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LTBP-3 promotes early metastatic events during cancer cell dissemination

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Abstract

Latent Transforming Growth Factor β (TGF β) Binding Proteins (LTBPs) are important for the secretion, activation and function of mature TGF β , especially so in cancer cell physiology. However, specific roles of the LTBPs remain understudied in the context of the primary tumor microenvironment. Herein, we investigated the role of LTBP-3 in the distinct processes involved in cancer metastasis. By using three human tumor cell lines of different tissue origin (epidermoid HEP-3 and prostate PC-3 carcinomas and HT-1080 fibrosarcoma) and several metastasis models conducted in both mammalian and avian settings, we show that LTBP-3 is involved in the early dissemination of primary cancer cells, namely in the intravasation step of the metastatic cascade. Knockdown of LTBP-3 in all tested cell lines led to significant inhibition of tumor cell intravasation, but did not affect primary tumor growth. LTBP-3 was dispensable in the late steps of carcinoma cell metastasis that follow tumor cell intravasation, including vascular arrest, extravasation and tissue colonization. However, LTBP-3 depletion diminished the angiogenesis-inducing potential of HEP-3 cells *in vivo*, which was restorable by exogenous delivery of LTBP-3 protein. A similar compensatory approach rescued the dampened intravasation of LTBP-3-deficient HEP-3 cells, suggesting that LTBP-3 regulates the induction of the intravasation-supporting angiogenic vasculature within developing primary tumors. Using our recently developed microtumor model, we confirmed that LTBP-3 loss resulted in the development of intratumoral vessels with an abnormal microarchitecture incompatible with efficient intravasation of HEP-3 carcinoma cells. Collectively, these findings demonstrate that LTBP-3 represents a novel oncotarget that has distinctive functions in the regulation of angiogenesis-dependent tumor cell intravasation, a critical process during early cancer dissemination. Our experimental data are also consistent with the survival prognostic value of *LTBP3* expression in early stage head and neck squamous cell carcinomas, further indicating a specific role for LTBP-3 in cancer progression towards metastatic disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Keywords

LTBP; TGF β ; metastasis; tumor cell intravasation; tumor angiogenesis; intratumoral vasculature

INTRODUCTION

Metastasis, a major cause of cancer-related deaths, can be visualized as a multi-step process that includes early events occurring at the primary tumor site, such as epithelial-mesenchymal transition (EMT)¹⁻³, tumor-induced angiogenesis⁴⁻⁶ and tumor cell intravasation (i.e. entry of escaping tumor cells into the circulation mainly *via* intratumoral blood vessels).⁷ These early events are often paralleled by invasion of expanding tumor cells into the adjacent stroma, followed by later events, such as apoptosis avoidance and vascular arrest of the intravasated cells, their escape from immune surveillance, extravasation of the survived tumor cells into the secondary stroma, and outgrowth of extravasated tumor cells into overt metastases.⁸⁻¹² Because only few therapies efficiently target metastatic tumors and halt their deadly expansion, the investigation of specific mechanisms underlying early steps of cancer metastasis and discovery of new oncotargets represent an important task in cancer research.

Consistent with the complexity of metastasis, various types of molecules have been implicated in early steps of the metastatic cascade, including chemokines, signal transducers, transcription factors, proteases and adhesion molecules.^{10,13,14} Some of these molecules have direct and profound effects on tumor progression and development of a specific tumor microenvironment favoring metastasis. A significant mediator of events in the microenvironment is the cytokine transforming growth factor beta (TGF β), which has both restraining and promoting effects on tumor progression.¹⁵⁻¹⁸ For many epithelial cells, TGF β acts as an inhibitor of cell growth and thus, functions as a tumor suppressor *in vivo*. However, in the later stages of tumor development, TGF β can function as a potentiator of cell growth and dissemination.¹⁹⁻²¹

The intracellular and extracellular biology of TGF β is complex. TGF β , found as three isoforms (TGF β -1, -2, and -3), is synthesized as a TGF β pro-protein monomer containing two distinct regions: the propeptide sequence, called the latency-associated protein (LAP), and the mature TGF β sequence. In the rough endoplasmic reticulum, the pro-TGF β homodimer is formed and bound covalently to an LTBP molecule, generating a tripartite large latent complex (LLC). LLC formation involves disulfide bonding between the LTBP molecule and each of the two LAP regions of pro-TGF β .²²⁻²⁴ During maturation in the Golgi, pro-TGF β is cleaved by furin, generating a mature TGF β and LAP. Mature TGF β and LAP homodimers remain associated, constituting a small latent complex (SLC) covalently bound to LTBP. Therefore, all TGF β is secreted as a latent complex. Among the four LTBP isoforms, LTBP-1 and -3 efficiently bind to SLC containing any of the three TGF β isoforms, whereas LTBP-4 binds only TGF β 1-SLC and LTBP-2 does not bind SLC.²³⁻²⁵

The formation of the LLC is crucial for secretion of latent TGF β , its binding to the extracellular matrix (ECM) and for release of mature TGF β from the ECM.²⁶⁻²⁹ The LLC, comprising the LTBP molecule and mature but inactive TGF β dimer is secreted from the

cell. In the extracellular milieu, LLC is anchored *via* LTBP to fibronectin or fibrillin.^{30,31} TGF β binding to LAP precludes the interaction of TGF β with its receptor, TGFR, and therefore, TGF β must be released from LAP (a process referred as to activation) to bind TGFR and induce TGFR-mediated cell signaling.²⁵ LTBP-1, -3, and -4 are important for modulating TGF β functions,^{32–34} whereas LTBP-2 and -4 possess TGF β -independent activities that regulate the organization of the ECM.^{29,35}

Given the pleiotropic nature of TGF β functions in cancer progression and the importance of LTBPs in the overall regulation of TGF β activity, LTBP involvement in the metastatic cascade has received surprisingly little attention and the potential roles of individual members of the LTBP family in cancer cell dissemination remain unresolved. A few papers describe variations in expression of LTBP family members in a limited number of cancer types,^{36–39} but only 2 publications have functionally linked individual members of the LTBP family with different *in vivo* aspects of cancer cell biology. Thus, high levels of LTBP-3 correlated with poor outcome in a subset of human breast cancer patients, whereas RNA knockdown causally linked LTBP-3 with metastatic spread of breast cancer cells in mice.⁴⁰ The knockdown approach has also linked LTBP-2 with inhibited invasion of thyroid carcinoma cells *in vitro* and their growth *in vivo*.⁴¹ However, no studies have documented specific role(s) of LTBPs in cancer cell dissemination or indicated whether other types of cancer cells require LTBPs for metastasis.

Herein, using three human tumor cell lines of distinct tissue origin, two spontaneous metastasis models and two experimental metastasis models, we have demonstrated LTBP-3 is required for efficient hematogenous dissemination of primary cancer cells. Importantly, loss of LTBP-3 did not affect proliferation of tumor cells or their capacity to colonize secondary tissues, but significantly inhibited the ability of LTBP-3-deficient cells to complete early steps in the metastatic cascade, including induction of angiogenesis and intravasation. Furthermore, the intravasation-promoting role of LTBP-3 in metastasis might be independent of TGF β . Our data indicate that LTBP-3 acts as an autonomous regulator of early steps of the metastatic cascade and therefore LTBP-3 represents a potential oncotarget for therapeutic intervention into metastatic spread of cancer. Finally, we show the prognostic value of *LTBP3* expression for survival of cancer patients with early stage head and neck squamous cell carcinomas, further corroborating our findings on a specific role for LTBP-3 in cancer progression towards metastatic disease.

RESULTS

Expression of LTBP-3 in human tumor cells and its downregulation by siRNA

To examine the functional role of LTBP-3 in different steps of the metastatic cascade, we employed siRNA silencing to downregulate the expression of LTBP-3 in human epidermoid HEP-3 and prostate PC-3 carcinomas and HT-1080 fibrosarcoma. All three cell lines secrete LTBP-3 with an expected apparent mol. wt. of ~160–180 kDa (Figure 1). Following treatment with LTBP-3-specific siRNA (siLT3), all tested cell types displayed a substantial (>90–95%) and sustained (5–6 days) reduction in secreted LTBP-3 compared to cells treated with control siRNA (siCtrl) (Figure 1). Importantly, this significant downregulation of LTBP-3 was observed with 5 distinct siRNAs, all targeting unique sequences in *LTPB3*

transcripts, thereby reaffirming the specificity of siLT3 treatment (Supplementary Figure 1). The sequences of siLT3 duplexes are presented in Table 1 in the Supplemental Information. These LTBP-3-targeting siRNA were used throughout this study in both *in vitro* and *in vivo* experiments and all siLT3 constructs demonstrated similar functional effects associated with the deficiency of secreted LTBP-3 protein. Specificity of LTBP-3 targeting was also confirmed by the lack of any LTBP-3 downregulation by siRNA constructs against a transmembrane molecule CD44 or an intracellular protein RCL, while expression of LTBP-3 was knocked down completely in parallel cultures treated with siLT3 (Supplementary Figures 2A, B). In agreement with the transient nature of siRNA treatment, LTBP-3 secretion returned to control levels approximately 8–10 days after transfection (Supplementary Figure 2C).

To investigate whether downregulation of LTBP-3 was accompanied by compensation in expression of other genes of the LTBP family, we analyzed the expression of *LTBP1*, *LTBP2* and *LTBP4* in HEp-3 cells transfected with siLT3. RT-qPCR data demonstrate that expression levels of these 3 LTBP genes were not affected by downregulation of *LTBP3* (Supplementary Figure 3). In addition, we analyzed putative changes in the secretion of LTBP-1 and LTBP-3 proteins secreted after reciprocal treatment of cancer cells with LTBP-3 or LTBP-1 siRNAs (Supplementary Figure 4). This protein secretion analysis revealed no changes in the secretion of LTBP-3 in response to treatment with siLT1 in both HEp-3 and HT-1080 cells and also demonstrated no changes in LTBP-1 levels in HEp-3 cells treated with siLT3. Together, our mRNA and protein expression data provide no evidence for any putative compensation of LTBP-3 deficiency in siLT3-treated cancer cells.

LTBP-3 is involved in dissemination of human tumor cells in an avian model of spontaneous metastasis

The role of LTBP-3 in spontaneous metastatic dissemination of human carcinoma cells was analyzed in two animal model systems employing either chick embryos or immunodeficient mice. In the chick embryo spontaneous metastasis model, human tumor cells are grafted on the chorioallantoic membrane (CAM), where aggressive cancer cells generate primary tumors and induce formation of angiogenic blood vessels.⁷ Tumor cells intravasate into intratumoral blood vessels, reach the general circulation and after a rapid cycle within the embryo, the majority of circulating tumor cells are trapped in the fine capillary network of the CAM ectoderm plexus. Therefore, the CAM tissue serves as the main repository of intravasated cells, although a small fraction of intravasated cells also arrest in internal organs, including liver and lungs. For quantification of intravasated human cells, the portions of the CAM distal to primary tumor (2–3 cm from tumor border) are harvested and analyzed by extremely sensitive human-specific *Alu*-qPCR.^{42–46}

On the CAM, LTBP-3-silenced cells from the three tumor cell lines formed highly vascularized primary tumors similar in size to siCtrl tumors (Figures 2a, d and g). These results indicate that decreasing LTBP-3 production and secretion does not diminish primary tumor growth over 5–7 days, consistent with the lack of inhibitory effects of siLT3 treatment on overall cell morphology and cell proliferation (Supplementary Figures 5 and 6A). However, LTBP-3 silencing caused ~70%, 65% and 50% decrease in the number of HEp-3,

PC-3 and HT-1080 intravasated cells, respectively, as demonstrated by qPCR of human-specific *Alu*-sequences (Figures 2b, e and h; graphs on the left). When analyzed as combined fold differences calculated in individual experiments (7 experiments for HEp-3 cells, 6 experiments for PC-3 cells and 5 experiments for HT-1080 cells), LTBP-3 knockdown resulted in 53% ($P<0.01$), 55% ($P<0.01$) and 52% ($P<0.01$) inhibition of HEp-3, PC-3 and HT-1080 cell intravasation, respectively (Figures 2b, e and h, graphs on the right). Thus, two methods of statistical analysis confirmed inhibition of intravasation ability by LTBP-3 silencing. Furthermore, this inhibitory pattern of siLT3 treatment on intravasation was reproduced for all 3 types of tumor cells in the extent of their metastatic dissemination into the liver from control *versus* LTBP-3-deficient tumors (Figures 2c, f and i).

These results indicate that LTBP-3 is involved in spontaneous dissemination of human cancer cells and that expression and secretion of LTBP-3 is essential for efficient intravasation in cancer cells of three distinct histological types.

LTBP-3 expression is not required for tissue colonization in an experimental metastasis model

Extravasation and secondary tissue colonization constitute late steps in the metastatic cascade, which can determine the overall outcome of spontaneous metastasis. To distinguish between requirements for LTBP-3 during early versus late metastatic events, we examined whether LTBP-3 expression in cancer cells was critical for tissue colonization. We employed an experimental metastasis model, in which tumor cells are inoculated directly into the circulation, thereby bypassing all early steps of the metastatic cascade, including primary tumor formation and intravasation.

When inoculated into the allantoic vein of day 12 chick embryos, LTBP-3 silenced HEp-3 and PC-3 cells exhibited similar levels of CAM colonization compared to their siCtrl-treated counterparts as determined by *Alu*-qPCR (Figures 3a and b). Furthermore, tissue colonization by siCtrl and siLT3 GFP-tagged HEp-3 cells was very similar morphologically when analyzed by immunofluorescence microscopy (Figure 3c), indicating that later events in metastasis, which follow intravasation and include vascular arrest, extravasation and colony formation, are not affected by substantial downregulation of LTBP-3 production in both HEp-3 and PC-3 cells. This conclusion is also supported by *in vitro* data showing that downregulation of LTBP-3 in HEp-3 cells did not affect their proliferation, migration and invasion (Supplementary Figures 6A, B, C). The lack of detrimental effects of LTBP-3 downregulation on the motility of HEp-3 cells was also corroborated *in vitro* by the expression analysis of EMT-related proteins such as twist, slug, fibronectin, β -catenin and E-cadherin, all of which are known to be involved in the regulation of cell locomotion. Whereas western blot analysis demonstrated that HEp-3 cells do not express E-cadherin, thus indicating their mesenchymal-like phenotype, it also showed no significant changes in any of these EMT-marker proteins in siLT3-treated versus control HEp-3 cells (Supplementary Figure 7).

Together, these data indicate that LTBP-3 deficiency does not affect the ability of carcinoma cells to complete late metastatic steps, further substantiating a significant role for LTBP-3 in early tumor cell dissemination.

Functional involvement of LTBP-3 in tumor cell-induced angiogenesis

One of the early metastatic events that precede the intravasation step during tumor cell dissemination is the formation of new tumor-induced blood vessels, i.e. angiogenesis. Therefore, we next examined whether angiogenesis-inducing ability of cancer cells was affected by loss of LTBP-3. Intratumoral angiogenic vessels provide the major vascular conduits for intravasation of primary tumor cells and their subsequent dissemination to secondary organs.⁷ Therefore, we examined the effect of LTBP-3 downregulation on the ability of HEP-3 cells to induce angiogenesis employing our quantitative *in vivo* assay.⁴⁷

HEP-3 cells, treated with control or LTBP-3-specific siRNA, were incorporated into 3-dimensional native collagen gels embedded between two small nylon meshes, generating “onplants” that were grafted on the CAM of chick embryos developing *ex ovo*. Within three days, newly formed vessels were scored and angiogenesis indices quantified as the ratio of grids filled with angiogenic blood-carrying vessels *versus* total number of grids examined in an onplant. As demonstrated in Figure 4a, siLT3 treatment significantly reduced the angiogenesis levels compared to the negative control ($P<0.01$), indicating that LTBP-3 secreted from tumor cells is involved in tumor angiogenesis.

We next sought to rescue the negative effect of LTBP-3 downregulation on tumor-induced angiogenesis. To this end, HEK-293 cells were stably transfected with human *LTBP3* alone (293-LT3), *TGFB1* alone (293-TGF β) or *LTBP3* and *TGFB1* together (293-LT3/TGF β). Negative control cells were generated by using empty vectors (293-EV) and were confirmed by western blotting to produce no detectable LTBP-3 or TGF β (Supplementary Figure 8A,B). Both 293-TGF β and 293-LT3 cells secreted very low levels of LTBP-3 as indicated by western blotting (Supplementary Figure 8A). These cells also produced relatively low levels of TGF β as demonstrated by a quantitative reporter assay and western blotting (Supplementary Figure 8B). In contrast, 293-LT3/TGF β cells secreted high levels of LTBP-3 and TGF β (Supplementary Figures 8A, B), suggesting the mutual importance of LTBP-3 and TGF β for their secretion. Silver staining demonstrated similar amounts of total proteins secreted by all four 293 cell types (Supplementary Figure 8C), pointing to the potential use of concentrated conditioned media (CMs) in rescue experiments. Of note, siLT3-treated HEP-3 cells secreted ~50% less total TGF β compared to siCtrl cells (Supplementary Figure 8D), also supporting that mutual expression of LTBP-3 and TGF β is critical for efficient secretion of each protein.

High levels of LTBP-3 in 10X LT3/TGF β -CM and the complete lack of LTBP-3 in EV-CM were confirmed by western blotting (Figure 4b, left). In contrast, silver staining indicated no major differences in the bulk of proteins in CMs, except for an enrichment of a 160–180 kDa silver-stained protein band in LT3/TGF β -CM likely representing secreted LTBP-3 (Figure 4b, right).

In angiogenesis assays, siLT3-treated HEP-3 cells were incorporated into collagen along with LT3/TGF β -CM (serving as a specific enriched source of LTBP-3 protein) or EV-CM (serving as a negative control), and the levels of induced angiogenesis were compared with those of siCtrl-treated cells (Figure 4c). Similar to the data shown in Figure 4a, downregulation of LTBP-3 significantly diminished angiogenesis-inducing capacity of

HEp-3 cells and these dampened levels of angiogenesis were rescued with LT3/TGF β -CM but not with EV-CM (Figure 4c).

These data further indicated that the inhibition of tumor-induced angiogenesis in siLT3-treated HEp-3 cells was related to the LTBP-3 protein deficiency and prompted us to employ this rescue approach in our primary tumor intravasation model since the development of intratumoral angiogenic vessels is required for efficient tumor cell dissemination.⁷

LTBP-3 protein is functionally involved in tumor cell intravasation

LTBP-3-enriched LT3/TFG β -CM was used to rescue intravasation of LTBP-3-silenced HEp-3 cells. In these experiments, siLT3-treated HEp-3 cells were grafted on the CAM along with LT3/TGF β -CM or EV-CM (10 μ l of 10X-CM per cell inoculum). Topical additions of the corresponding CMs were conducted on days 1 through 3 after tumor cell grafting. The siLT3 cells treated with plain serum-free (SF) medium provided negative control for CM treatments, whereas siCtrl-treated HEp-3 cells served as a positive control for primary tumor development and high levels of tumor cell intravasation. On day 5 after cell grafting, primary tumors were excised to determine tumor weight and distal CAM tissue was analyzed by *Alu*-qPCR to determine the levels of intravasated HEp-3 cells in embryos bearing primary tumors with similar weight (Supplementary Figure 9).

When quantified as fold difference compared to the mean of negative control in 7 independent experiments, siLT3 treatment resulted in almost 75% inhibition of HEp-3 intravasation ($P < 0.05$; Figure 4d). Strikingly, the dampened levels of intravasation were increased more than 2.5 fold by addition of LT3/TGF β -CM, which rescued intravasation to near siCtrl levels ($P < 0.05$), but there was no such increase if EV-CM was used (Figure 4d). Importantly, no increase in intravasation occurred when TGF β -CM was used for rescue, even if TGF β -CM was added at 2.5 fold excess of LT3/TGF β -CM (Figure 4d), suggesting that the latent TGF β , non-complexed to LTBP-3, is incapable of rescuing LTBP-3 deficiency. In addition, TGF β -CM slightly inhibited growth of siLT3 tumors (Supplementary Figure 9), indicating a possible suppressive role of TGF β not complexed with LTBP-3.

LTBP-3 regulates the angiogenesis-dependent tumor cell intravasation

To associate the LTBP-3-dependent development of an intravasation-supporting intratumoral angiogenic vasculature directly with the levels of tumor cell intravasation, we employed our microtumor model⁴⁸. In this model, tumor cells are grafted on the CAM within small droplets of native type I collagen. Five days later, the tumor-bearing embryos are injected with a fluorescent LCA, which is used to selectively highlight CAM vasculature.⁴⁹ LCA efficiently stains the surface of avian endothelial cells allowing for immunofluorescence imaging of live, non-fixed blood vessels in primary tumors developing from GFP-tagged tumor cells.^{43,45,46,48,50} Portions of distal CAM are harvested and analyzed by *Alu*-qPCR to quantify the levels of intravasated tumor cells that have disseminated from the microtumors, whereas intratumoral vasculature is visualized in a fluorescence microscope.

Within 5 days after grafting, GFP-tagged HEp-3 cells treated with siCtrl or siLT3 gave rise to morphologically similar primary microtumors ~1.5–2 mm in diameter with overall similar density of tumor-associated blood vessels (Figure 5a). However, epifluorescence microscopy

revealed that siLT3 tumors exhibited less dilated and less perfusable intratumoral vasculature compared to control tumors (Figure 5b). Following imaging, primary microtumors were analyzed for LTBP-3 expression by western blotting that confirmed the sustained downregulation of LTBP-3 in siLT3 microtumors at the time of intratumoral vessel microscopic examination (Figure 5c). Detailed image distribution analysis confirmed a significant decrease in the fraction of intratumoral vessels with lumen diameter of 15–40 μm and a reciprocal increase in thinner vessels (<15 μm in diameter) in the LTBP-3-deficient microtumors compared to the siCtrl tumors, where the vessels with 15–40 μm lumens constituted the majority (>60%) of all angiogenic vessels (Figure 5d). It is important that in conjunction with the downregulated LTBP-3, the levels of intravasation from LTBP-3-deficient tumors were substantially lower compared to control tumors (Figure 5e). In tandem with the deficiency in 15–40 μm intratumoral vessels, these data strongly indicate that LTBP-3 is functionally involved in tumor-induced angiogenesis and angiogenesis-dependent tumor cell intravasation.

LTBP-3 regulates dissemination of human carcinoma cells in a mouse model of spontaneous metastasis

To confirm that LTBP-3 expression and secretion are critical for spontaneous dissemination of cancer cells in a mammalian setting, we used our novel orthotopic model for head and neck cancer employing immunodeficient mice (Supplementary Figure 10). In this model, human head and neck carcinoma cells (e.g., HEP-3 cells) are inoculated into the buccal mucosa, a well-vascularized tissue, assuring rapid development of primary tumors. Inoculation into the buccal mucosa instead of the tongue, a usual place for orthotopic implantation for head and neck carcinomas,⁵¹ also allows for the growth of relatively large primary tumors without major interference with the ability of tumor-bearing mice to consume solid food during tumor development. Importantly, aggressive primary tumor cells disseminate to the lungs and mandibular lymph nodes (the main sites of hematogenous and lymphogenous metastasis, respectively, in this model), where they can be visualized microscopically and quantified by human-specific *Alu*-qPCR.

GFP-tagged HEP-3 cells were treated with siCtrl or siLT3 and inoculated into the buccal mucosa of NOD-SCID mice (Figure 6a). Within 12–14 days after grafting, both LTBP-3-silenced and control HEP-3 cells yielded primary tumors of similar size (Figure 6b). However, the spontaneous dissemination to the lung of siLTBP-3-silenced HEP-3 cells was inhibited by more than 60%, as visualized by immunofluorescence microscopy (Figure 6c) or quantified by *Alu*-qPCR (Figure 6d; $P=0.016$). Confirming the specificity and reproducibility of these findings, treatments of HEP-3 cells with different siLT3 constructs resulted in similar inhibitory effects of LTBP-3 downregulation on the levels of lung metastasis, but not on primary tumor growth (Supplementary Figure 11). In contrast, equal numbers of human cells were found in the lungs from mice injected i.v. into the tail vein with either siCtrl- or siLT3-treated HEP-3 cells (Figure 6e), demonstrating that tissue colonization did not depend on the levels of LTBP-3 produced by tumor cells.

These results are entirely consistent with the outcomes of the CAM spontaneous and experimental metastasis assays (Figures 2 and 3), thereby confirming a role of LTBP-3 in

early events in cancer cell metastatic dissemination, now demonstrated in two distinct animal models.

***LTBP3* is a survival prognosis gene in early-stage head and neck cancer**

Recently, *LTBP3* has been identified as one of a number of genes with copy number alterations in patients with oral squamous cell carcinomas.⁵² The data from our orthotopic murine model for head and neck cancer employing HEP-3 epidermoid carcinoma cells (Figure 6) indicated that expression of *LTBP-3* is important for the levels of spontaneous metastasis of cancer cells from primary buccal tumors to lungs, but not for late stages of metastatic colonization associated with vascular arrest, survival, extravasation and lung colonization, all of which follow the intravasation step.

To investigate whether the expression levels of *LTBP3* were associated with overall survival of patients with head and neck cancer, we analyzed The Cancer Genome Atlas (TCGA) Provisional dataset of head and neck squamous cell carcinomas (HNSC) (n=517; stages I-IV) for overall 10-year survival depending on relative levels of *LTBP3*. The Kaplan-Meier survival curves for median *LTBP3* expression value of >1135 (n=258) versus 1135 (n=259) indicated that lower levels of *LTBP3* expression are associated with slightly better survival rates (Figure 7a). These “low *LTBP3*” and “high *LTBP3*” survival curves become separated earlier (at ~30 months versus ~50 months) if survival analysis is performed for *LTBP3* expression cut-offs that were correspondingly increased to >1800 (n=136) and lowered to <700 (n=127) (Figure 7b). Importantly, when Kaplan-Meier survival curves are generated for these *LTBP3* expression cut-offs but also for the cohort of patients diagnosed only at stages I-III instead of stages I-IV, the data indicate that high levels of *LTBP3* expression are associated with much worse rates of survival compared to low *LTBP3* levels and this survival difference is of high significance ($P=0.028$), with the survival curves now separating at ~20 months (Figure 7c). Interestingly, a similar type of survival analysis from the same database does not indicate any overall survival benefit of low levels *LTBP1* or *LTBP2* gene expression with some but statistically insignificant benefit for low expression of *LTBP4* gene (Supplementary Figure 12), further highlighting the high specificity and significance of the inverse correlation of *LTBP3* expression with early stage cancer patients’ survival (Figure 7).

DISCUSSION

We have demonstrated that silencing of the TGF β -binding protein, *LTBP-3*, substantially reduces metastatic dissemination of two human carcinoma cell lines, HEP-3 and PC-3, and a human fibrosarcoma cell line, HT-1080, in two different live animal model systems, the chick embryo CAM and a mouse orthotopic implantation models. When combined with the earlier study by Naba *et al.* on human breast carcinomas,⁴⁰ the metastatic potential of four different tumor types has now been shown to depend on *LTBP-3*. The specificity of the *LTBP-3* silencing was verified with multiple *LTBP-3*-specific siRNA constructs that led to >90% reduction in *LTBP-3* secretion over 4–7 days. The metastasis-targeting specificity of *LTBP-3* silencing was demonstrated by a number of experimental approaches: *LTBP-3* siRNA had no effect on tumor cell proliferation, chemotactic migration and collagen invasion and, importantly, had little or no effect on primary tumor growth *in vivo*. However,

the diminished LTBP-3 production brought about a substantial, 50%-75% inhibition of tumor cell intravasation coordinated with a similar inhibition of cell dissemination to a secondary organ, liver, in the CAM model employing all 3 distinct tumor cell types tested and more than 60% inhibition of spontaneous metastasis to the lung in our orthotopic mouse model employing HEp-3 epidermoid carcinoma.

We also employed murine and avian experimental metastasis model systems, in which the outcomes of late steps in the metastatic cascade (vascular arrest, extravasation and secondary tissue metastatic outgrowth) were quantitatively compared between control and LTBP-3-silenced tumor cells. In these experiments, the LTBP-3-deficient HEp-3 and PC-3 cells did not differ from their LTBP-3-competent counterparts, showing equal colonization levels of mouse lung and chick embryo CAM. Not only did these experimental metastasis experiments indicate no deleterious, off-target effects of siLT3 on overall tumor cell functionality *in vivo*, but these results strongly affirmed that LTBP-3 silencing specifically affected early events in the metastatic cascade. Furthermore, our mouse orthotopic lung metastasis data actually indicate that in control animals the first tumor cell dissemination events that are measured 12 days after cell implantation occur in the developing primary tumors very early, namely within first days of primary tumor development. The immunofluorescence images in Figure 6 provide visual evidence for this conclusion: The colony size in the lungs of control animals appear larger than colonies in the lungs from siLT3 group, indicating that colony-forming cells had arrived to the lungs of control animals at earlier times and thus had more time to proliferate and grow in size compared to tumor cells disseminated from LTBP-3-deficient tumors. These data further indicate that knockdown of LTBP-3 affects an early step in cancer cell dissemination, namely the proposed intravasation step.

The most crucial evidence that LTBP-3 is functionally involved in tumor cell intravasation is that the diminished intravasation of LTBP-3-silenced cells was rescued by *in vivo* delivery of LTBP-3 protein to primary tumors. In agreement with previous publications indicating that LTBP-3 is secreted complexed with TGF β ,^{53,54} the release of LTBP-3 occurred only if LTBP-3 was co-transfected with TGF β . Importantly, only LTBP-3-enriched CM, but not control EV-CM or TGF β -CM devoid of LTBP-3, rescued the siLT3-dampened intravasation. This direct intravasation-rescuing ability of LTBP-3-containing CM strongly suggests that siLT3 treatment blocked a specific extracellular function of LTBP-3 within the primary tumor microenvironment.

Networks of newly formed angiogenic vessels constitute a critical component of the primary tumor microenvironment. Our findings showing the effects of LTBP-3-silencing on tumor cell-induced angiogenesis are indicative of the mechanism whereby LTBP-3 deficiency in primary tumors dampens intravasation. Thus, the knockdown of LTBP-3 in HEp-3 carcinoma cells caused a 50% inhibition of their angiogenesis-inducing capacity that was rescued by *in vivo* delivery of LTBP-3 protein. Similarly, LTBP-3-enriched CM delivered to primary tumors rescued *in vivo* the inhibited intravasation of LTBP-3-silenced HEp-3 cells.

Formation of a functional intratumoral vasculature is also a prerequisite for the initiation of intravasation.^{7,45,46} Therefore, the microarchitecture of intratumoral vasculature, namely the

lumen size of angiogenic vessels, their interconnectivity and permeability, are necessary for entry and subsequent dissemination of escaping primary tumor cells. In this regard, the LTBP-3-silenced HEP-3 cells are deficient in their ability to induce the development of intratumoral vessels with lumen sizes 15–40 μm and proper interconnectivity within the primary tumor. The outcome of LTBP-3 knockdown is that the intratumoral vasculature in LTBP-3-deficient tumors is significantly depopulated of blood vessels with lumens between 15 and 40 μm , a size necessary for efficient entry and occupancy of disseminating tumor cells^{45,46}. Reciprocally, the LTBP-3 knockdown caused an increase in the fraction of very thin, semi-collapsed or non-perfusible vessels with lumens $<15 \mu\text{m}$, a diameter too narrow to accommodate the volume of an intravasating tumor cell. Importantly, these LTBP-3-dependent microstructural changes in intratumoral angiogenic vessels were directly linked to the significantly diminished intravasation of LTBP-3-silenced tumor cells (Figure 5).

It is not possible at present to delineate the precise molecular mechanism of LTBP-3-mediated induction of intratumoral vasculature and maintenance of its intravasation-sustaining ability since the majority of tumor cell-secreted LTBP-3 is likely complexed with TGF β .^{53,54} To do so will require the isolation of LTBP-3 stripped of its TGF β or the development of mutant forms of LTBP-3 that are genetically manipulated to be incapable of binding TGF β but nevertheless secreted. Generation of such TGF β -less LTBP-3 would allow uncoupling the functional effects of TGF β versus LTBP-3 on tumor cell angiogenesis and tumor cell intravasation. Such efforts are now underway. The fact that the CM from LTBP-3-downregulated HEP-3 cells still contains TGF β is suggestive that TGF β is not limiting and that the diminution of metastasis reflects loss of a TGF β -independent activity of LTBP-3. Our experiments carried out with TGF β -CM used at 2.5-fold excess over LT3/TGF β -CM indicated that the latent TGF β secreted in the absence of LTBP-3 could not compensate for LTBP-3 deficiency in the tumor microenvironment (Figure 4), again highlighting the functional compensatory activity of LTBP-3. It is noteworthy that LTBP-2 and -4 both possess TGF β -independent activities.^{29,35} Likewise, LTBP-3 appears to possess specific functions that are either independent of TGF β functionality or required for TGF β to act properly within the primary tumor microenvironment. The rescuing effects of the exogenously-added LTBP-3 on both tumor-induced angiogenesis and tumor cell intravasation also indicate that the LTBP-3 that might have been produced by the host stromal cells was not able to compensate for the LTBP-3 deficiency in cancer cells, once more emphasizing the importance of expression and secretion of tumor cell-derived LTBP-3 within the complex microenvironment of the developing primary tumor.

The special significance of LTBP-3 expression in cancer metastasis was also corroborated by our survival analysis for head and neck cancer, which indicated that among the 4 members of the LTBP family only *LTBP3* gene expression levels were significantly associated with poor prognosis for patients diagnosed with tumors at relatively early stages of development (stages I-III), preceding stage IV when metastatic cancer cells had already accomplished their hematogenous metastasis to the secondary site(s). These clinical data nicely corroborate our findings obtained in live animal models on the significance of LTBP-3 production and secretion in cancer cells for their early metastatic dissemination. In contrast to *LTBP3*, expression levels of *LTBP1* and *LTBP2* appear to positively correlate with survival rates of head and neck cancer patients. This tendency towards survival correlation

with higher levels of *LTBP1* expression is consistent with the view that *LTBP1* might act as a tumor suppressor.⁵⁵

In conclusion, our study identified LTBP-3 as a novel oncotarget protein involved in specific processes during cancer metastasis, namely in the induction of tumor angiogenesis resulting in the formation of the intratumoral vasculature capable of supporting active tumor cell intravasation. Importantly, our data are consistent with the survival prognostic value of *LTBP3* expression in early stage head and neck tumors, further indicating a specific role for LTBP-3 in cancer progression towards metastatic disease. Since inhibition of pleiotropic TGF β signaling in cancer therapy has proven to be challenging,^{15,21,56,57} the targeting of LTBP-3 functions upstream of the TGF β molecule might provide a more efficient approach to prevent early tumor cell dissemination.

MATERIALS AND METHODS

Human tumor cell lines and culture conditions

High-disseminating tumor variants were selected from parental human fibrosarcoma HT-1080, prostate carcinoma PC-3 (ATCC) and head and neck epidermoid carcinoma HEp-3.^{44,45,58} Generation of HEK-293 cell transfectants expressing LTBP-3, TGF β or LTBP-3/TGF β is described in Supplementary Information. Cells were routinely cultured in DMEM supplemented with 10% FBS (Peak serum, Colorado, USA).

Downregulation of LTBP-3

Small interfering RNA (siRNA) duplexes against human LTBP-3 siRNA were from Santa Cruz (sc-106921A-C; pool of three unique siRNAs), Ambion (AM16708) and Invitrogen (10620318 and 10620319). Corresponding negative control siRNA were from the same companies. Transfections were conducted with RNAiMax (Invitrogen), according to the manufacturer's instructions. The nucleotide sequences of all 5 siRNA duplexes are presented in Table 1 in the Supplemental Information.

Animal studies

All experiments involving animals were conducted in accordance with the Animal Protocol approved by TSRI Animal Care and Use Committee (IACUC). Standard chick embryo angiogenesis model, chick embryo spontaneous and experimental metastasis models and mouse experimental metastasis model are described in detail in the **Supplemental Information**. Group allocations were done without blinding during experiments or when assessing the outcomes of experiments.

CAM microtumor model

The assays were conducted as described.^{45,46,48} Briefly, GFP-tagged HEp-3 cells, transfected with siCtrl or siLT3, were suspended at 1×10^7 cells per mL within 2.2 mg/mL neutralized type I collagen (Becton Dickinson). Six 10- μ L droplets of cell-containing collagen mixtures were placed separately on the top of the CAM. After 5 days, Rhodamine-conjugated LCA was inoculated i.v. to highlight the vasculature (25 μ g per embryo). Within 5–10 min, several pieces of the CAM tissue distal to the microtumors were excised and

processed for *Alu*-qPCR to quantify the number of intravasated tumor cells, after which the primary microtumors were visualized in an Olympus microscope. Images were acquired using Pictureframe software and analyzed using ImageJ for the lumen size distribution of intratumoral blood vessels. Following image acquisition, microtumors were analyzed by western blotting for the levels of LTBP-3 protein.

Mouse orthotopic model for spontaneous metastasis of human head and neck cancer

In this novel model employing either immunodeficient NOD-SCID or *nu/nu* mice, human head and neck carcinoma cells are orthotopically implanted into the buccal lining of the upper lip, instead of standard injections of tumor cells into the tongue, allowing for development of primary tumors without major interference with the food consumption by tumor-bearing mice till primary tumors reach ~0.5–0.6 g in weight. The lung is the main organ for hematogenous dissemination of HEP-3 cells in our orthotopic model: No disseminated human cells have been detected by *Alu*-qPCR in the liver, spleen, bone marrow, kidney, prostate or brain of HEP-3 tumor-bearing mice.

GFP-tagged HEP-3 cells were inoculated into 8 week-old NOD-SCID female mice at 5×10^5 cells per site (one site per mouse). After 10–12 days, HEP-3 tumors reached the end-point size and the mice were sacrificed. Primary tumors were excised and weighed. Lungs were excised and analyzed in an Olympus fluorescence microscope for the overall levels of lung colonization. Following imaging, lung tissue was processed for quantification of human tumor cells by *Alu*-qPCR.

Statistical analysis

Data processing and statistical analyses were conducted using GraphPad Prism. The data are presented as means \pm s.e.m. Sample sizes for power of 90% and a significance level of 5% using a two-tailed unpaired *t*-test were determined based on the formulas provided in ⁵⁹. The outliers were identified based on Grubbs' test provided by GraphPad Software. Where indicated, the data from individual experiments were normalized relative to the control of a particular experiment and then fold differenced pooled for statistical analyses. The data sets were compared with the non-paired two-tailed Student's *t*-test. *P* values <0.05 were considered statistically significant.

Analysis for overall 10-year survival depending on the levels of *LTBP* gene expression in head and neck cancer

Kaplan-Meier analysis was used to determine the prognostic significance of *LTBP3* gene expression in head and neck cancer progression. Overall 10-year survival curves for patients with head and neck squamous cell carcinomas were generated for the four members of the LTBP family (*LTBP1*, *LTBP2*, *LTBP3* and *LTBP4*) with different cut-off values of their expression in primary tumors and different stages of cancer (I-IV) at the time of diagnosis using the data from The Cancer Genome Atlas (TCGA) Research Network (TCGA, Provisional: <http://www.cbioportal.org/>). Statistical analyses were performed using the statistical package provided by cBioPortal and *P* values were calculated to test the associations between the selected cut-off level of *LTBP* gene expression and cancer stage as a clinico-pathological parameter. *P* values <0.05 were considered significant.

Standard procedures, including western blotting, silver staining, measurement of total TGF β , cell proliferation, Transwell migration, collagen invasion and *A/u*-qPCR, are described in the Supplementary Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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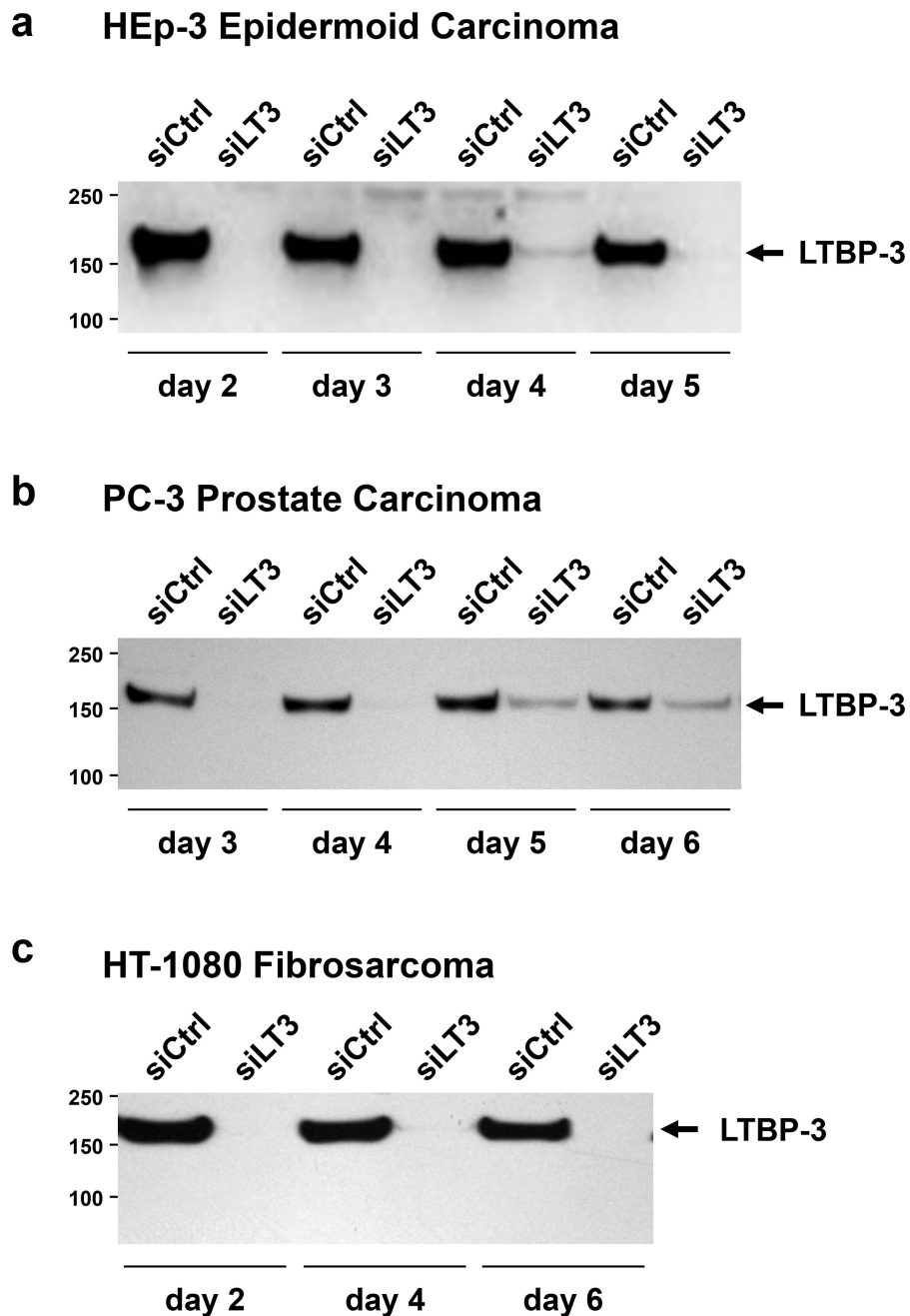


Figure 1. Secretion of LTBP-3 by human tumor cells and its downregulation by siRNA
 High disseminating variants of human epidermoid carcinoma HEP-3 (a), prostate carcinoma PC-3 (b) and fibrosarcoma HT-1080 (c) cells were treated with control siRNA (siCtrl) or siRNA targeting LTBP-3 (siLT3). At the days indicated at the bottom of images (days after siRNA transfection), culture medium was changed for SF-DMEM. Equal volumes of 24 hr-CMs (SF-medium conditioned by the same number of tumor cells) were analyzed for the levels of LTBP-3 protein (~ 160–180 kDa) by western blotting under reducing conditions. Positions of molecular weight markers in kDa are indicated on the left.

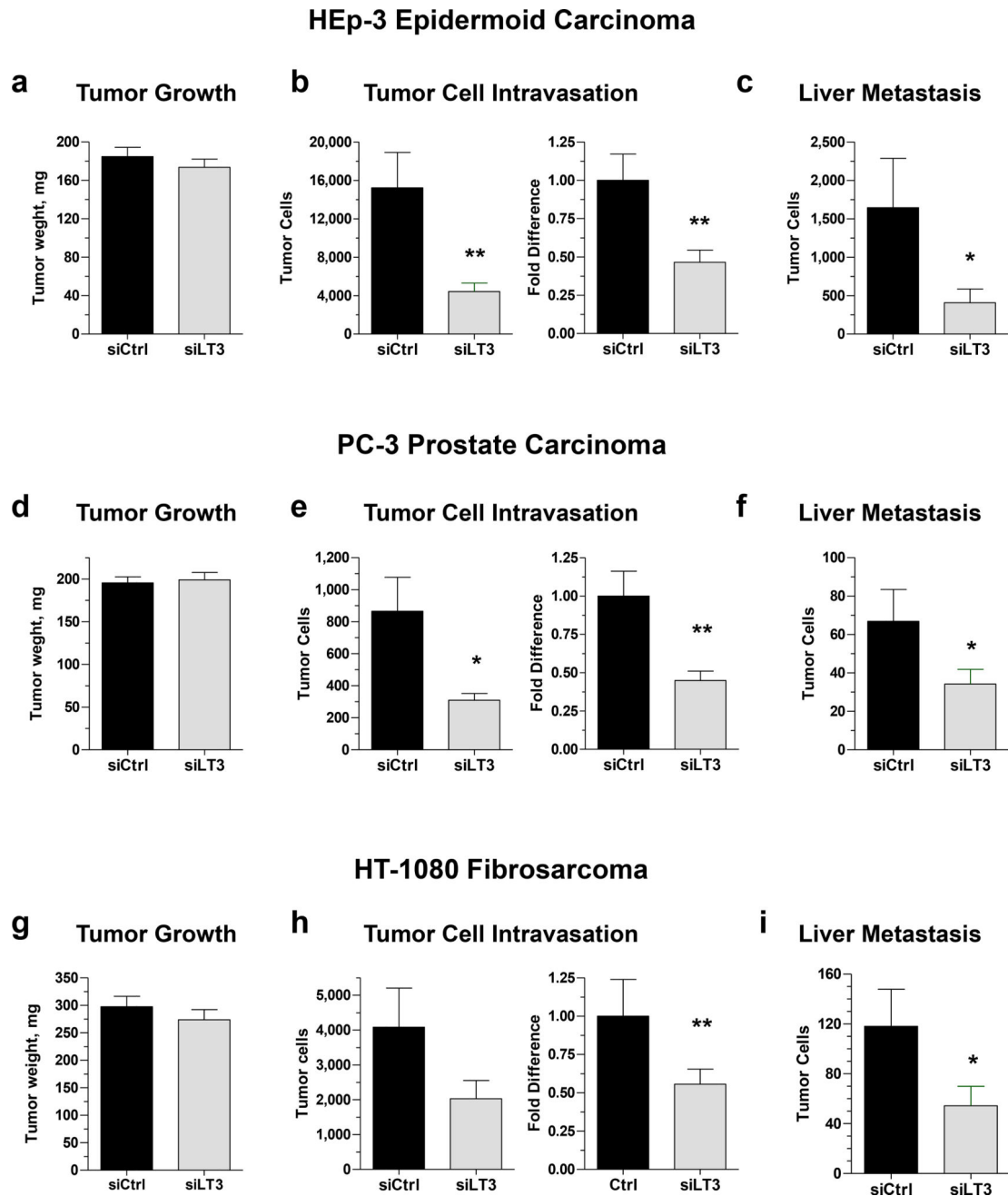


Figure 2. Spontaneous dissemination of human tumor cells depends on LTBP-3 expression
 HEp-3 (a–c), PC-3 (d–f) and HT-1080 (g–i) cells were treated with control siRNA (siCtrl) or siRNA targeting LTBP-3 (siLT3). The day after transfection, the cells were grafted on the CAM of 10 day-old chick embryos at 0.6×10^6 (HEp-3), 2.0×10^6 (PC-3) or 0.5×10^6 (HT-1080) cells per embryo. On day 5 after cell grafting, primary tumors were excised and weighed to determine the efficiency of tumor growth (a, d, and g). Portions of distal CAMs were harvested and processed to determine by *Alu*-qPCR the number of primary tumor cells intravasated to the CAM vasculature (b, e, and h; graphs on the left). A total of 47 to 63 siCtrl embryos and 40 to 52 siLT3 embryos were analyzed for each cell type in 6

independent experiments for Hep-3 and PC-3 cells and 5 independent experiments for HT-1080 cells. To analyze the differences between intravasation efficiency across independent experiments, fold differences were determined for each data point relative to the mean of siCtrl group in each individual experiment and then combined for statistical analyses (**b, e, and h; graphs on the right**). Dissemination of tumor cells from primary tumors to the liver was analyzed in 3 independent experiments for Hep-3 and PC-3 cells (employing a total of 15 to 41 embryos per treatment/cell type) and 2 independent experiments for HT-1080 cells (employing a total of 20 and 12 embryos per siCtrl and siLT3 groups, respectively) (**c, f and i**). Data are presented as means \pm s.e.m. * $P < 0.05$. ** $P < 0.01$.

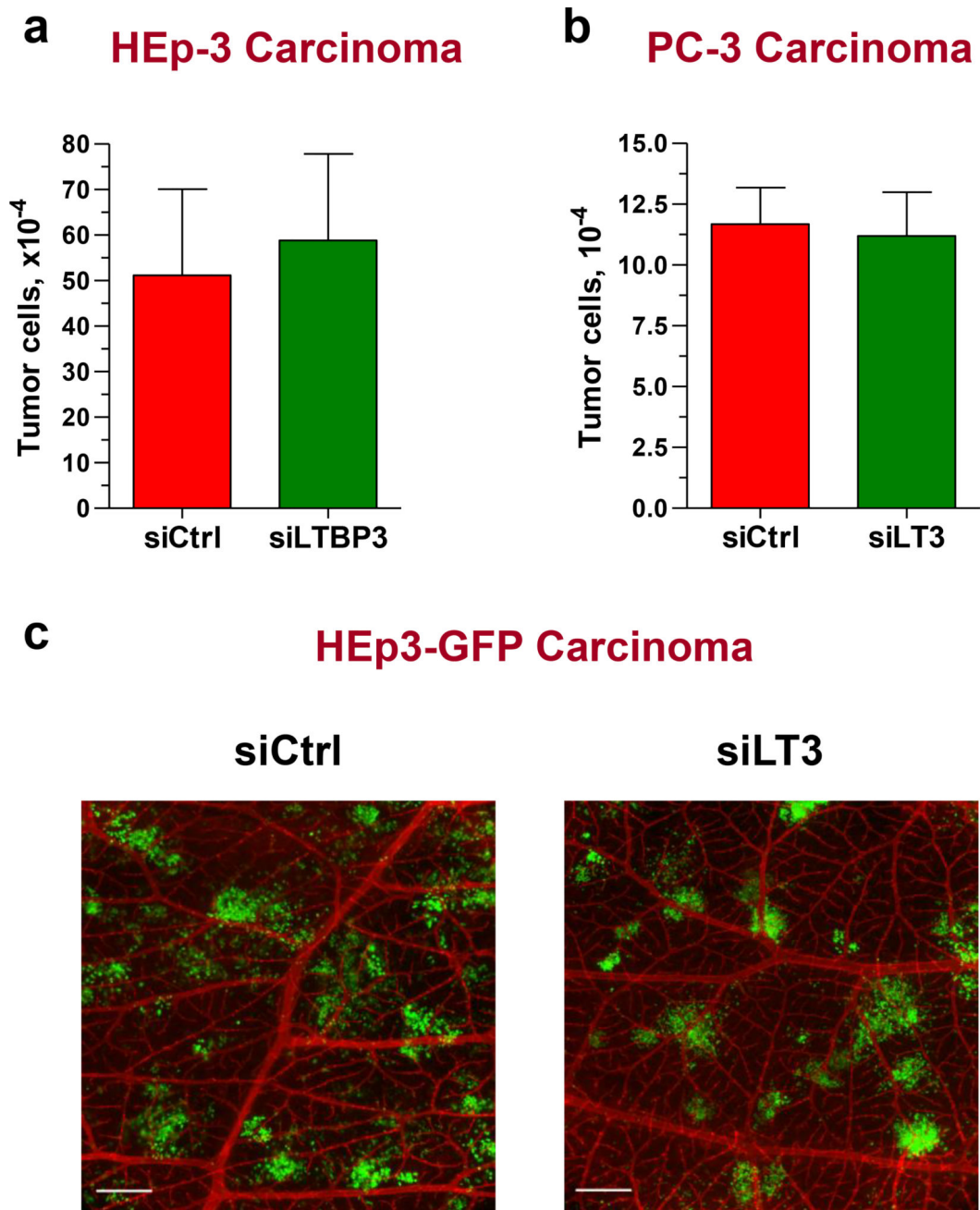


Figure 3. Levels of LTBP-3 expression do not affect tissue colonization ability of human tumor cells

HEp-3 (a) and PC-3 (b) cells were treated with control siRNA (siCtrl) or siRNA targeting LTBP-3 (siLT3). The following day after transfection, the cells were inoculated i.v. into the allantoic vein of 12 day-old chick embryos at 1×10^5 cells per embryo. On day 5 after cell injections, the CAM tissue was harvested and processed by *Alu*-qPCR to determine the efficiency of tumor cell colonization in the experimental metastasis model. Colonization capacity was determined in 7 independent experiments for HEp-3 cells (employing a total of 65 control and 62 siLT3 embryos) and 3 independent experiments for PC-3 (employing a total of 28 and 31 embryos per siCtrl or siLT3 treatments, respectively).

(c) Immunofluorescence microscopy of CAM tissue colonization. GFP-tagged HEp-3 cells were treated with siCtrl or siLT3 and inoculated i.v. into the allantoic vein of 12 day-old embryos. On day 5, the embryos were injected with Rhodamine-LCA to highlight the vasculature. After 5–10 min incubation, portions of the CAM were analyzed in a fluorescence microscope at the original 200X magnification (20X objective × 10X eyepiece). Scale bars, 50 μm.

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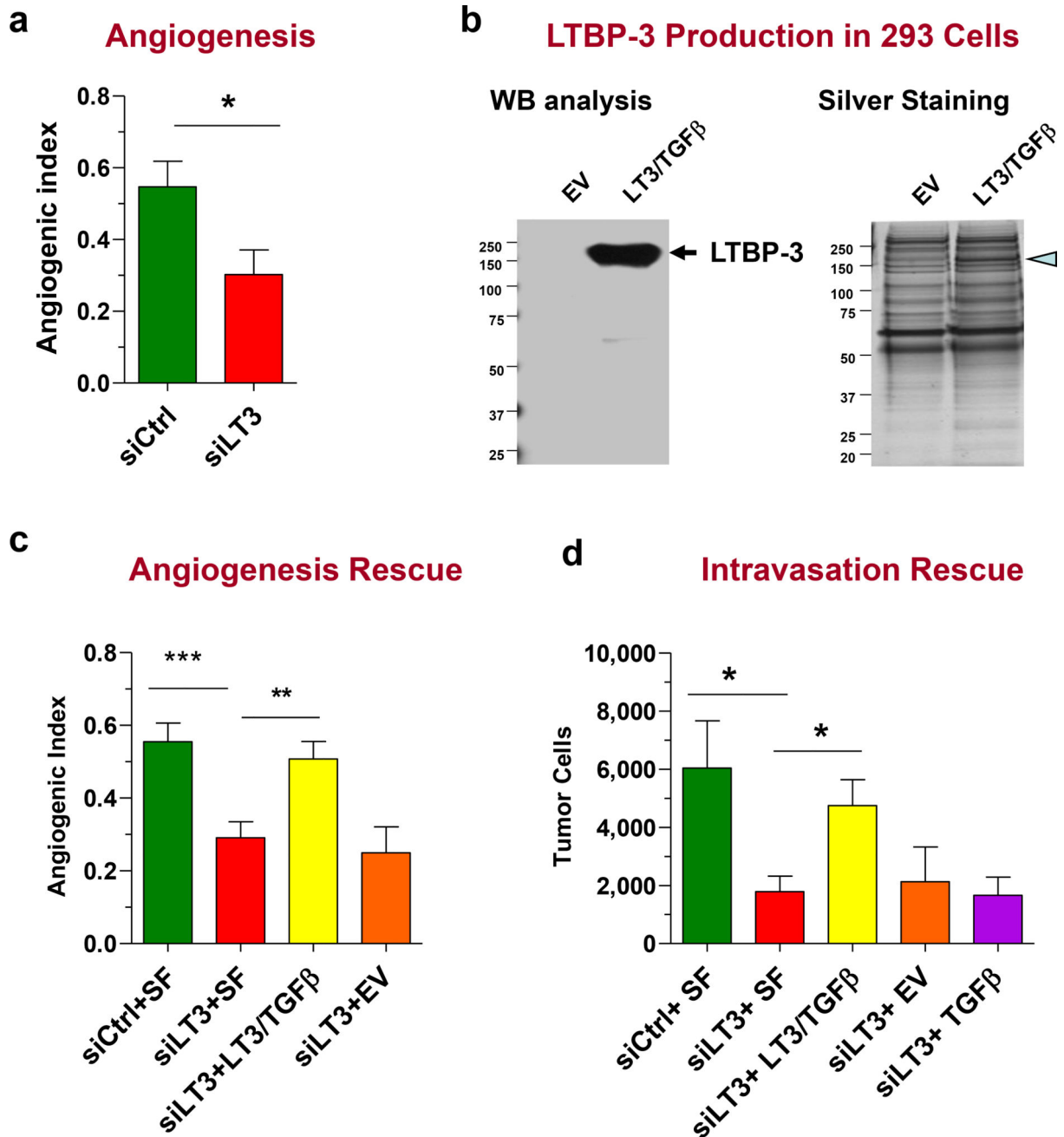


Figure 4. LTBP-3 protein is involved in tumor angiogenesis and tumor cell intravasation
 (a) Angiogenic capacity of tumor cells depends on LTBP-3 expression. HEp-3 cells were treated with siCtrl or siLT3 and employed in an *in vivo* angiogenesis model 2 days after siRNA transfection. The tumor cells were incorporated into 3-D collagen onplants grafted on the CAM of 10 day-old chick embryos developing *ex ovo*. A total of 3×10^4 cells were incorporated in 30 μ l-collagen onplants and 6 onplants were grafted per embryo, 6 embryos per variant. On day 3 after grafting, the angiogenic blood vessels developed within 3-D collagen gels were scored and angiogenesis indices determined for each individual onplant as the fraction of grids filled with the newly-formed vessels versus all grids of an onplant

mesh. Data are presented as means \pm s.e.m; a total of 29 and 30 onplants were scored for each siRNA treatment in 2 independent experiments. * P <0.05.

(b) Analysis of total proteins secreted by 293 cells co-expressing LTBP-3 and TGF β . 10X-CMs from 293-EV and 293-LT3/TGF β cells (6 μ l/lane) were analyzed by western blotting for LTBP-3 (*left*) or silver staining (*right*) under reducing conditions. Whereas western blot indicates the abundance of LTBP-3 protein secreted by the 293-LT3/TGF β cells, silver staining indicates similar patterns of total proteins separated by SDS-PAGE with the exception of a ~160–180 kDa protein band that could represent the secreted LTBP-3 (*blue arrowhead on the right*).

(c) LTBP-3 protein is functionally involved in tumor angiogenesis. HEp-3 cells were treated with siCtrl or siLT3 and employed in angiogenesis assays as described in (A). LTBP3-deficient cells were incorporated into 3-D collagen gels along with the 10X EV or LT3/TGF β CMs (brought to a final 1X concentration with PBS). From 53 to 62 onplants were scored for each treatment variant in 2 independent experiments. Data are presented as means \pm s.e.m. * P <0.05, ** P <0.01, *** P <0.005.

(d) LTBP-3 protein is functionally involved in tumor cell intravasation. HEp-3 cells were treated with siCtrl or siLT3 and employed in intravasation assays as described in Figure 2. On day 1 after tumor cell grafting, the “siLT3” embryos were randomly divided into 4 groups for daily treatments of developing microtumors (days 1–3) with 10X CMs (10 μ L of EV-CM or LT3/TGF β -CM and 25 μ L of TGF β -CM) or 10 μ l SF-DMEM as vehicle control (all brought to a total volume of 100 μ l with PBS). The siCtrl tumors also received 3 daily additions of vehicle control. On day 5, primary tumors were excised and weighed, whereas the distal CAM tissue was analyzed by *Alu*-qPCR for the actual number of intravasated cells. CAM data are presented for embryos bearing tumors of similar tumor weight (see Supplementary Figure 7). Data are presented as means \pm s.e.m. calculated from the pooled data of 2 to 7 independent experiments each employing from 6 to 8 embryos per treatment condition. A total of 54, 33, 89, 15 and 12 embryos were analyzed for siCtrl+SF, siLT3+SF, siLT3+LT3/TGF β -CM, siLT3+EV-CM, and siLT3+TGF β -CM, respectively. * P <0.05.

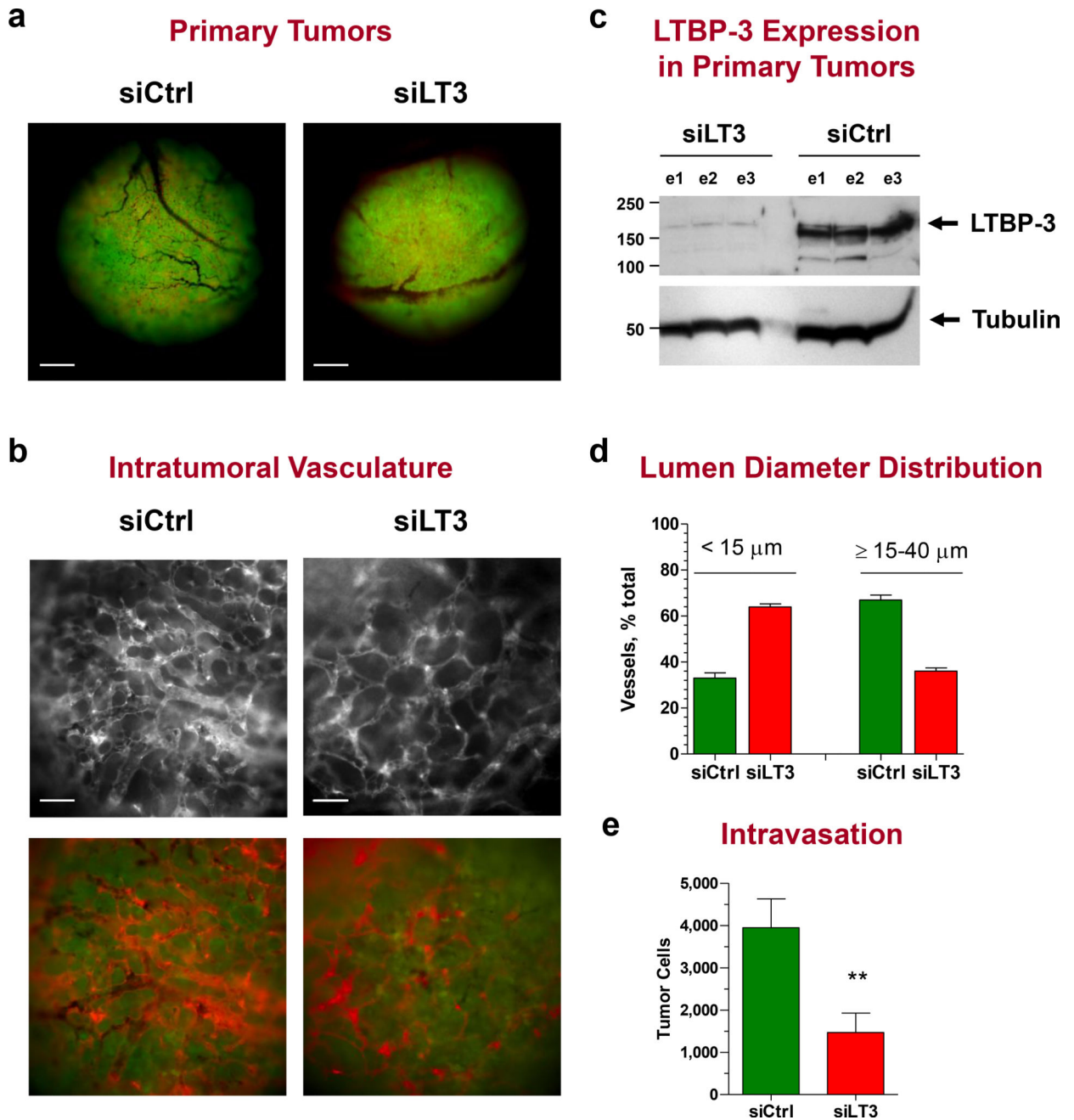


Figure 5. LTBP-3 regulates the development of intravasation-sustaining intratumoral vasculature

(a) Microtumors developed from HEP-3 cells treated with siCtrl or siLT3. GFP-tagged HEP-3 cells (*green*) were grafted on the CAM of chick embryos. Five days later, tumor-bearing embryos were inoculated with Rhodamine-LCA to highlight the vasculature (*red*). Scale bars, 250 μm.

(b) Angiogenic vessels within siCtrl and siLT3 tumors were imaged after injection of Rhodamine LCA into HEP-3-GFP tumor-bearing embryos at 200X original magnification (20X objective × 10X eyepiece). Scale bars, 50 μm. Intratumoral vasculature was imaged

monochromatically in a red fluorescence channel (*top*) and then merged with the image of tumor cells acquired in green immunofluorescence channel (*bottom*).

(*c*) Western blot analysis of LTBP-3 protein (*top*) in siCtrl and siLT3 microtumors collected from tumor-bearing embryos (*e1–e3*). Five to 6 microtumors were pooled from each individual embryo. Analysis of human tubulin (*bottom*) was conducted in the same samples to validate equal protein load of human-specific tissue. Positions of molecular weight markers in kDa are indicated on the left.

(*d*) Lumen diameter distribution within siCtrl and siLT3 intratumoral vasculature. Images of LCA-stained intratumoral vasculature were analyzed with Image J software for the size of intratumoral vessels in siCtrl and siLT3 primary tumors (n=16 for each tumor type). Bars represent the fraction of vessels with a lumen size range (indicated above corresponding bars).

(*e*) Levels of tumor cell intravasation determined by *Alu*-qPCR in siCtrl and siLT3 embryos indicate positive correlation with the proportion of intratumoral vessels with the >15–40 μm lumen size (shown in D). Data are presented as means \pm s.e.m. calculated from 2 independent experiments, collectively involving 11 embryos for each siRNA treatment.

** $P < 0.01$.

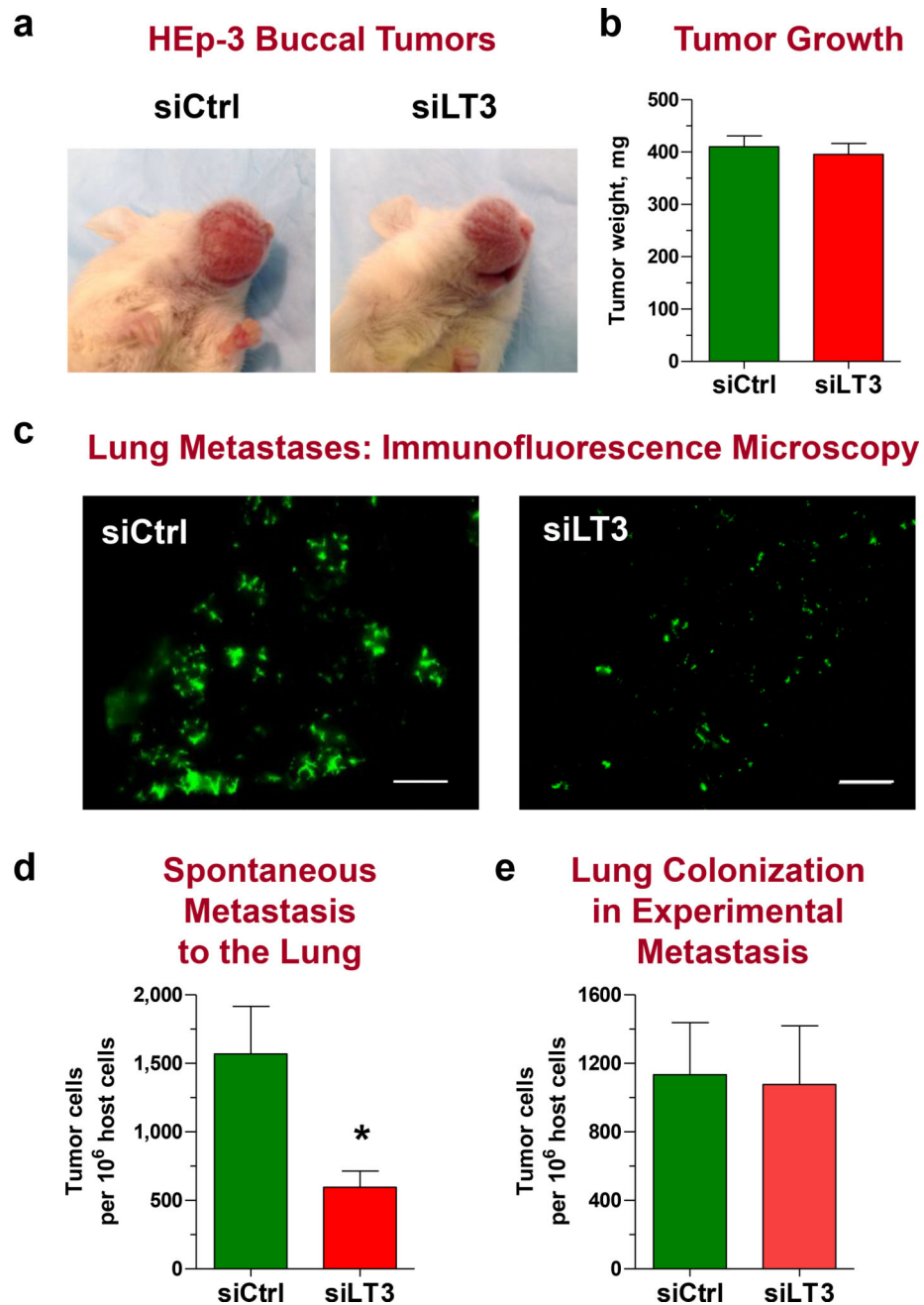


Figure 6. Levels of LTBP-3 affect the capacity of HEp-3 cells to disseminate in an orthotopic spontaneous metastasis model for head and neck carcinomas

GFP-tagged HEp-3 cells were treated with control siRNA (siCtrl) or siRNA targeting LTBP-3 (siLT3). The capacity of siRNA-treated HEp-3 cells to spontaneously disseminate in a mammalian setting was determined in an orthotopic model employing NOD-SCID mice. Control and LTBP-3-deficient HEp-3 cells were implanted into the buccal mucosa at 0.5×10^6 cells per site (one site per mouse). Primary tumors, fully formed by day 12 after cell implantations (a), were excised and weighed (b). The lungs were excised and imaged in a fluorescence microscope (c) and processed by *Alu*-qPCR to determine the number of human cells that metastasized into the lung tissue (d). The data are expressed as means \pm s.e.m.

from 5 independent experiments employing collectively 30 and 27 mice in siCtrl and siLT3 groups, respectively. * $P < 0.05$. Control siCtrl and LTBP-3-deficient siLT3 cells were also compared for their lung colonization capacity in an experimental metastasis model (e) and found not different statistically from each other ($P > 0.05$). Two independent experiments were performed, involving collectively 14 and 16 mice for siCtrl and siLT3 treatment, respectively.

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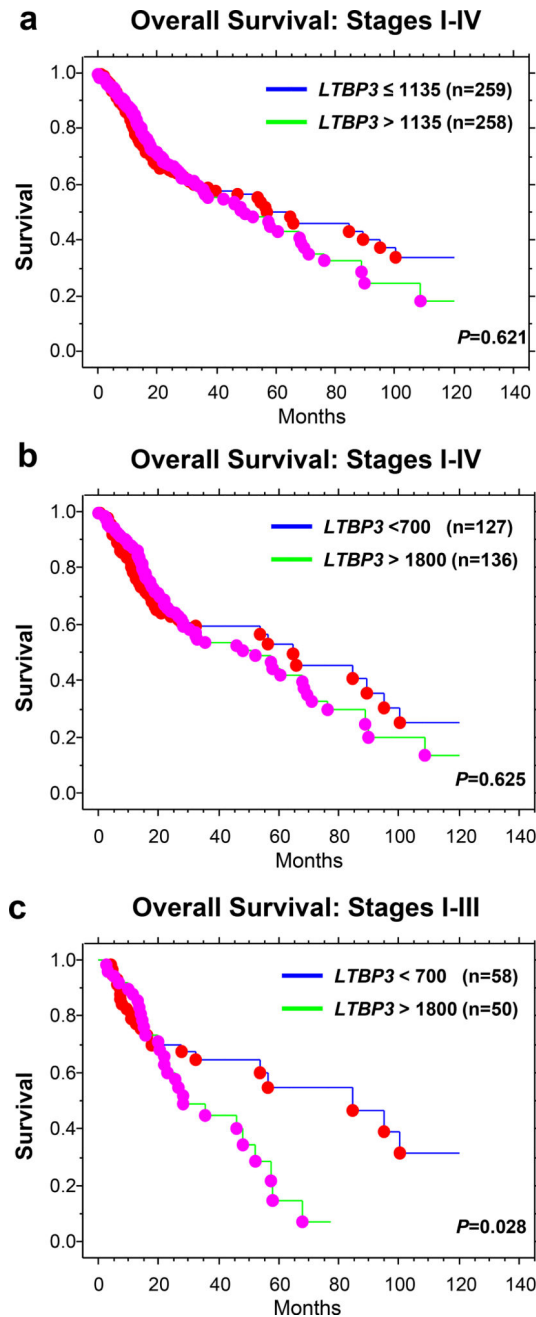


Figure 7. Overall survival analysis for significance of *LTBP3* gene expression in head and neck cancer

Overall 10-year survival curves were generated by Kaplan-Meier analysis for different cut-off values of *LTBP3* gene expression levels and different stages of head and neck tumors in patients from TCGA dataset (n=517).

(a) Median level of expression (1135) was used as a cut-off for all tumors, stages I through IV, in the database.

(b) Kaplan-Meier analysis was conducted for stage I-IV tumors with relatively high (>1800) and low (<700) levels of *LTBP3* expression.

(c) Kaplan-Meier analysis was conducted for stage I–III patients with relatively high (>1800) and low (<700) levels of *LTBP3* expression.

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