TGFβ signaling in cancer progression

Liu, S.

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Chapter 3

Invasive Behavior of Human Breast Cancer Cells in Embryonic Zebrafish

Jiang Ren[1, *], Sijia Liu[1, *], Chao Cui[1,] and Peter ten Dijke[1]

[1]Department of Molecular Cell Biology, Leiden University Medical Center, The Netherlands

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Chapter 3

Abstract:

In many cases cancer patients do not die of a primary tumor but rather because of metastasis. Although numerous rodent models are available for studying cancer metastasis *in vivo*, other efficient, reliable, low-cost models are needed to quickly access the potential effect of (epi)genetic changes or pharmacological compounds. As such, we illustrate and explain the feasibility of xenograft models using human breast cancer cells injected into zebrafish embryos that support this goal. Under the microscope, fluorescent protein or chemically labeled human breast cancer cells are transplanted into transgenic zebrafish embryos Tg (*fli:GFP*) at the site of the perivitelline space or duct of Cuvier (Doc) 48 h after fertilization. Shortly afterwards, the temporal-spatial process of cancer cell invasion, dissemination, and metastasis in the living fish body is visualized under a fluorescent microscope. The models using different injection sites, i.e. perivetelline space or Doc, are complementary to one another, reflecting the early stage (intravastion step) and late stage (extravasation step) in the multistep metastatic cascade of events. Moreover, peritumoral and intratumoral angiogenesis can be observed with injection into the perivitelline space. The entire experimental period is no more than 8 days. These two models combine cell labeling, micro-transplantation, and fluorescence imaging techniques, enabling rapid evaluation of cancer metastasis in response to genetic and pharmacological manipulations.

Introduction:

Overt cancer metastasis in the clinic comprises a series of complex and multi-step events known as the ‘metastatic cascade’. The cascade has been extensively reviewed and can be dissected into successive steps: local invasion, intravasation, dissemination, arrest, extravasation, and colonization (1,2). Better understanding of the pathogenesis of cancer metastasis and the development of potential treatment strategies *in vivo* require robust host models of cancer cell spread. Rodent models are well established and widely used to evaluate metastasis (3), but these approach have low efficiency, ethical limitations, and are costly as a forefront model to determine whether a particular manipulation could affect the metastatic phenotype. Other efficient, reliable, low-cost models are needed to quickly access the potential effect of (epi)genetic changes or pharmacological compounds. Due to the high genetic homology to humans and transparency of the embryos, the zebrafish (*Dano rerio*) has emerged as an important vertebrate model and is being applied increasingly in studying developmental processes, microbe-host interactions, human disease, drug screening, and other areas (4). The cancer metastasis models established in zebrafish may provide ideal solutions to the shortcomings of rodent models (5,6).

Although spontaneous neoplasia is scarcely discovered in wild zebrafish (7), there are several longstanding techniques to induce desired cancer in zebrafish. Carcinogen-induced gene mutations or signaling pathways-activation can model carcinogenesis histologically and molecularly resembling human disease in zebrafish (7-9). By taking advantage of diverse forward and reverse genetic manipulations of oncogenes or tumor suppressors, (transgenic) zebrafish also have enabled potential studies of cancer formation and maintenance (6,10). The induced cancer models in zebrafish cover a broad spectrum of cancer types in digestive, reproductive, blood, nervous systems, as well as the epithelium(6).

The utilization of zebrafish in cancer research has expanded recently by the establishment of human tumor cell xenograft models in this organism. This was first reported with human metastatic melanoma cells that were successfully engrafted in zebrafish embryos at blastula stage in 2005 (11). Several independent laboratories have validated the feasibility of this pioneering work by introduction of a diverse range of mammalian cancer cells lines into zebrafish at various sites and developmental stages (5). For example, injection near the blastodisk and blastocyst of blastula stage, injection into the yolk sac, perivitelline space, duct of Cuvier (Doc), and posterior cardinal vein of 6 h to 5 day old embryo, and injection into peritoneal cavity of 30 days old immunosuppressed larvae (5,12). Additionally, allogeneic tumor transplantations were also reported in zebrafish (12,13). One of the great advantages of using xenografts is that the engrafted cancer cells can be easily fluorescently labeled and distinguished from normal cells. Hence, investigation into dynamic behaviors of microtumor formation (14), cell invasion and metastasis (15-17), tumor-induced angiogenesis (15,18), and interaction between cancer cells and host factors (17) can be clearly visualized in the live fish body, especially when transgenic zebrafish lines are applied (5).

Inspired by the high potential of zebrafish xenograft models in evaluating metastasis, we demonstrated transvascular extravasation properties of different breast cancer cell lines into tail fin area of Tg (*fli:GFP*) zebrafish embryos by Doc injection (16). The role of transforming growth factor-β (TGF-β) (16) and bone morphogenetic protein (BMP) (19) signaling pathway in pro-/anti-breast cancer cell invasion and metastasis were also investigated in this model. Moreover, we also recapitulated the intravasation ablity of various breast cancer cell lines into circulation using xenograft zebrafish model by perivitelline space injection.

This article presents detailed protocols for zebrafish xenograft models based upon injection of human breast cancer cells into perivitelline space or Doc injection. Using high-resolution fluorescence imaging, we show the representative process of intravastion into blood vessel and invasive behavior of different human breast cancer cells from blood vessel into avascular tailfin area.

Protocol:

All research using zebrafish, including housing and experiments, was carried out according to the international guidelines and approved by the local Institutional Committee for Animal Welfare (Dier Ethische Commissie (DEC) of the Leiden University Medical Center. Note: As summarized in Figure 1, the protocol is roughly dissected into four steps, embryo collection (Figure 1A), microinjection (Figure 1B), screening (Figure 1C), and analysis (Figure 1D).

1. Prepare the injection needles

Prepare injection needles with borosilicate glass microcapillary. Put the microcapillary in a micropipette puller device with the following settings: air pressure 500; heat 650; pull 100; velocity 200; time 40. Keep the injection needles in a needle holder plate until used for injection.

2. Prepare of the fluorescent genetically labeled breast cancer cells for injection

1. Culture human breast cancer MDA-MB-231 cells at 37 °C in DMEM-high glucose media containing L-glutamine, 10% fetal bovine serum and 1:100 Penicillin-Streptomycin-Glutamine.
2. Culture breast epithelial cell line MCF10A (M1), MCF10Aras (M2) at 37 °C in DMEM/F12 media containing L-glutamine, with 5% horse serum, 20 ng/mL epidermal growth factor, 10 mg/mL insulin, 100 ng/mL cholera enterotoxin, 0.5 mg/mL hydrocortisone, and 1:100 Pen-Strep.
3. Produce mCherry lentivirus by co-transfecting PLKO-mCherry, pCMV-VSVG, pMDLg-RRE (gag/pol), and pRSV-REV plasmid into HEK293T cells. Harvest cell supernatants 48 h after transfection and store at -80 °C.
4. Infect MDA-MB-231 and M2 cells at 30% confluence for 24 h with lentiviral supernatants diluted 1:1 with normal culture medium in the presence of 5 ng/mL polybrene.
5. Select single cell clones by diluting cells in a 96-well plate, which allows the outgrowth of isolated cell clones, until obtaining the stable mCherry-expressing cell lines.
6. Culture one T75 flask of cells for injection. Harvest the cells at 80% confluence by 0.5% trypsin-EDTA treatment. Wash the cells with 1× PBS 2-3 times.
7. Re-suspend the cells in about 200 µL PBS. Store at 4 °C for less than 5 h before injection.

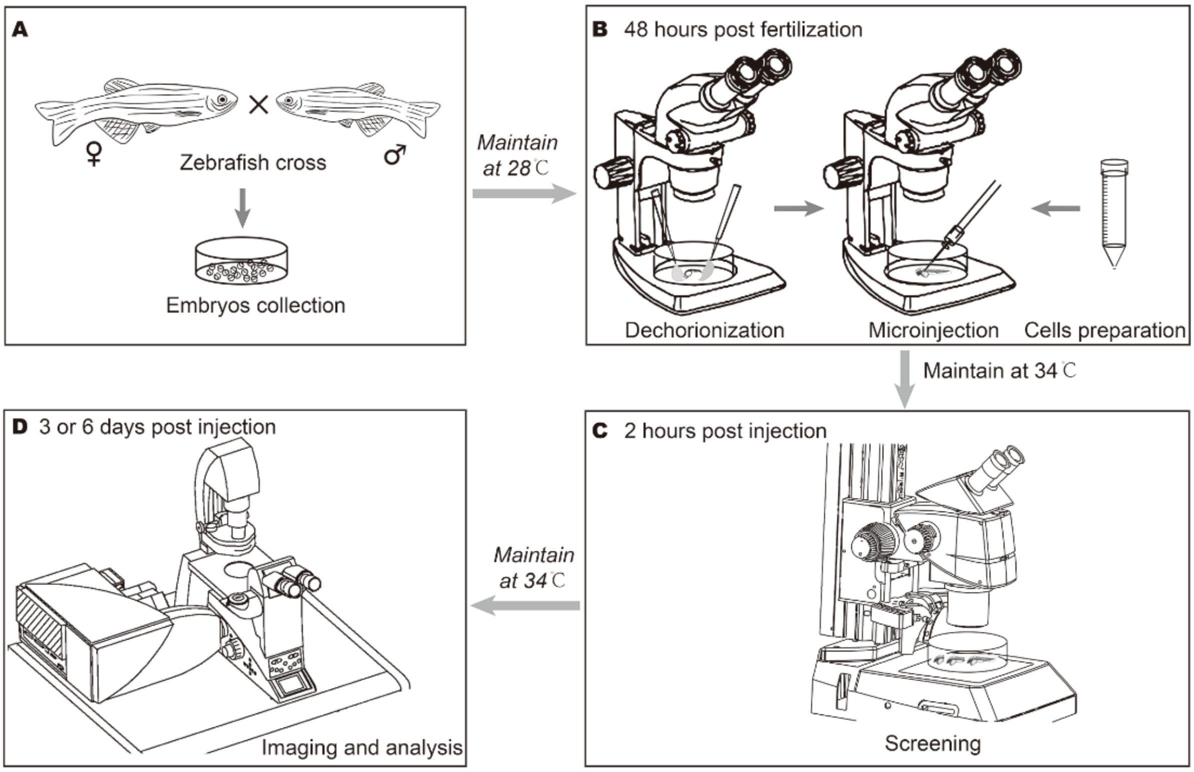

Figure 1. Main steps for investigating the invasive behavior of breast cancer cells in embryonic zebrafish. (A) After crossing parental zebrafish overnight, Tg (*fli1:GFP*) zebrafish embryos were collected the following morning and maintained at 28 °C. (B) The embryos were dechorionated with fine tweezers under a stereo microscope 48 h post fertilization (hpf). The labeled breast cancer cells were collected and re-suspended in a small amount of PBS. After well-preparation, suspended cells were loaded into one needle. Approximately 400

cells were injected into the duct of Cuvier (Doc) of the perivitelline space under a stereo microscope. The injected embryos were maintained at 34 °C. (C) 2 hours post-njection (hpi), the embryos were subjected to careful screening under a fluorescence stereo microscope. The embryos were maintained at 34 °C for 3 or 6 d. During the interval, embryos could be subjected to designed treatment. (D) Cancer cell dissemination by perivitelline space injection or invasion by Doc injection was detected, counted, and imaged by confocal microscopy 3 or 6 days post-njection (dpi).

3. Prepare Zebrafish Embryos for Injection

1. Set up zebrafish breeding pairs and collect embryos as shown in a previous Jove article by Rosen et al (20).
2. Select the embryos that are at 0-4 hpf by removing the unfertilized and abnormal embryos. Keep the embryos in a petri-dish filled with egg water (60 µg/mL sea salts; ~60 embryos/dish) and incubate at 28 °C.
3. Dechorionate the embryos with fine tweezers at 48 hpf.
4. Anesthetize the embryos with 200 µg/mL 3-aminobenzoic acid buffer approximately 10 min prior to injection, but no longer than 2 h prior to injection.

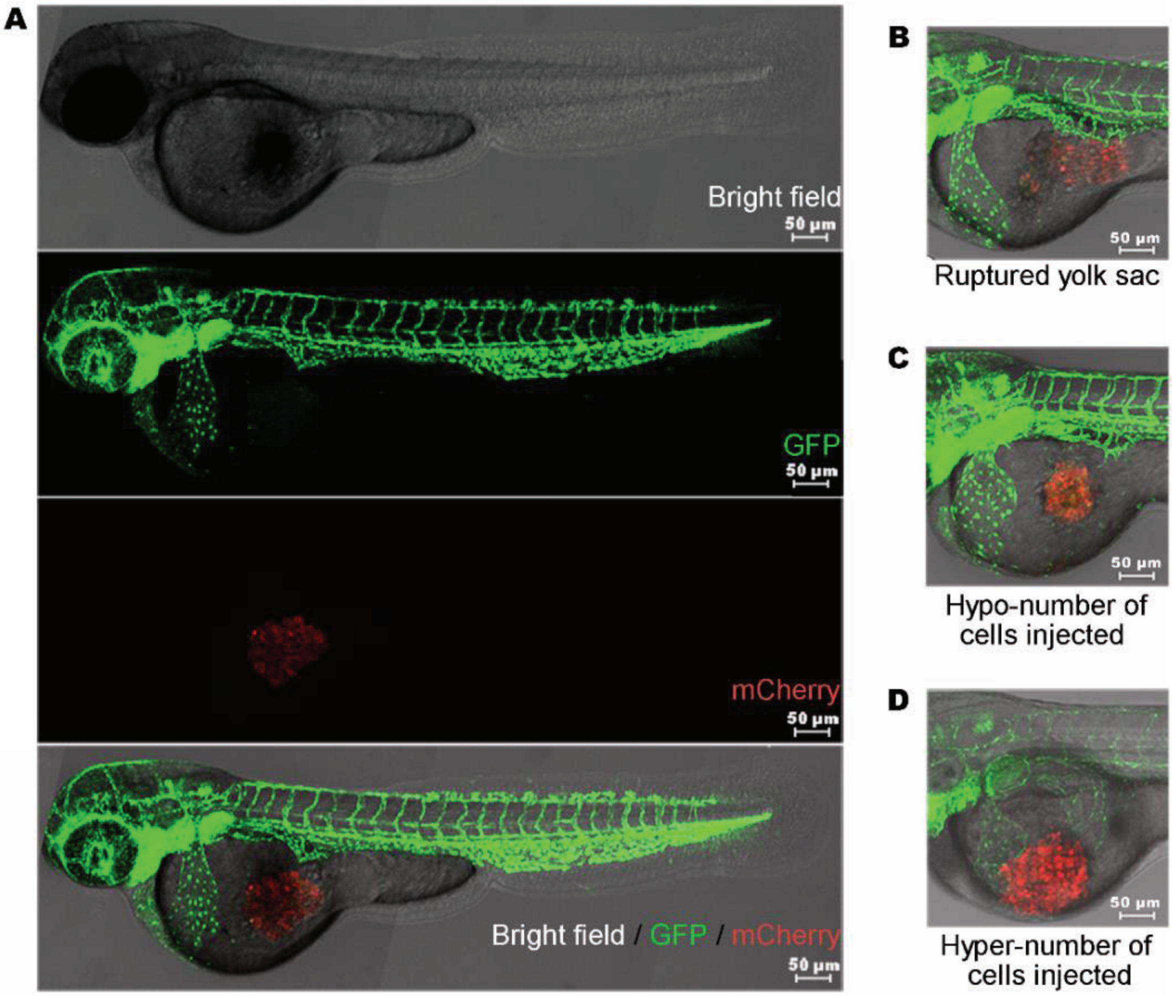

Figure 2. Perivitelline space injection site and common errors. (A) Approximately 400 mCherry-labeled cells (MDA-MB-231) were injected into the perivitelline space. The Brightfield (upper most), green vasculature (middle upper), and red cell mass (middle lower) of injected zebrafish embryos were captured by confocal microscope. The merged image (lower most) of three channels shows the stereo location of the cell mass in the embryo. (B) The cells did not target the perivitelline space appropriately. The yolk sac was ruptured. (C) A hypo-number of injected cells (much less than 400). (D) Hyper-number of injected cells (much more than 400). The cell mass was too close to the duct of Cuvier, which has a broad blood stream. Scale bar = 50 µm.

4. Perivitelline space injection of human breast cancer cells

1. Load 15 μL of the cell suspension into an injection needle. Mount the needle onto the micromanipulator and break off the needle tip with fine tweezers to obtain a tip opening diameter of 5-10 μm.
2. Use a pneumatic picopump and a manipulator to perform microinjection. Adjust the picopump to inject 400 cells each time. Prior to injection, count the cell numbers manually by injecting the cells on the top of a dish containing 1% agarose.
3. Line up anesthetized embryos (2-3 days post fertilization (dpf)) on a flat 1% agarose injecting plate.
4. Orient the injection plate by hand during injections to place the embryos in the preferred position for inserting the needle (*i.e.*, diagonally).
5. Point the needle tip to the injection site and gently insert the needle tip into the perivitelline space between the yolk sac and the periderm of the zebrafish embryo (Figure 2A).
6. Inject approximately 400 mCherry-labeled tumor cells. Make sure that the yolk sac is not ruptured to avoid implantation into the yolk sac.

5. Doc injection of human breast cancer cells

1. Prepare injection needle and zebrafish embryos as described previously.
2. Use a 45° needle angle so that the Doc can be approached from the dorsal side of the embryo.
3. Insert the needle into the starting point of the Doc (Figure 3A) just dorsal to where the duct starts broadening over the yolk sac and inject ~400 cells. The injection is correct if the volume within the duct expands directly after the pulse and the yolk sac.
   Note: Several consecutive injections can be performed without extracting the needle.
4. Transfer the injected zebrafish embryos to egg water.
   Note: As considerable variation exists among individual zebrafish embryos; relatively large number of zebrafish embryos should be injected with cancer cells.
5. Maintain the zebrafish embryos at 34 °C to accommodate the optimal temperature requirements for fish and mammalian cells.

6. Screen the injected embryos.

1. Screen each fish under a fluorescence stereo microscope at 2 h post-injection (hpi) for perivitelline space injection (Figure 2) or at 2-24 hpi for Doc injection (Figure 2), to ensure all the embryos are injected with similar number of tumor cells. Remove the embryos with injection errors, such as rupture (Figure 2B) or injection (Figure 3B) of yolk sac, and pick out embryos with a hypo- (Figure 2C and Figure 3B) or hyper- (Figure 2D and Figure 3B) number of injected cells. Keep only the embryos with approximately 400 cells in culture.
2. Rule out the possibility that cells are introduced directly into the circulation during the injection process by removing the embryos with cells already in the circulation from further analysis. Also remove any embryo with a cell mass close to the Doc (Figure 2D).

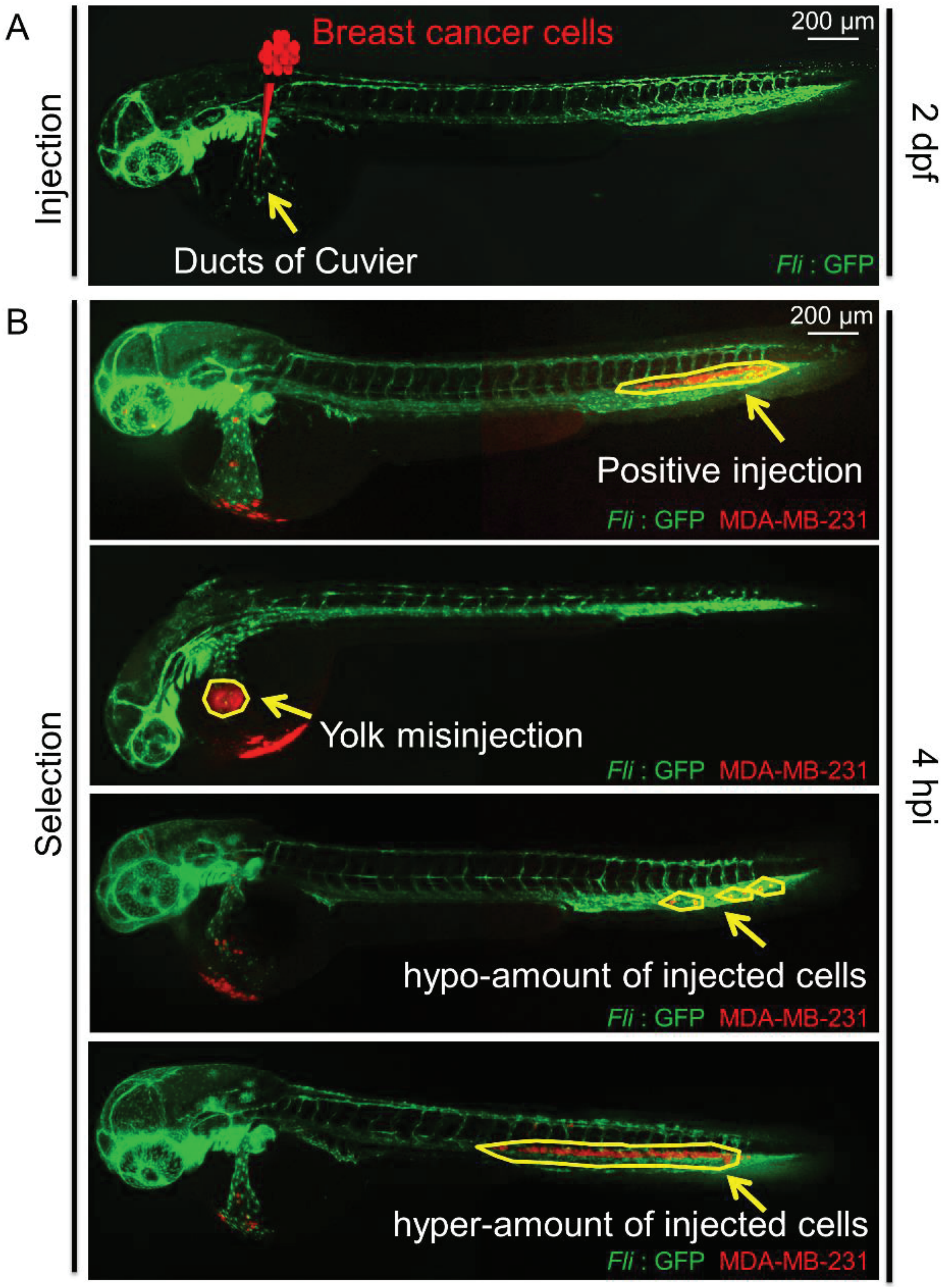

Figure 3. Overview of duct of Cuvier (Doc) injection. (A) Schematic of Doc injection at 2 days post-fertilization (dpf) with breast cancer cells in zebrafish embryos. Arrow indicates Doc. (B) Examples of positive injection with ~400 breast cancer cells, negative injections including the yolk mis-injection and incorrect number of cells injection at 4 hpi. Arrows and circles indicate injected cells.

7. Image and analyze the metastatic process.

1. Collect several anesthetized embryos with a wide-tip Pasteur pipette, and transfer them onto the glass bottom of a polystyrene dish.
2. Remove excess water and keep a limited amount of egg water. Manipulate the embryo into position with a hair loop tool, and place a cover on top of the glass.
3. Use an inverted confocal microscope in combination with water-immersion or long-distance dry objectives. The embryo should be positioned so that the region of interest is as close to the objective as possible.
4. Perform imaging immediately after anesthesia to reduce the risk of embryo death due to liquid evaporation.
    1. Capture signals from GFP-labeled vasculature and mCherry labeled tumor cells at the same position of the embryos to co-register injected cells with blood vessels by merging the two imaging channels.
    2. For each zebrafish embryo, collect two different sets of images from the head region and tail region.
5. Quantify the number of disseminated cells.
    1. For perivitelline space injection, count the cells that disseminated from the cell mass toward 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.
    2. For Doc injection, count the number of individual cells that invade the collagen fibers of the tail fin from circulation (MDA-MB-231) or the number of clusters formed by cells collectively (M2).
6. To study invasion and metastasis in more detail, confocal microscopy is highly recommended.
    1. Use low magnification (× 4 objective) to image the whole body and obtain an overview of the tumor cell dissemination pattern.
       Note: Higher magnification (× 20 and × 40 objectives) is suitable for studying intra- and peri-tumoral angiogenesis and precise localization of disseminated cells in the embryo body.
    2. Use a 488-nm laser to scan the zebrafish embryo vasculature, and a 543-nm laser to scan implanted tumor cells labeled with red fluorescence. Obtain a high-quality image, by scanning each embryo in eight to ten steps. Scan and average each step six times.
7. Carefully place the embryo back into the egg water if it is required for further experiments.

8. Perform statistical analysis using one-way analysis of variance (ANOVA) followed by post hoc analysis.

Representative Results:

In the embryonic xenograft zebrafish model with perivitelline space injection, the hematogenous dissemination of labeled cancer cells in the fish body is considered as active migration. This process can be detected and quantified under a fluorescent microscope as described in the methods above. To illustrate this xenograft model we followed the dissemination process of different breast cancer cell lines with known (or lack of) invasion/metastasis potential according to *in vitro* and *in vivo* mouse studies, including the benign normal breast epithelial M1 cells, HRAS-transformed premalignant M2 cells, and highly metastatic MDA-MB-231 cells 1 day post-njection (dpi) onward.

A high-resolution confocal microscopy image showed that MDA-MB-231 cells (red) exhibit an aggressive phenotype with irregular borders in the perivitelline space. Pseudopodial-like protrusions and invasive fronts were also frequently present (Figure 4A, left). A few cells disseminated into the blood circulation as early as 1 dpi (Figure 4A, right). At 2 dpi, clear dissemination was observed in the distal parts of the fish (Figure 4A, right). The number of disseminated cells increased further at 3 dpi (Figure 4A and B). In contrast, when M2 cells were challenged in zebrafish, they exhibited modest spread in the fish body after 2 dpi (Figure 4C). They also showed increased dissemination with time lapse (Figure 4D).

As shown in Figure 4E and F, M1 cells infrequently disseminated into the zebrafish circulation, and even active local migration within the perivitelline space was infrequent during the period of observation. The M1 cell mass was virtually detained at the original injection site. If defining positive dissemination or metastasis as >5 cells in the fish body (4), MDA-MB-231 and M2 cell metastasis was observed in 92% and 57% of fish, respectively, at 3 dpi (Figure 4G).

In contrast, no positive dissemination was observed with M1 cells. Therefore, this zebrafish model of human cancer cell progression accurately reflects the relative level of metastatic potential of the different cells in mice. Neovascularization (green) that sprouted from the subintestinal plexus of the embryonic zebrafish and penetrated into the MDA-MB-231 or M2 cell mass, was also present vividly through perivitelline space injection (Figure 4A and C, left). Consistent with the disability in dissemination, only slight neovascularization was detected upon M1 cell implantation (Figure 4E).

In the embryonic xenograft zebrafish model with mCherry-labeled MDA-MB-231 cells Doc injection, the labeled cancer cells in the tail fin of zebrafish is considered as active extravasation. The mCherry-labeled MDA-MB-231 cells were injected at 2 dpf. At 3 dpi, the cells started to migrate out of the vessels to the tail fin, which is enriched with collagen. Single MDA-MB-231 cells migrated one by one independently from the vessels to the distant tail fin (Figure 5A). At 6 dpi, invasion can be quantified by counting the number of cells that have migrated into the tail fin tissue. n mCherry-labeled M2 cells Doc injection model, injection was also performed at 2 dpf. However, a clustered phenotype is observed during the active extravasation process. At 1 dpi, M2 cells started to migrate out from the vessels into the caudal hematopoietic tissue (CHT) of the zebrafish. At 2 dpi the migrated M2 cells started to form a cluster between the vessels in the CHT (Figure 5B). Quantification of the M2 invasive cell clusters number in CHT region can be conducted at 6 dpi.

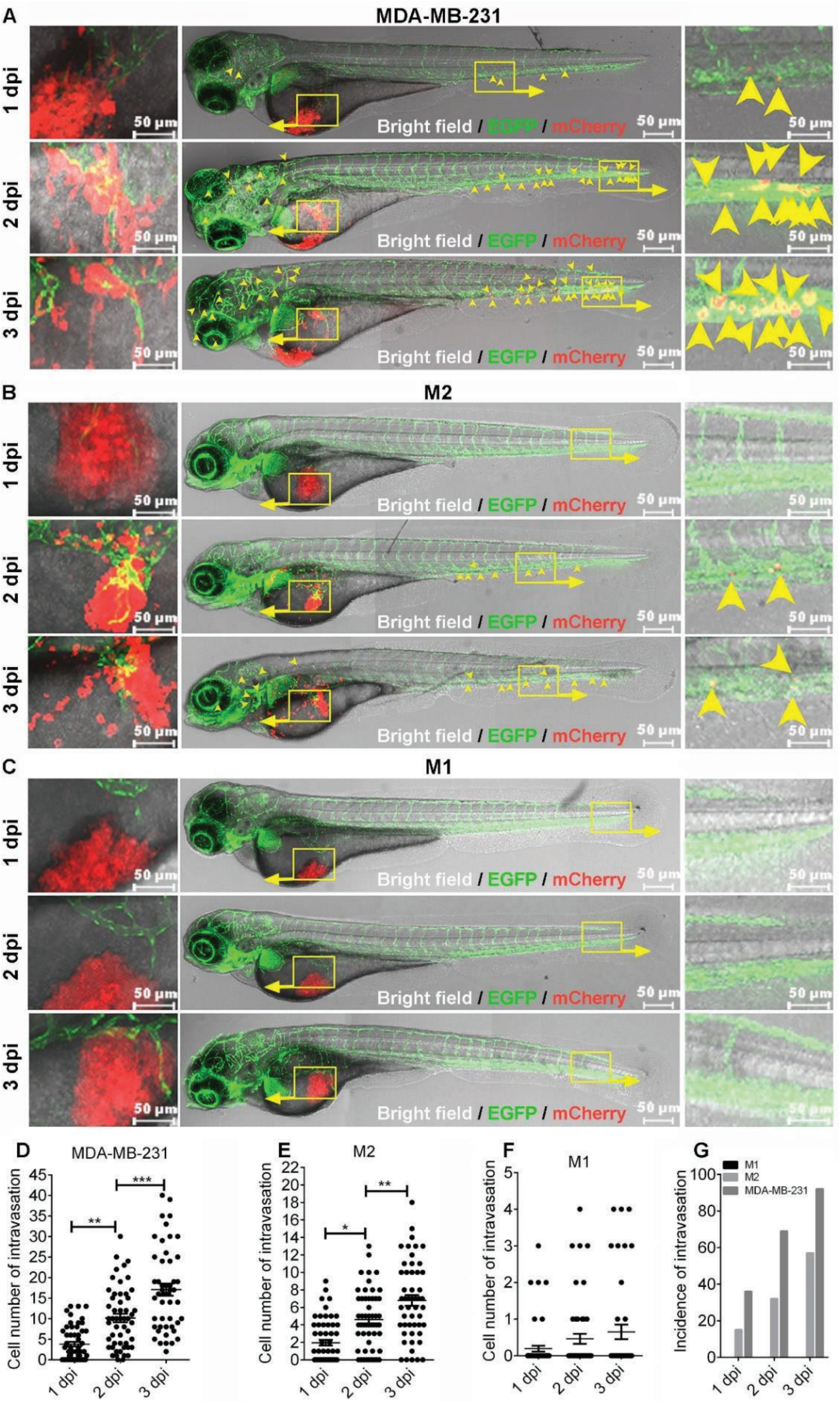
A
MDA-MB-231
1 dpi
2 dpi
3 dpi
50 μm
Bright field / EGFP / mCherry
B
M2
C
M1
D
MDA-MB-231
Cell number of intravasation
0
5
10
15
20
25
30
35
40
45
**
***
1 dpi
2 dpi
3 dpi
E
M2
Cell number of intravasation
0
2
4
6
8
10
12
14
16
18
20
22
*
**
F
M1
Cell number of intravasation
0
1
2
3
4
5
G
M1
M2
MDA-MB-231
Incidence of intravasation
0
20
40
60
80
100

Figure 4. Comparison of dissemination ability among various breast cell lines. Approximately 400 mCherry-labeled MDA-MB-231, MCF10Aras (M2), or MCF10A (M1) cells were injected into the perivitelline space of zebrafish embryos 48 hpf. The injected embryos were followed for 3 days. (A, C, and E) High-resolution micrographs showing the representative migration and dissemination process of MDA-MB-231 (A), M2 (C), and M1 (E) cells in individual embryonic bodies 1, 2, and 3 days post-injection (dpi). Left, cell migration in the perivitelline space (red) and the peritumoral and intratumoral vasculature (green). Yellow signals indicate the overlap of microvessels and cells. Right, yellow arrowheads indicate single disseminated cells. Scale bar = 50 µm (B, D, and G) Quantification of the number of disseminated cells in each embryonic body at 1, 2, 3 dpi. Results are expressed as the Mean ± SEM. Results from one-way analysis of variance (ANOVA) followed by the post hoc analysis are shown. P <0.05 was accepted as statistically significant (*0.01 < P <0.05; **0.001 < P <0.01; *** P <0.001. (F) Comparison of the incidence of metastasis for MDA-MB-231, M2, and M1 cells in embryonic bodies at 1, 2, 3 dpi.

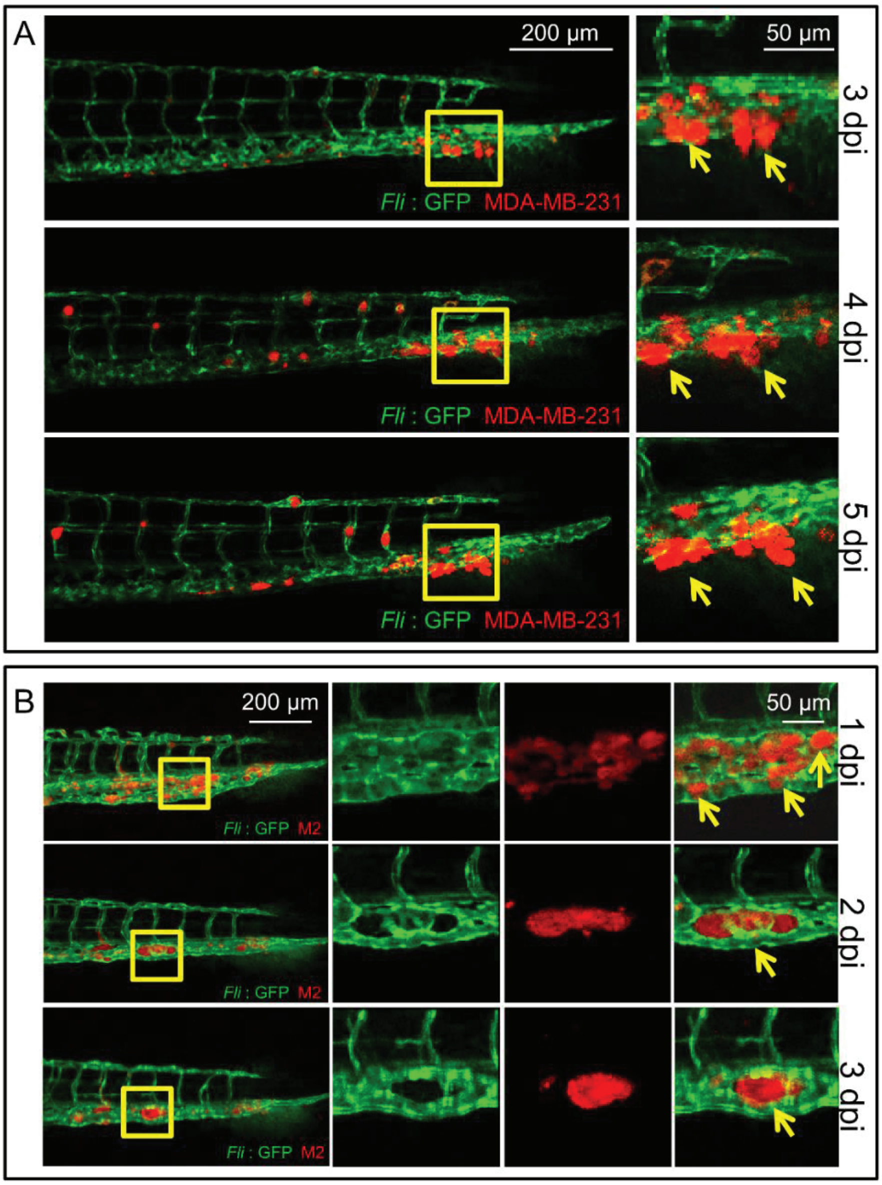

Figure 5. Different behavior of MDA-MB-231 and M2 cell metastasis in zebrafish with duct of Cuvier injection. (A) Representative confocal images of the zebrafish followed at 3, 4, 5 dpi to show the single cell migration behavior of the MDA-MB-231 cells in zebrafish. Arrows indicate invasive MDA-MB-231 cells that migrated out of the vessels to the tail fins. Scale bar = 200 µm in the left column, 50 µm in the right column. (B) Representative confocal images of the zebrafish followed at 1, 2, 3 dpi to show the cell cluster migration behavior of M2 cells in zebrafish. Arrows indicate invasive M2 cells that migrated out of the vessels to the caudal hematopoietic tissue (CHT) and formed a cluster between the vessels.

Discussion:

Here, we described two methods for investigating the invasive behavior of breast cancer cells in Tg (*fli1:GFP*) zebrafish embryos based on perivitelline space and Doc injection. By injecting cancer cells labeled with chemical dye or fluorescent protein into transgenic zebrafish embryos, the dynamic and spatial characteristics of invasion and metastasis can be clearly tracked in real-time at the single cell or cluster level under a fluorescence microscope. In most cases, the rapid progression of metastasis in zebrafish ensures that the assay can be performed within 1 week after transplantation. Moreover, powerful statistics can be obtained with large cohorts of fish.

Early and late events of the metastatic cascade could be simulated and recapitulated by injecting cancer cells into the perivitelline space or Doc, respectively. The perivitelline space is the confined space between the periderm of the fish and the yolk sac, which allows one to monitor dissemination of single tumor cells from primary sites in the living body. After implantation, the cancer cells undergo local migration and invasion within the perivitelline space (considered the primary site), then intravasate into blood vessels and disseminate along with the circulation. At the head and tail fin (considered distant target sites), cancer cells accumulate in narrow capillary beds and extravasate. Therefore, the number of cells that are found at the distant sites in the fish body is a measurement of metastatic capability. In addition, more extravasated cells could be observed at later time points, which is also shown in the Doc injection assay.

The Doc is an enlarged common cardinal vein with an extensive blood stream (21). Directly targeting the Doc as an injection site introduces cancer cells into the circulatory system. In practice, breast cancer cells diffuse throughout the embryonic body via the blood stream instantly after Doc injection. The cells then arrest at the caudal vein and dorsal aorta. Extravasation, invasion, and micrometastasis formation can be observed successively within 6 days. As reported previously(16), metastatic MDA-MB-231 cells and premalignant mammary M2 cells exhibit different invasive phenotypes. MDA-MB-231 cells undergo single cell invasion of the collagen matrix-rich tail fin. Thus, the invasion potential of MDA-MB-231 cells can be measured by counting the number of cells that have extravasated and invaded the tail fin tissue. In contrast, M2 cells form clusters of different sizes and undergo collective invasion of the CHT. Quantitating the invasion potential of M2 cells by counting the number of clusters in this protocol is difficult, and is preferably performed by making a 3D image using confocal microscopy and determining the volume of clustered tumor cells.

The technical challenge in cancer cell microinjection is successfully targeting the perivitelline space or Doc. The microinjection of large numbers of embryos is a tedious procedure requiring a highly skillful and patient operator. Factors that contribute to variations in the results in individual fish include the developmental stage of the embryo when injecting, differences in the number of cells injected, and the leakage of cells into the yolk sac. Though rare, the manipulation could unintentionally but inevitably penetrate the vasculature and introduce cells into the circulatory system directly, especially in perivitelline space injection. To further reduce variation and ensure the reliability of analyses, microscopic examination is necessary to exclude unqualified fish at a given time point throughout the process. In addition, blinded analysis by a professional without knowledge of the setting is strongly suggested to achieve unbiased quantification.

In summary, the two models we introduced here shed light on visualizing the processes of cell invasion and metastasis *in vivo* without invasive procedures. Although we only studied breast cancer cells in the two models regarding metastatic potential, they could be extrapolated to other types of cancer. Moreover, the models could have broader applications in excavating the mechanisms and new molecular targets controlling cancer cell metastasis using (epi)genetic manipulation. Due to the higher penetrability of zebrafish embryos by small-molecule compounds as compared to the feeding or injection of rodents (22), the two presented models also have advantages in high-throughput screening of potential new anti-invasion/metastasis drugs.

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Materials

Name	Company	cat #	Comments
Agarose	MP Biomedicals	AGAF0500	
Borosilicate glass capillary	Harvard Apparatus	300038	
Cholera enterotoxin	Calbiochem	227035	
Confocal microscope	Leica	SP5 STED	
DMEM-high glucose media containing L-glutamine	ThermoFisher Scientific	11965092	
DMEM/F-12 media containing L-glutamine	ThermoFisher Scientific	21041025	
Dumont #5 forceps	Fine Science Tools Inc	11252-20	
Epidermal growth factor	Merck Millipore	01-107	
Fetal bovine serum	ThermoFisher Scientific	16140071	
Fluorescent stereo microscope	Leica	M165 FC	
HEK293T cell line	American Type Culture Collection	CRL-1573	
Hydrocortisone	SigmaAldrich	227035	
Horse serum	ThermoFisher Scientific	26050088	
Insulin	SigmaAldrich	I-6634	
MCF10A (M1) cell line			Kindly provided by Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, MI, USA)
MCF10Aras (M2) cell line			
MDA-MB-231 cell line	American Type Culture Collection	CRM-HTB-26	
Manual micromanipulator	World Precision Instruments	M3301R	
Micropipette puller	Sutter Instruments	P-97	
Wide-tip Pasteur pipette (0,5-20 ul)	Eppendorf	F276456I	
pCMV-VSVG plasmid			Kindly provided by Prof. Dr. Rob Hoeben (Leiden University Medical Center, Leiden, The Netherlands)
pMDLg-RRE (gag/pol) plasmid			
pRSV-REV plasmid			
Penicillin-Streptomycin (10,000	ThermoFisher Scientific	15140122	

U/mL)			
PLV-mCherry plasmid	Addgene	36084	
Pneumatic picoPump	World Precision Instruments	SYS-PV820	
Polybrene	SigmaAldrich	107689	
Prism 4 software	GraphPad Software		
Stereo microscope	Leica	MZ16FA	
Tg (*fli:EGFP*) zebrafish strain			Kindly provided by Dr. Ewa Snaar-Jagalska (Institute of Biology, Leiden University, Leiden, The Netherlands)
Tris-base	SigmaAldrich	11814273001	
Tricaine (3-aminobenzoic acid)	SigmaAldrich	A-5040	
Trypsin-EDTA (0.5%)	ThermoFisher Scientific	15400054	
Petri dishes, polystyrene (60 × 15 mm)	SigmaAldrich	P5481-500EA	
Polystyrene dish with glass bottom	WillCo	GWST-5040	

The video of this article can be found at https: https://www.jove.com/video/55459/

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