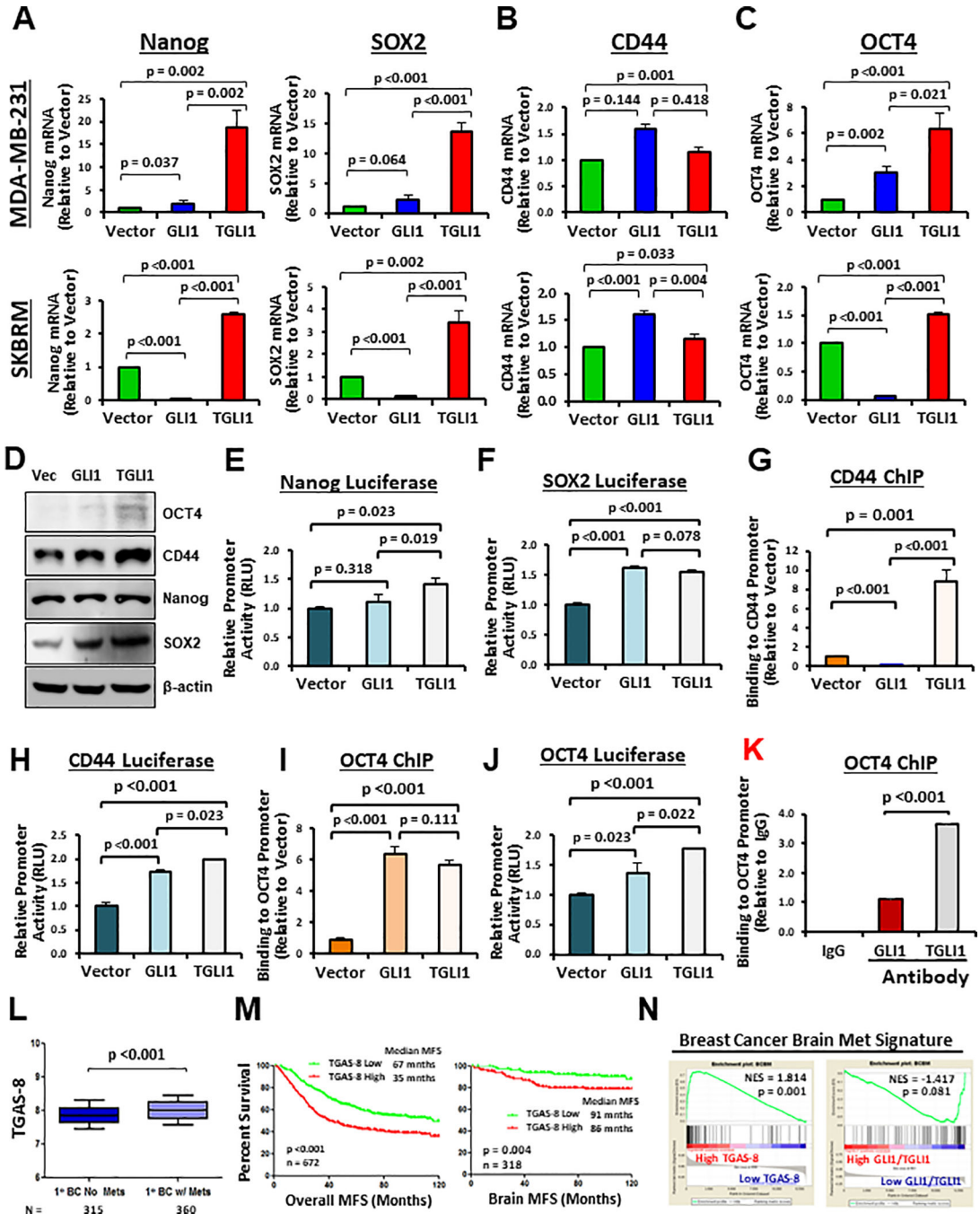

Figure 6: TGLI1 upregulates stemness genes in breast cancer cells; Eight-gene TGLI1 activation signature (tGAS-8) is predictive of breast cancer metastasis and radioresistance.

A-C) TGLI1 upregulates expression of four stemness genes in two breast cancer cell lines of different subtypes. Quantitative RT-PCR was performed using transfected MDA-MB-231and SKBRM cells. **D)** Protein levels of all four stemness genes were enhanced by TGLI1 in SKBRM cells. Western blotting was conducted. **E&F)** SKBR3 cells were transiently transfected with promoter luciferase reporters and vector, GLI1 or TGLI1 plasmids, stimulated with SHH ligand (100 ng/mL) for 4 hrs and promoter transactivation was

measured by luciferase assay. **E)** Relative transactivation of the Nanog promoter by GLI1 or TGLI1. **F)** Relative transactivation of the SOX2 promoter by GLI1 or TGLI1. **G)** Relative binding of GLI1 or TGLI1 to the CD44 promoter as measured by the ChIP assay using transfected SKBR3 cells. **H)** Relative transactivation of the CD44 promoter by GLI1 or TGLI1 in SKBR3 cells. **I)** Relative binding of GLI1 or TGLI1 to the OCT4 promoter in SKBR3 cells as determined by the ChIP assay. qPCR was performed using primers spanning the OCT4 gene promoter. **J)** Relative transactivation of the OCT4 promoter by GLI1 or TGLI1 in SKBR3 cells as determined by the luciferase reporter assay. **K)** Relative binding of GLI1 or TGLI1 to the OCT4 promoter in MDA-MB-231 cells with dual overexpression of GLI1 and TGLI1 as determined by the ChIP assay using GLI1-specific and TGLI1-specific antibodies developed and validated in our laboratory. Student's *t*-test was used to determine p-values. **L-M)** The same GEO cohort of 710 breast tumors from Figure 3 was used. **L)** Metastatic breast tumors had higher tGAS-8 than non-metastatic counterparts. **M)** Patients with high tGAS-8 have a shortened overall MFS and brain MFS compared to those with low tGAS-8. **N)** GSEA that determined whether breast tumors with high tGAS-8 or high TGLI1/GLI1 mRNA were enriched for breast cancer brain metastasis signature. NES, normalized enrichment score.

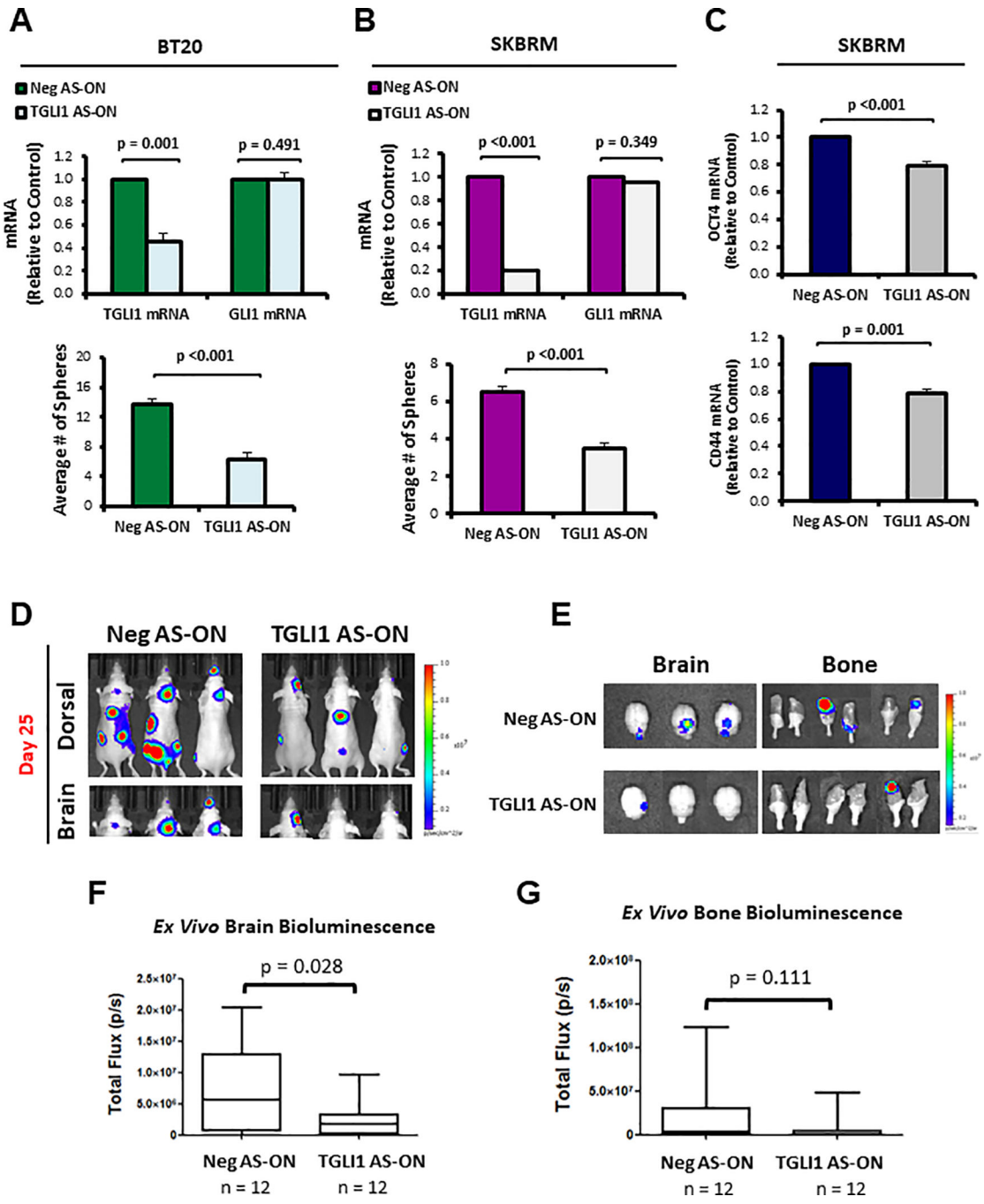

Figure 7: Knockdown of TGLI1 suppresses breast cancer stem cells *in vitro* and reduces BCBM *in vivo*.

Antisense oligonucleotides (AS-ON) were used to knockdown TGLI1. **A-B)** Selective knockdown of TGLI1 as indicated by qRT-PCR and effects of TGLI1 knockdown on mammosphere formation in **A)** BT-20 cells and **B)** SKBRM cells. **C)** Decreased expression of OCT4 and SOX2 with TGLI1 knockdown. Quantitative RT-PCR was performed using transfected SKBRM cells. In Panels D-G, athymic mice were inoculated with SKBRM cells transfected with negative control oligos or TGLI1-AS-ON through intracardiac injections

(12 mice per group). **D)** Bioluminescent images of female athymic mice 25 days post intracardiac injection of transfected SKBRM cells. Dorsal and brain views are shown. **E)** Representative *ex vivo* IVIS images of brain and bone are shown. **F)** Brain *ex vivo* bioluminescence was quantified. **G)** Bone *ex vivo* bioluminescence was quantified. Student's *t*-test was used to compute p-values.