Establishment of Embryonic Zebrafish Xenograft Assays to Investigate TGF-β Family Signaling in Human Breast Cancer Progression

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Citation

Snaar-Jagalska, B. E., & Dijke, P. ten. (2022). Establishment of Embryonic Zebrafish Xenograft Assays to Investigate TGF-β Family Signaling in Human Breast Cancer Progression. *Methods In Molecular Biology (Clifton, N.j.)*, *2488*, 67-80. doi:10.1007/978-1-0716-2277-3_6

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Chapter 6

Establishment of Embryonic Zebrafish Xenograft Assays to Investigate TGF-β Family Signaling in Human Breast Cancer Progression

Chao Li, Jin Ma, Arwin Groenewoud, Jiang Ren, Sijia Liu, B. Ewa Snaar-Jagalska, and Peter ten Dijke

Abstract

Transforming growth factor-β (TGF-β) family members have pivotal functions in controlling breast cancer progression, acting not only on cancer cells but also on other cells within the tumor microenvironment. Here we describe embryonic zebrafish xenograft assays to investigate how TGF-β family signaling controls breast cancer cell intravasation, extravasation and regulates tumor angiogenesis. Fluorescently mCherry-labeled breast cancer cells are injected in the perivitelline space or Duct of Cuvier of Tg (*fli:EGFP*) transgenic Casper zebrafish embryos, in which the zebrafish express enhanced green fluorescent protein in the entire vasculature. The dynamic responses of migratory and invasive human cancer cells, and the induction of new blood vessel formation by the cancer cells in zebrafish host, are visualized using a fluorescent microscope. These assays provide efficient, reliable, low-cost models to investigate the effect of (epi)genetic modulators and pharmacological compounds that perturb the activity of TGF-β family signaling components on breast cancer cell metastasis and angiogenesis.

Key words Angiogenesis, Bone morphogenetic protein, Duct of Cuvier, Human breast cancer, Invasion, Perivitelline space, Signaling, Transforming growth factor-β, Zebrafish embryo

1 Introduction

Transforming growth factor-β1 (TGF-β1) is the prototype of a large family of structurally and functionally related pleiotropic cytokines, which include the closely related TGF-β2 and TGF-β3 isoforms, and more distantly related activins and bone morphogenetic proteins (BMPs) [1]. These secreted factors signal via selective transmembrane receptors that are endowed with serine/threonine kinase activity, and their intracellular SMAD effector proteins [2]. Whereas TGF-β and activin receptors signal via, and induce the phosphorylation of, receptor-regulated SMAD2 and SMAD3, BMPs do so via receptor-regulated SMAD1, SMAD5, and SMAD8

Zhike Zi and Xuedong Liu (eds.), *TGF-Beta Signaling: Methods and Protocols*, Methods in Molecular Biology, vol. 2488,
https://doi.org/10.1007/978-1-0716-2277-3_6,
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[3]. Mis-regulation of TGF-β family signaling has been causally associated with numerous diseases, including breast cancer [4]. TGF-β has a dichotomous role during breast cancer progression. In initial phases TGF-β acts as tumor suppressor by stimulating a growth inhibitory response on normal and pre-malignant breast cells, but in later phases, when breast cancer cells have become non-responsive to cytostatic effects of TGF-β, TGF-β instead functions as a tumor promotor [5]. It does so by acting directly on breast cancer cells by stimulating their so-called epithelial to mesenchymal transition (EMT), and by acting indirectly on tumor microenvironmental cells mediating cancer cell immune evasion [6] and promoting tumor angiogenesis [7]. BMPs can oppose the action of TGF-βs, and promote epithelial identity [8].

Here we focus on embryonic zebrafish (*Danio rerio*) xenograft assays to interrogate the role of TGF-β family signaling components in breast cancer cell invasion and tumor angiogenesis. Xenografting human cancer cells was first performed by Lee and co-workers by injecting metastatic melanoma cells into zebrafish embryos at blastula stage [9]. Many studies thereafter have extended these studies using a large variety of different (human) cancer cell lines and more recently patient tissues, including from breast, prostate, colon, pancreatic, and brain, and also using different injection routes, including yolk sac, perivitelline space, Duct of Cuvier (Doc), and hindbrain ventricle [10–15]. We found that the transparent zebrafish xenograft assays with its advantages (including rapid speed, cost-effectiveness, easy in vivo (life) tracking and monitoring of cancer cell behavior and microenvironmental changes, immature immune system allowing cancer cells to survive) and limitations (including heterologous nature of experimental system that is conducted at not-optimal temperature for host and mammalian cells inducing possible metabolic changes [12, 16, 17]) serve as reliable models to investigate the effect of (epi)genetic modulators and pharmacological compounds that perturb the activity of TGF-β family signaling components on breast cancer progression [18, 19]. The models not only extended results obtained using in vitro cultured breast cancer cells and endothelial cells to an in vivo level, but also fulfilled an important filter to select the most critical regulators for further validation in the expensive, time consuming and administratively demanding mouse models.

TGF-β is an attractive target for cancer therapy. However, besides tumor promotion, TGF-β is also crucial for healthy tissue maintenance, making it a challenging target for cancer therapy. This explains the severe toxicities that are observed when TGF-β signaling is entirely blocked by directly inhibiting ligand/receptor function or transforming growth factor β type I receptor (TβRI) kinase activity [20–22], despite indeed showing clinical anti-cancer benefit [23–25]. To overcome the limitation of current TGF-β inhibitors, we have explored the possibility to indirectly inhibit TGF-β

signaling by targeting pivotal activators of pro-oncogenic TGF-β signaling. First, we needed to identify such factors, and for that we performed mass spectroscopy-based proteomics for SMAD interactors [26, 27] or genetic gain- or loss-of-function screens using TGF-β family SMAD-dependent transcriptional reporters screens ($CAGA_{12}$-Luc [28] and BRE-Luc [29]) as read outs [30–32]. Thereafter, we mined mRNA expression or mutation databases to investigate whether the identified genes/proteins responses are mis-expressed/regulated in diseases, including breast tumor tissues. Subsequently, selected genes were experimentally mis-expressed, or if possible the activity of encoded proteins pharmacologically manipulated in in vitro cultured cell models, and effects on cell migration, invasion or epithelial to mesenchymal transition (EMT) investigated. Of genes/proteins that showed relevant phenotypic responses, subsequent in vivo functional studies were performed using embryonic zebrafish assays for an in vivo assessment of their role in breast cancer cell invasion. This was done by mis-expressing genes in breast cancer cells, or if possible by adding pharmacological modulators of the targets to the egg water of the zebrafish. By injecting fluorescently labeled cancer cells into perivitelline space or Doc, the intravasation and extravasation of breast cancer cells could be investigated. Some of the targets were thereafter further tested and explored in mouse xenograft models, and in each case the results in zebrafish were confirmed and validated [19, 30, 31, 33–36]. For some of the druggable targets, we then tested the effect of pharmacological intervention and we could show that their targeting inhibited breast cancer invasion and metastasis [19, 33, 37, 38].

To measure tumor angiogenesis using zebrafish xenografting of breast cancer cells, we injected cancer cells into the avascular perivitelline space and measured the influx of blood vessels as a measure of tumor angiogenesis. The effect of pharmacological agents that target the TGF-β family signaling pathways on tumor angiogenesis was analyzed by adding the compounds to the egg water of the zebrafish [18].

Before describing the materials, methods, notes for troubleshooting and references of related techniques, we highlight in short two examples, how the embryonic zebrafish xenograft assays using perivitelline space or Doc injection were used to demonstrate a specific role of TGF-β family members in breast cancer progression. The effect of compounds on the behavior of xenografted cancer cells can be simply measured by adding the compound to the egg water. In the first example, to demonstrate the critical role of TβRI kinase activity in mediating breast cancer cell extravasation [19], we challenged zebrafish embryos that were injected with mCherry-labeled MDA-MB-231 cells into Doc with a small molecule TβRI kinase inhibitor. We found that treatment with TβRI kinase inhibitor blocked TGF-β/Smad2 signaling in cancer cells and mitigated

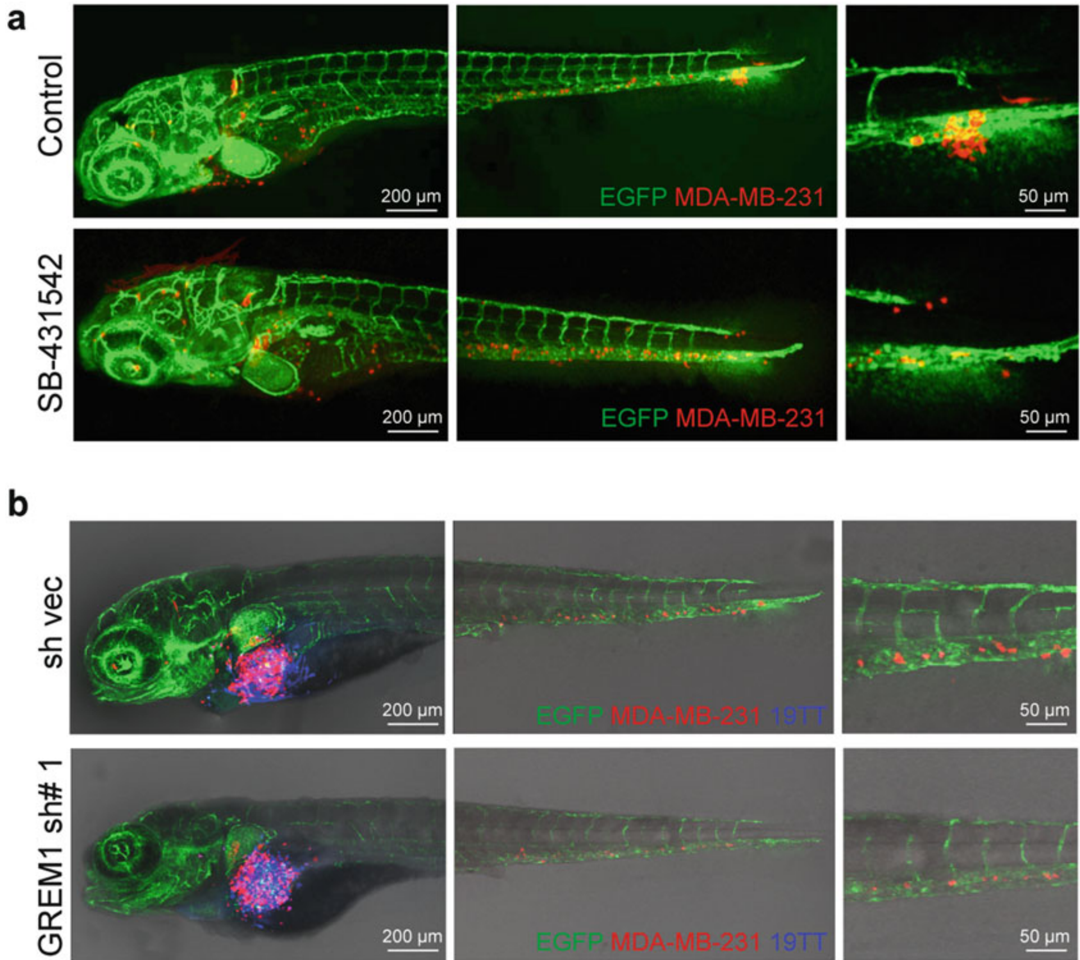

Fig. 1 Embryonic zebrafish xenograft assays to interrogate how TGF-β family members control extravasation or intravasation of breast cancer cells. (**a**) Fluorescence images of zebrafish embryos that are injected at Doc with red fluorescently mCherry-labeled MDA-MB-231 cells and the cancer cells have extravasated into the avascular tailfin 5 days after injection. MDA-MB-231 cells have left the vasculature and are located within the collagen fibers of the tail fin (upper panel). Upon treatment of zebrafish embryos with a small molecule TβRI kinase inhibitor (SB-431542) the intravasation of MDA-MB-231 cells was inhibited (lower panel). The 5 μM SB-431542 was added directly to the fish water every second day. Scale bar = 50 μm. (**b**) Perivitelline space co-injection of MDA-MB-231 cells and 19TT breast cancer-associated fibroblasts (CAFs) with or without Grem1 knockdown. Green, endothelium of zebrafish; red, mCherry-labeled MDA-MB-231 cells; blue, AmCyan-labeled CAFs. The Grem1 knockdown attenuated the number on intravasated cells. This is adapted from reference [39]

MDA-MB-231 cell extravasation (Fig. 1a). In the second example, we interrogated the role of Gremlin1, a secreted antagonist of BMP, in the activation of cancer-associated fibroblasts (CAFs), and how this contributes to breast cancer cells intravasation [39]. We found that co-injection of breast CAFs, which express high levels of Gremlin 1, activate the intravasation of mCherry-

labeled MDA-MB-231 breast cancer cells. Genetic depletion of Gremlin 1 in CAFs, which interferes with CAF activation, decreased this ability (Fig. 1b).

2 Materials

2.1 Cell Culture

1. mCherry-labeled MDA-MB-231 human breast cancer cells were cultured in a 5% CO_2 humidified incubator at 37 °C and were regularly checked for the absence of mycoplasma infection.
2. Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (FBS) and 100 units/mL penicillin and 100 μg/mL streptomycin.
3. Phosphate buffered saline (PBS).
4. Trypsin-EDTA (0.25%): 0.25% trypsin and 0.02% EDTA in PBS, pH = 7.4.

2.2 Zebrafish Injection

1. The Tg (*fli*:EGFP) Casper zebrafish strain is a transgenic zebrafish line in which the EGFP is driven by the *fli* promoter that is active in endothelial cells and hematopoietic cells [40, 41].
2. Egg water: 60 μg/mL sea salts (Instant Ocean, SS15-10) in tap water.
3. 1% or 1.5% (w/v) agarose dishes.
4. 28 °C and 33 °C incubators.
5. Microloaders (Eppendorf, 5242 956.003).
6. Borosilicate glass microcapillaries (Harvard apparatus, 30-0038) (1.0 OD × 0.78 ID×100 L mm) are used for making needles.
7. Micropipette puller (Sutter Instruments, P-97) (Fig. 2a).
8. Tricaine stock solution (4 mg/mL, 100×): 400 mg of tricaine powder (Sigma-Aldrich, A-5040) in 97.9 mL of double-distilled water and added 2.1 mL of 1 M Tris-base. The final pH of the solution was adjusted to 7.4.
9. Devices that are used for injection: Stereomicroscope (Leica M50), Micro manipulator (World Precision Instruments, M3301R), Pneumatic PicoPump Pv820 (Fig. 2b).
10. Embryo fixation: 4% paraformaldehyde.
11. Imaging: Confocal microscope (SP5 STED, Leica Microsystems).

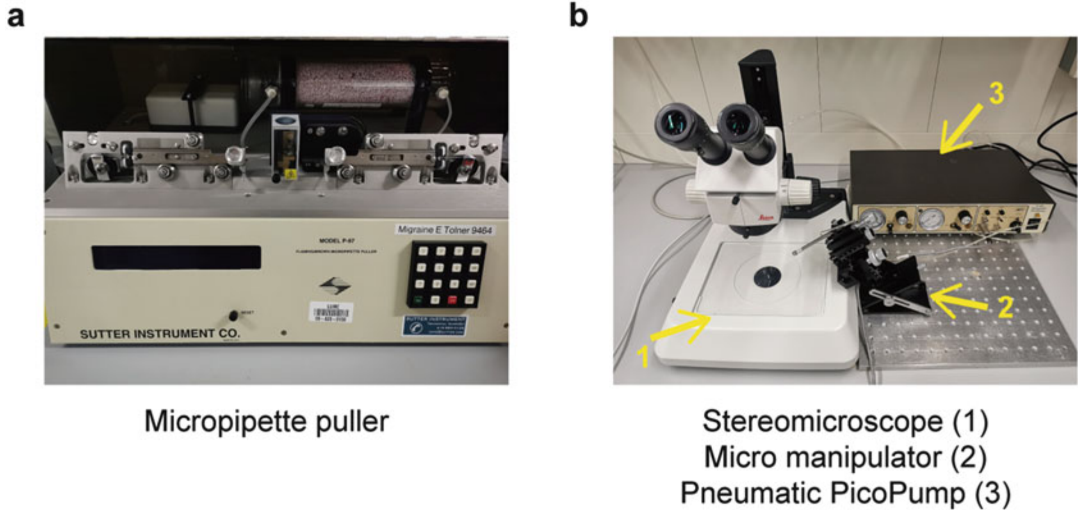

Fig. 2 Zebrafish injection devices. (**a**) Micropipette puller is used to pull one borosilicate glass microcapillary into two injection needles. (**b**) The stereomicroscope, micro manipulator, and pneumatic picopump are combined to perform zebrafish injection. A foot pedal is used to control the power of air pump during the injection (It is not shown in the figure)

3 Methods

3.1 Preparation of Injection Needles

1. Use borosilicate glass microcapillaries for making injection needles.
2. Place the microcapillary in the micropipette puller device according to the following settings: air pressure = 500; heat = 650; pull = 100; velocity = 200; time = 40. One microcapillary can be pulled and used to prepare two needles.
3. After needle preparation place them carefully in a needle holder plate to avoid needle damage before use.

3.2 Zebrafish Embryos Preparation

1. Set up zebrafish breeding pairs with a ratio of one female to one male and leave them for mating in tanks overnight (*see* **Note 1**). Collect embryos the next morning (Fig. 3) (*see* **Note 2**).
2. Remove the unfertilized and unhealthy embryos and only keep the 0–4 h post-fertilization (hpf) embryos (before sphere stage). Maintain the embryos in a Petri dish full of egg water (60 μg/mL sea salts; about 60 embryos/dish) and incubate in a 28 °C incubator.
3. Dechorionate the embryos with fine tweezers under a microscope, at 48 hpf.
4. Anesthetize the embryos by transferring them into 40 μg/mL tricaine (3-aminobenzoic acid)-containing egg water for at

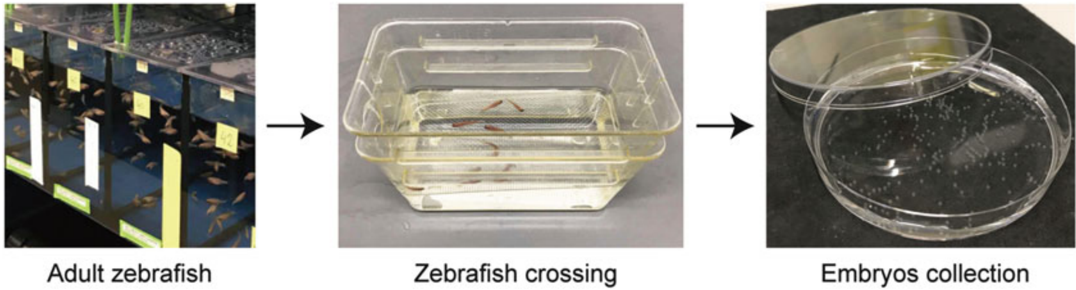

Fig. 3 Zebrafish embryos preparation. Pick up the adult zebrafish breeding pairs from the tanks (left) and divide zebrafish randomly into cross boxes and let them mate freely overnight (middle). Then collect the embryos with a spoon net at the bottom of the cross boxes and culture the embryos in petri dishes with egg water at 28 °C (right)

least 2 min before injection. This cannot be extended for more than 2 h.

3.3 Preparation of the Fluorescent Labeled Cancer Cells

1. Harvest mCherry-labeled human breast cancer MDA-MB-231 cells with 0.25% trypsin-EDTA when the cells reach 80% confluence (*see* **Notes 3** and **4**).
2. Transfer the cells into a sterile 15 ml tube and centrifuge at 200 × *g* for 4 min at room temperature. Wash the cells 2–3 times with 1× PBS.
3. Resuspend the cells in about 200 μL of PBS to reach an appropriate density around 2.5×10^8 cells/ml for injection. Keep the cells on ice for less than 5 h (*see* **Note 5**).

3.4 Injection Preparation

1. Prepare the injection needles. Carefully break the tip of needle with a fine tweezer. Check under the stereomicroscope whether a tip opening has a diameter of 10–20 μm (*see* **Note 6**).
2. Resuspend the cells in a 15 ml tube thoroughly to make an evenly distributed, single cell suspension before use (*see* **Note 7**).
3. Pipette about 15 μL cell suspension into the glass capillary needle using a long tip (microloader). Then, insert the needle into micro manipulator (*see* **Note 8**).
4. Warm a 1% or 1.5% (w/v) agarose dish to 28 °C. Transfer the anesthetized zebrafish onto the agarose dish and cover in egg water to avoid the zebrafish embryos from drying out (*see* **Note 9**).
5. Adjust the needle tip angle to 45°. A pneumatic picopump and a manipulator are used to conduct the microinjection. Adjust the eject pressure and pneumatic pulse length to make sure around 400 cells can be delivered to the embryo each time (*see* **Note 10**).

3.5 Doc Injection

1. Approach the Doc from the dorsal side of the embryo. Insert the needle into the anterior point of the Doc (Fig. 4), where the dorsal begins to widen above the yolk sac. Approximately 400 cells are injected into the Doc (*see* **Note 11**).
2. Repeat injection of other embryos. About 10 embryos are injected per experimental condition. After all embryos are injected, transfer the injected zebrafish embryos to egg water (*see* **Notes 12** and **13**).
3. Select the injected embryos by screening them for mCherry-labeled MDA-MB-231 cells in the circulation, using a fluorescent microscope.
4. Maintain the zebrafish embryos at 33 °C for 5 days to investigate tumor extravasation (Fig. 1a). Count the number of individual cells (MDA-MB-231) that have invaded the collagen fibers of the tailfin from circulation (*see* **Note 14**).

3.6 Perivitelline Space Injection

1. Adjust the direction of the agarose dish by hand to orientate the embryo diagonally to the insert the needle.
2. Insert the needle tip from the dorsal side of the embryo into the perivitelline space between the yolk sac and periderm of the zebrafish embryo (Fig. 5a).
3. Inject around 400 tumor cells into the perivitelline space. Avoid mis-injection and rupture of the yolk sac (*see* **Note 15**).
4. Check the injected zebrafish embryos under a fluorescent microscope 2 h post-injection. Remove mis-injected embryos, such as those are injected or ruptured with cancer cells in the yolk sac. Select the embryos that are injected with approximately same number of cancer cells.
5. Maintain the zebrafish embryos at 33 °C for 3 days to observe tumor cell-induced angiogenesis (Fig. 5b) (*see* **Note 16**).
6. Image analysis and quantification of the tumor angiogenesis by measuring the formed vessel length at the perivitelline space.
7. Maintain the zebrafish embryos at 33 °C for 6 days to observe tumor cells intravasation (Fig. 5c) (*see* **Note 17**).
8. Image analysis and quantification of intravasation by counting the number of cancer cells in the zebrafish circulation. Count the number of cells in each fish that have spread from the cell mass in the perivitelline space towards the embryonic fish body within the head and tail regions; the regions are beyond the boundaries of the heart cavity frontally, on top of the swim bladder dorsally, and beyond the urogenital opening caudally.

3.7 Statistical Analysis

For statistical analysis a two-way analysis of variance (ANOVA) followed by post hoc analysis is used for extravasation and

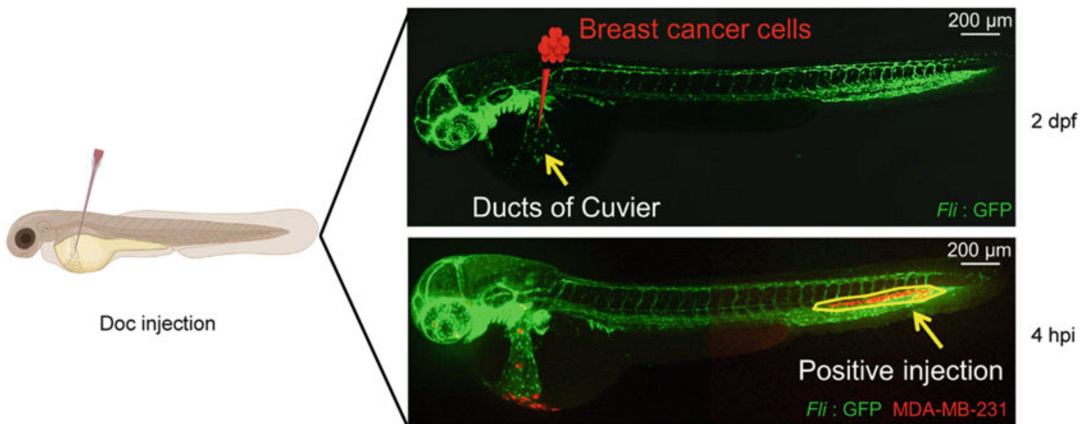

Fig. 4 Doc injection. Zebrafish Doc injected with fluorescently labeled breast cancer cells at 2 days post-fertilization (dpf). The arrow in the triangle area besides the yolk indicates the Doc site (the right upper picture). The right lower picture is an example of positive injection. Around 400 breast cancer cells (MDA-MB-231) were injected into the Doc. Most of the cells can be found in tail region around 4 h post-injection (hpi). The yellow arrow and circle indicate injected cells which circulated from Doc to the tail region. This is adapted from ref. 18

intravasation. Unpaired two-sided Student's t-tests were used to calculate tumor angiogenesis statistical differences (*see* **Note 18**).

4 Notes

1. For setting up the zebrafish intercrosses, each tank should not contain more than 10 zebrafish. Zebrafish can also be set up in a ratio of two females to one male to increase total embryo production. Do not cross the same tank of zebrafish too often (more than one every 7 days) as it would lead to lower quality and reduced numbers of the eggs.
2. There is a standard light regime of 14 h light and 10 h dark in the zebrafish facility. Light turns on at 8:00 and turns off at 22:00. For collecting of the embryos, leaving the zebrafish in the tank for few hours after the room lights turn on in the morning can lead to more embryos.
3. Cancer cells can be labeled biochemically with a dye or fluorescently following transfection/transduction with a vector driving the expression of a fluorescent protein, such as mCherry. We greatly favor the use of fluorescently labeled cells. Only living cells remain fluorescent. When cancer cells are labeled chemically with a dye, care should be taken as damaged cancer cells may still retain dye and these cells should not be included in the analysis. Moreover, dye particles may also take on the shape of cells and diluted after cell division.

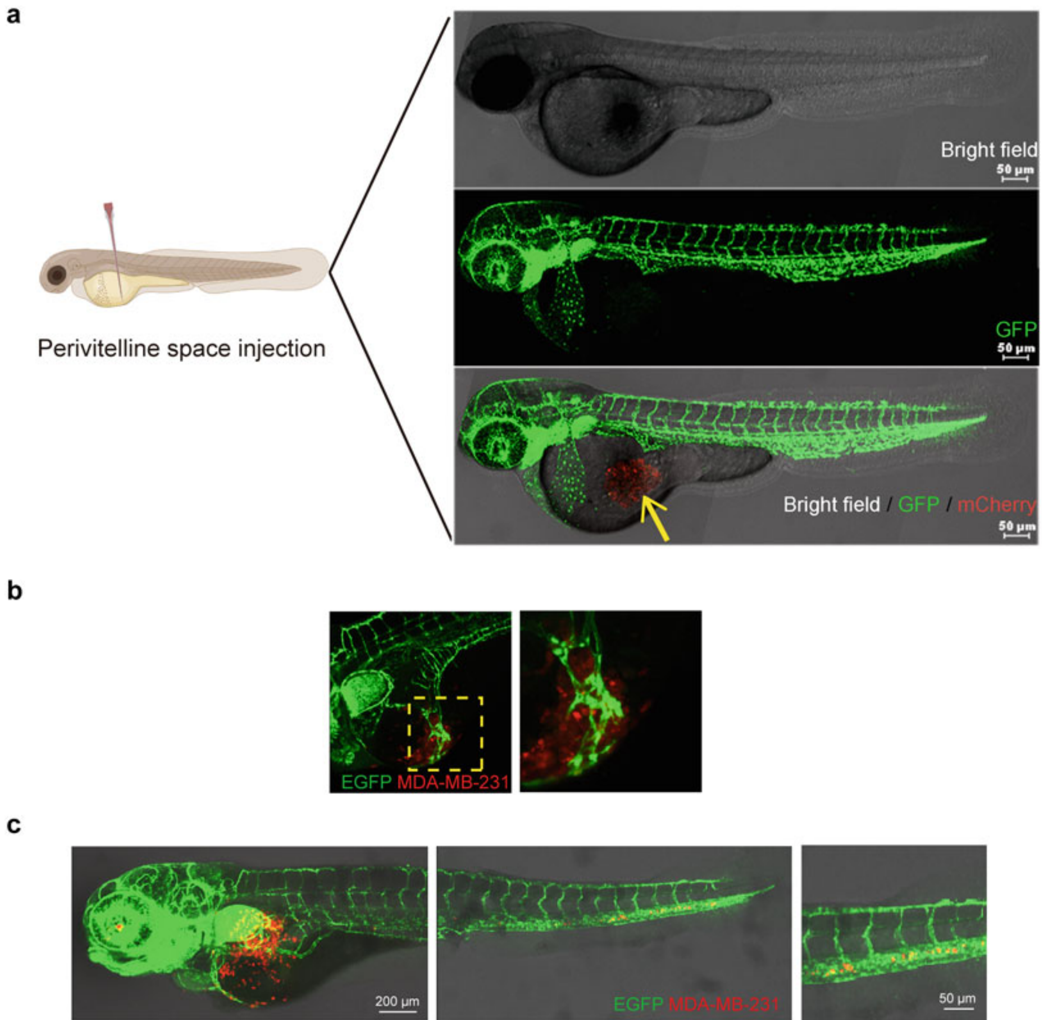

Fig. 5 Perivitelline space injection. (**a**) Around 400 breast cancer cells (mCherry-labeled MDA-MB-231) were injected into the perivitelline space. Yellow arrow indicates the cell mass injection at the perivitelline space. This is adapted from reference (18). (**b**) Tumor angiogenesis in zebrafish. After 3 days of breast cancer cells injection in perivitelline space, new blood vessels formed in sub-intestinal vein (SIV) plexus. (**c**) After 6 days post-injection (dpi), MDA-MB-231 cells intravasation could be detected. Cells were found in the head and tail regions of zebrafish embryos. This is adapted from ref. 39

4. The optimal time length of trypsin digestion for each cell type is different. It's necessary to digest for enough time to obtain single cells. Use a microscope to ensure that a single cell suspension is formed. Avoid over-trypsinization of cells, which makes the cell suspension sticky that easily clog the needles during injection.
5. Different cancer cells show different invasion patterns. MDA-MB-231 cells are highly aggressive cells and these cells invade into the avascular tail fin area in a single cell manner.

When pre-malignant cells such as MCF10A-RAS cells are injected into Doc, they extravasate but remain clustered in between the vessels.

6. The diameter of tip opening depends on the cell type. The circular diameter of most human cells is approximately 10–20 μm. It's better to cut the tip of the needle such that it is flat not beveled. This will protect the yolk of zebrafish by decreasing the risk of mechanical damage with the protruding edge of the needle during the injection.
7. The cells should evenly distribute in a PBS suspension before loading into the needle. If not, the number of cells would be unequal when injecting the cells into the embryos.
8. Prevent the formation of air bubbles in the needle by loading the cells completely in the bottom of the needle.
9. Keep an appropriate amount of egg water in the dish. Too little water will make the embryos dry out quickly.
10. Ensure the correct number of cells are manually injected. In the initial phase of injection, cell counting can be performed by injecting the cells onto the top of a petri dish containing 1% agarose to ensure the correct number of cells are injected (approximately 400 cells).

    Sometimes needles become clogged. In this case, try to eject the blockage by increasing the pressure or cut the needle further to get a wider opening. However, when the diameter of the opening is too big, it can lead to more severe wounding and damage to the zebrafish embryo. When increasing the needle opening size make sure to decrease the pressure to compensate for the lowering of resistance in the needle.
11. Multiple injections can be performed with the same needle when the same cancer cell line is injected.
12. As there is considerable individual variation between zebrafish embryos, and some of the embryos may die after injection because of injection damage, sufficient numbers (approximately 100, though it depends on death rate of the injected embryos) of zebrafish embryos should be injected for each condition to make sure that at least 30 embryos survive for statistical analysis. The experiments need to be repeated a minimum of 3 times.
13. The technical challenges of injection can be overcome by practice. The Doc injections are much easier to perform than perivitelline space injections. Keep the groups of embryos as homogeneous as possible (determined by microscope examination) and select staged embryos to minimize the variation of the results.

14. 33 °C is a compromise to accommodate conditions that are reasonable for zebrafish (usually maintained at 28 °C) and mammalian cells (usually cultured at 37 °C). After the injection the egg water should be refreshed immediately to prevent the contamination by the dead cells attached to the zebrafish embryos. During the experiment, the dead zebrafishes should be removed every day and the egg water should be changed every 1–2 day.
15. It is recommended to take mock injection with buffer as a control to avoid the effect of the wound.
16. When testing the effect of compounds that are added to the egg water, first perform a titration to determine the maximal dose that does not induce toxicity (malformed embryos). The stability of the compound may vary, and egg water containing compounds may need to be refreshed every day or every other day in case the compound has a limited stability.
17. Select only zebrafish embryos that are injected with cancer cells within the perivitelline space, and that do not have cells already in circulation immediately following injection.
18. It is greatly recommended to perform the analysis in a blinded manner, meaning that the person analyzing the zebrafish embryos does not know which treatment group he or she is analyzing. Ideally, two people should independently score the embryos.

Acknowledgements

Chao Li and Jin Ma contributed equally to this chapter. Studies on TGF-β signaling in cancer are supported by the Cancer Genomics Centre Netherlands (CGC.NL) and ZonMW grant (09120012010061). Chao Li and Jin Ma are supported by the Chinese Scholarship Council. We thank David Baker for proof-reading of manuscript. We also thank the authors of Cancer-associated fibroblast-derived Gremlin 1 promotes breast cancer progression: Jiang Ren, Marcel Smid, Josephine Iaria, Daniela C F Salvatori, Hans van Dam, Hong Jian Zhu, John W M Martens, Peter ten Dijke for the reuse of figures, which is licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

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