Development of an in vitro vascular network using zebrafish embryonic cells

Ibrahim, M.

Chapter 3

Zebrafish blastocyst cell culture and differentiation of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells

Muhammad Ibrahim and Michael K. Richardson

Abstract

Different strategies have been developed to induce specific differentiation pathways in zebrafish embryonic cell culture. Our ultimate goal is to develop *in vitro* vascular networks using zebrafish embryonic cells. Therefore, we need to establish the basal, optimal conditions under which endothelial cells can be maintained in zebrafish embryonic cell culture. To achieve this aim, we describe here procedures for culture of zebrafish blastocyst cells and the quantification of putative endothelial cells in these cultures. To determine the basic nutrient requirements for zebrafish primary cells, we first cultured the blastocyst fragments (explants) in LDF medium (combination of Leibowitz's L-15, Dulbecco's modified Eagle's and Ham's F12 media) supplemented with different concentrations of heat-inactivated fetal bovine serum (FBS) or zebrafish embryo extract (ZEE). In the second experiment we analysed the development of putative endothelial cells (expressing green fluorescence protein), under basal culture conditions, in trypsinized blastocyst cell cultures derived from the transgenic zebrafish *fli:GFP* and *kdrl:GFP* lines. In the third experiment we analysed the characteristic and growth of blastocyst secondary cell cultures established by passaging primary cultures. The blastocyst explant cultures showed significant expansion in the area covered by cells in media supplemented with 15% FBS and 60 µg/mL ZEE. The trypsinized blastocyst cells formed embryoid body (EB) aggregates in culture which contained different cell types including *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells. The percentage of these GFP^{+} cells dropped in cultures after day 4, which is probably because of the thriving fibroblast-like cells emigrating from the EBs. These fibroblast-like cells were maintained for 8 passages with 12 population doublings. These cells showed a homogeneous morphology and higher growth rate compared to the primary blastocyst cells.

Introduction

In many vertebrates, embryonic stem cells (ESCs) are cells isolated from early embryos [1]. These ESCs may form, when cultured, embryoid bodies (EBs) containing endoderm and mesoderm cells which differentiate into different cell types including hematopoietic, endothelial, muscle and neural cells [2]. This differentiating property of ESCs can be used to study the differentiation and lineage commitment events in embryos [2]. Mouse ESCs have been extensively studied and used in various experiments [3]. Because of the their considerable potential, techniques for the culture of pluripotent ESCs are being developed in other mammals [4] including humans [5]. However, the use of these species in research is relatively expensive and raise ethical concerns [6]. As an alternative, the zebrafish can be used for ESC research because of it has advantages over mammalian cells as a culture system [7].

The manipulation of zebrafish is relatively simple and cell isolation procedures are well described [7]. Zebrafish are small, have a short generation time of about 2 to 3 months and one female produces about 200 eggs per week [8]. Zebrafish eggs are externally fertilized so there is no need to kill the mother for embryo isolation as in the mouse. Furthermore, zebrafish cells can be maintained at a lower temperature (26 °C) in atmospheric CO_2, in contrast to mammalian cells [7]. Being a relatively new research model, few cell cultures have been developed for zebrafish [9-17]. The methods used to culture zebrafish cells often vary between researchers. One of the important inconsistencies is the composition of the culture medium (Table 1). It will be important in this field to define a standard medium for efficient growth of zebrafish embryonic cell culture.

A commonly-used zebrafish cell culture medium is composed of LDF (combined Leibowitz's L-15, Dulbecco's modified Eagle's medium (DMEM) and Ham's F12) with added HEPES buffer (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), sodium bicarbonate, sodium pyruvate and antibiotics [18]. The medium is usually supplemented with fetal bovine serum (FBS), fish serum, fish embryo extract and basic fibroblast growth factor (bFGF) [7]. The concentrations of these ingredients, and the type of antibiotic used, may vary for different zebrafish cell culture experiments (Table 1). Primary cells from zebrafish embryos tend to require a nutrient-rich medium for their growth [7]. Therefore, the media are sometimes enriched with additional supplements and growth factors, including L-glutamine [19], bovine insulin [20], trout plasma, human epidermal growth factor (hEGF) [21] and murine epidermal growth factor (mEGF) [22].

When studying cellular differentiation in zebrafish ESC culture, transgenic zebrafish lines expressing green fluorescence protein (GFP) under *fli-1* [23] and *kdrl* [24, 25] promoters, may be used as a marker to observe real time differentiation of vascular progenitor cells.

Fli-1 (Friend leukemia virus integration site 1) is a transcription factor gene highly expressed in endothelial cells, hematopoietic cells and lymphoid tissue in mouse [26].

Table 1: Composition of various media used for zebrafish cell culture.

Culture	Units	Zebrafish cell type											
Constituents		EFB	EC	EC	EC	EC	SC	ESN	EMC	FB	EC	EFB	EC
Substrate		P	FC	P	FC	FC	P	L	L	P	PL	C1	G
Leibowitz's L-15	%	-	52	85	50	50	83	85.9	25	-	50	-	-
DMEM	%	45	21	-	35	35	-	-	25	85	35	84	85
Ham's F12	%	45	9	-	15	15	-	-	-	-	15	-	-
HBSS	%	-	-	-	-	-	-	-	50	-	-	-	-
HEPES	mM	15	15	-	-	15	-	4	15	-	15	-	20
Sodium bicarbonate	mg/mL	1.2	0.11	-	-	0.15	-	-	-	-	0.15	-	-
Sodium pyruvate	mM	0.5		-	-	-	-	-	12.5	-	-	-	1
Sodium selenite	nM	-	10	-	10	-	-	-	-	-	10	-	2
Calcium chloride	mM	-		0.8	-	-	-	0.16	0.8	-	-	-	-
2-mercaptoethanol	µM	-	-	-	-	-	-	-	-	-	-	-	100
Antibiotics													
Penicillin	µg/mL	-	72	50	-	120	-	-	60	60	120	-	-
Streptomycin	µg/mL	-	120	50	-	200	-	-	100	100	200	-	-
Ampicillin	µg/mL	-	15	-	-	25	-	-	-	-	25	25	-
Kanamycin	µg/mL	-	-	-	-	-	-	-	100	-	-	-	-
Gentamycin	mg/mL	-	-	10	-	-	-	-	0.05	-	-	-	-
Pen-strep	%	-	-	-	-	-	1	0.4	-	-	-	1	100#
Antimycotic mix*	%	-	-	-	-	-	1	-	-	-	-	-	-
Amphotericin	µg/mL	-	-	-	-	-	-	-	25	-	-	-	-
Serum	%												
FBS		10	14.5	15	5	5	15	2	10	15	1	15	15
Carp serum		-	-	-			-	-	5	-	-	-	-
Trout serum		-	-	-	0.4	1	-	-	-	-	0.5	1	1
Trout plasma	%	-	1	-	-	-	-	-	-	-	-	-	-
Embryo extract	µg/mL**												
Trout		-	-	-	50	50	-	-	-	-	40	-	-
Zebrafish		-	50	-	-	-	-	-	10	-	-	50	1##
Growth factors	ng/mL												
bFGF		-	50	-	50	50	-	-	-	-	-	50	10
hEGF		-	50	-	-	50	-	-	-	-	-	25	-
mEGF		-	-	-	50	-	-	-	-	-	-	-	-
l-glutamine	mM	2.5	-	-	-	-	-	-	2	3	-	-	2
Bovine insulin	µg/mL	-	10	-	10	10	-	-	5	-	10	10	-
NE amino acids	mM	-	-	-	-	-	-	-	-	-	-	-	1
Reference		[19]	[21]	[17]	[22]	[11]	[14]	[10]	[12]	[13]	[16]	[20]	[27]

Abbreviations: bFGF, basic fibroblast growth factor; C1, collagen type-I; DMEM, Dulbecco's modified Eagle's medium; EC, embryonic cells; EFB, embryonic fibroblasts; EMC, embryonic muscle cells; ESN, embryonic spinal neurons; FB, fibroblasts; FBS, fetal bovine serum; FC, growth arrested feeder cell line (RTS34st cells); HBSS, Hank's balanced salt solution; hEGF, human epidermal growth factor; HEPES, (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid); L, laminin; mEGF, murine epidermal growth factor; NE, non-essential; P, polystyrene; PL, poly-D-lysine; SC, spleen cells.
KEY: *, antimycotic mix from Gibco; **, concentration of embryo extracts means the amount of total proteins in the extract; #, concentration 100 U/mL; ##, concentration 1 embryo/mL; -, not added.

In zebrafish embryos *fli-1* is expressed during early vasculogenesis and the expression persists throughout vascular development [28]. The transgenic zebrafish (*fli:GFP*) has

been shown to express GFP in endothelial and hematopoietic cells, as well as in cells on the yolk sac and neural crest cells i.e. mesenchyme and jaw cartilages [23]. *Kdrl*, also known as *flk-1* or VEGFR2, is a vascular endothelial growth factor receptor, expressed particularly in endothelial cells during vascular development [25, 29].

Zebrafish embryos at the blastocyst stage of development (approximately 3.5 hours post fertilization or hpf) are usually dechorionated and trypsinized to isolate ESCs. Culturing blastocyst explants is equally important in studying cellular interactions, organization and lineage commitment in early embryos [30]. Embryo explant culture has also been applied to invertebrate embryos such as *Caenorhabditis elegans* [31] and the sea urchin [32]. However, in most of the procedures for ESC research, the embryos are dissociated (trypsinized) into single cells for culturing. These early embryonic cells are used to establish pluripotent ESC cultures which may be used for various applications e.g. genetic manipulation of cells and organisms and production of germ line chimeras [21]. By culturing these ESCs under differentiation conditions, specific cell lines can also be developed [12, 17].

In order to maintain the ESCs in a pluripotent, undifferentiated state, they may be grown on a feeder monolayer of growth-arrested cells [33]. Different feeder cell lines used to culture zebrafish embryonic cells include rainbow trout spleen cells (RTS34st) [11, 22], zebrafish spleen stromal cells (ZSSJ) [14], buffalo rat liver cells [34] and zebrafish embryonic fibroblasts [18]. For specific differentiation of ESCs, the feeder layer is replaced by coating the culture plate with adhesive molecules. Fibronectin, laminin, collagen and poly-D-lysine are the molecules known to promote attachment and differentiation of zebrafish cells [10, 12, 20]. Zebrafish embryonic cells have also been successfully cultured on polystyrene culture dishes without any feeder layer or substrate coating [17].

In this chapter we have optimized the culture conditions for zebrafish blastocyst cells in the following steps: (1) The optimization of FBS and zebrafish embryo extract (ZEE) concentrations in the media for the expansion of blastocyst explant cultures. (2) The quantification of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells under basic medium conditions, in the blastocyst cell culture developed from the transgenic zebrafish embryos. (3) The effect of different concentrations of FBS, ZEE and gelatin substratum on the growth and viability of blastocyst secondary cell cultures.

Materials and methods

Zebrafish rearing and mating setup for embryo isolation

All the animal experiments were performed according to the Netherland Experiments on Animals Act [35], based on guidelines laid by the Council of European Union [36]. Adult

zebrafish were maintained, as previously described [37], in 5 liter tanks having continuously circulating egg water ("Instant Ocean" sea salt 60 µg/mL demi water), on 14 h light: 10 h dark cycle. Temperature of the water and air was controlled at 26 and 23 °C, respectively. Wild type zebrafish embryos were used to characterize general growth of blastocyst cells. Transgenic *fli:GFP* and *kdrl:GFP* embryos were used to analyse differentiation of blastocyst cells into putative endothelial and hematopoietic cells. To obtain embryos, adult male and female fish, at a proportion of 1:1, were transferred to small breeding tanks in the evening. The zebrafish usually laid eggs when light turns on. The eggs were collected at the bottom of the tank, separated from adults using a cotton mesh to protect the eggs from being eaten.

Embryos were transferred to a temperature controlled room (28 °C) and were distributed in 9 cm Petri dishes at a final density of 100 embryos per dish, after removing dead and unfertilized eggs. The embryos were washed thoroughly with clean egg water to remove any debris.

Sterilization of embryos

Embryos were allowed to develop to the high blastula stage of Kimmel et al. [38] (approximately 3 hpf) at 28 °C. These embryos were then sterilized using 70% ethanol for 10 sec and 0.05% bleach solution (Table 2) for 2 × 5 min. Between the sterilization steps the embryos were washed with LDF medium (Table 2). The sterilization procedure described in Ref. [21] was followed. The embryos were finally left in 0.5 mL of LDF medium and any dead embryos or embryos with visible contamination inside the chorion were removed. These embryos were dechorionated using a pair of No. 5 watchmaker's forceps for the isolation of blastocyst cells.

Overview of experiments

The procedure of cell isolation and culture from embryos is shown in Figure 1. Three different experiments were performed (Figure 1). In the first experiment the wild type blastocysts were triturated gently with a P-200 Gilson micropipette (Gilson, B.V., Europe: Den Haag) without enzymatic digestion to form fragments (explants). These explants were cultured in LDF medium (Table 2) supplemented with different amounts of FBS (Invitrogen, 10500) and ZEE to optimize the concentration of these nutrients for zebrafish primary cell culture (see below, Experiment 1: Primary explant cultures). In the second experiment (see below, Experiment 2: Quantification of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures) trypsinized blastocyst cells isolated from *fli:GFP* and *kdrl:GFP* embryos were cultured to analyse the generation of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in these cultures. In the third experiment (see below, Experiment 3: Secondary blastocyst cell culture) the effect of FBS and ZEE supplementation and gelatin substratum was analysed on the growth and

viability of blastocyst secondary cell cultures, established after several passages from the primary cultures.

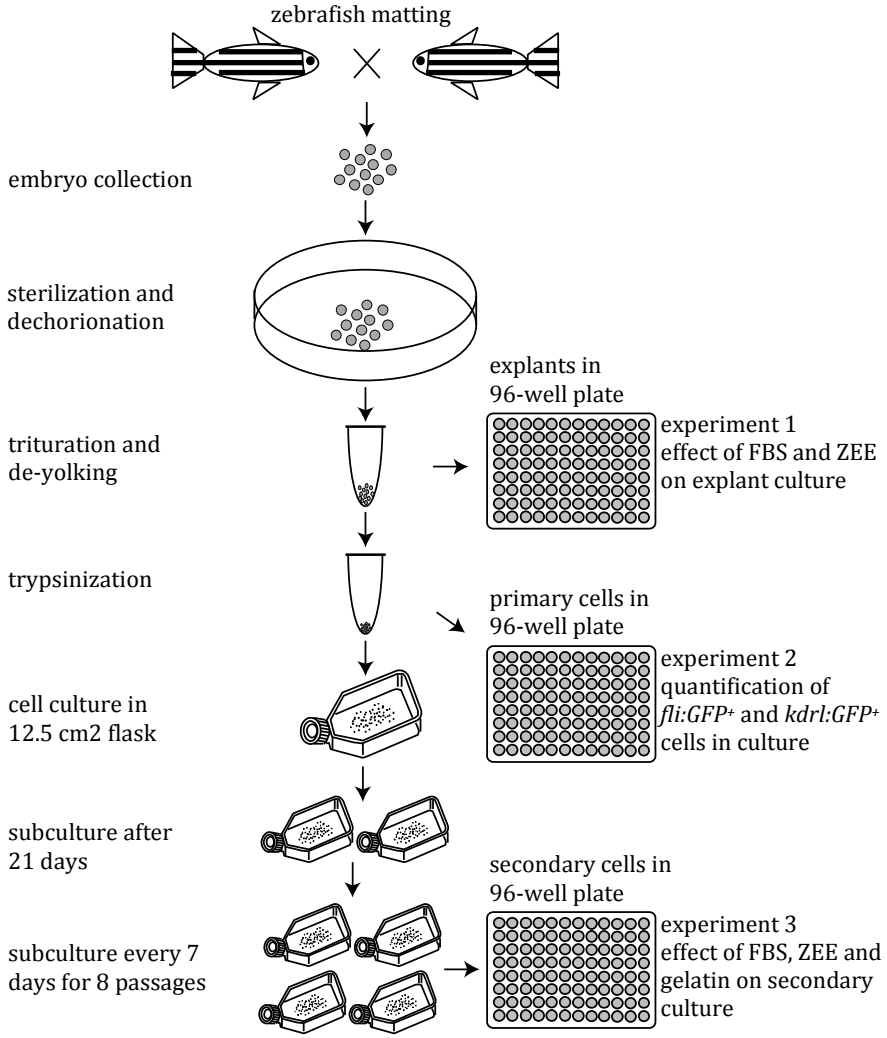

Figure 1: Schematic diagram of isolation and culture procedures of zebrafish blastocyst cells and the experiments performed.
Abbreviations: FBS, fetal bovine serum; ZEE, zebrafish embryo extract.

Medium preparation

A basic LDF medium was prepared as shown in Table 2. All of the solutions used in LDF medium were supplied from Invitrogen (Gibco). Media were reconstituted with different concentrations of FBS (5, 10, 15 and 20%) and ZEE (15, 30, 45 and 60 µg total protein/mL) in separate tubes, before adding into culture wells. FBS (10%) contained in all media having different concentrations of ZEE; similarly, ZEE (50 µg/mL) contained in

all media having different concentrations of FBS. Cells were cultured in each distinct medium in six wells (replicates) of the 96 well microplate. The FBS used in the experiments was qualified, heat inactivated, E.U.-approved, South America Origin from Invitrogen, catalog number 10500. ZEE was prepared using 36 hpf wild type zebrafish embryos using the following procedure.

Preparation of zebrafish embryo extract

The ZEE was prepared according to the procedure described in Ref. [21]. The 36 hpf wild type zebrafish embryos were washed thoroughly with egg water. About 500 embryos suspended in 0.5 mL LDF medium were transferred to a 2 mL Eppendorf tube having a 5 mm glass bead. The embryos were homogenized in a QIAGEN TissueLyser II at 30 oscillations/min for 1 min. After homogenization, 1 mL LDF was added to the embryo lysate. The tubes were then centrifuged at 20,000 g for 10 min at 4 °C. The supernatant was transferred to a new tube, and the total protein content of the extract was quantified using bovine serum albumin protein assay kit (Thermo scientific, product 23227). Finally, the extract was diluted to a final concentration of 1 mg total protein/mL, filter sterilized and stored in aliquots at -20 °C.

Experiment 1: Primary explant culture

The wild type embryos were dechorionated using sterile No. 5 watchmaker forceps and each set of 50 embryos was then transferred to a separate 1.5 mL sterile Eppendorf tube. The embryos were triturated using a P-200 Gilson micropipette, in the LDF medium, to fragmentize the blastocysts. The tubes were then centrifuged at 300 g for 2 min and the supernatant, including the yolk, was discarded. The blastocyst fragments (explants) were washed with CMF-PBS (calcium magnesium free – phosphate buffered saline) and then with LDF to remove any remaining yolk particles. Finally, the explants were re-suspended in 300 µL of LDF medium containing 10% FBS, and distributed in a 96-well plate such that, on average, the explants from 2 blastocysts were plated per well. Finally 200 µL LDF media, having varying concentrations of FBS and ZEE (as described above in Medium preparation) were added to the wells. The cultures were maintained in an incubator at 28 °C in atmospheric CO_2 until data collection.

Trypsinized blastocyst cell culture

The concentrations of FBS and ZEE in the medium optimized for explant culture in experiment 1 (above) was used to culture the completely dissociated (trypsinized) blastocyst cells. For this purpose, the embryos were sterilized, dechorionated and de-yolked as described above. Then, the blastocysts were given a rinse with CMF-PBS and incubated in 1 mL of 0.25% trypsin solution (Table 2) for 2 min. The trypsin-blastocysts solution was triturated several times using p-1000 Gilson micropipette, to facilitate

dissociation of cells. The trypsin was deactivated by adding 0.1 mL of FBS or 1 mL of trypsin inhibitor (Invitrogen, catalogue number R-007-100). The solution was centrifuged at 300 g for 3 min and the supernatant was discarded.

Table 2: Solutions and Media.

Reagent (supplier, catalog number)	Final Concentration
LDF medium	
Leibowitz's L-15 (Invitrogen, 11415) : DMEM (Invitrogen, 11966) : Ham's F12 (Invitrogen, 21765)	55 : 32.5 : 12.5
HEPES (Invitrogen, 15630)	15 mM
Antibiotic antimycotic mix (Invitrogen, 15240)	1%
$NaHCO_3$	0.015%
CMF-PBS (Invitrogen, 14190)	
KCl	2.7 mM
KH_2PO_4	1.5 mM
NaCl	138 mM
Na_2HPO_4-7H2O	8.06 mM
Trypsin solution for embryos	
Trypsin 2.5% (Invitrogen, 15090)	0.25%
CMF-PBS	99.75%
EDTA	1 mM
Trypsin solution for cultured cells	
Trypsin 10X (Invitrogen, 15400)	0.05%
CMF-PBS	99.95%
EDTA	1 mM
Bleach solution	
Sodium hypochlorite 10-15% (Sigma, 425044)	0.05%
Sterile distilled H_2O	99.95%
Gelatin solution	
Gelatin (Sigma, G1890)	2%
Sterile distilled H_2O	98%

Abbreviations: CMF-PBS, calcium magnesium free phosphate buffer saline; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

The cell pellet was washed three times with LDF medium containing 10% FBS and finally suspended in 300 µL of the same medium. The concertation of the cells in the isolates was determined by counting in a heamocytometer. The *fli:GFP* or *kdrl:GFP* blastocyst cells (for Experiment 2: Quantification of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in cultures, see below) were plated at a density of 17000 cells per well in a 96 well plate, with or without gelatin coating. Another batch of wild type blastocyst cells isolated using the same procedure was cultured in 12.5 cm^2 flasks (for Experiment 3: Blastocyst secondary cell culture, see below) at a density of 6×10^5 cells per flask for sub-culturing.

After allowing approximately 1 h for the cells to attach to the surface, 200 µL or 2 mL of LDF medium containing 15% FBS and 50 µg/mL ZEE was added per well of the 96 well plate or per 12.5 cm^2 flask, respectively. These cultures were maintained at 28 °C in 0.5% CO_2 until data collection or sub-culturing.

Media refreshment

To optimize the time for media refreshment, in a separate experiment, the zebrafish blastocyst explants were cultured without replacement of media. We changed to a refreshment interval of 4 days for the primary cultures when we observed a decrease in the surface area covered by the emigrating cells around the explants after day 4 in the non-replacement cultures. For the secondary cultures the medium was refreshed weekly.

Experiment 2: Quantification of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in culture

Two different cultures were established to analyse spontaneous differentiation in primary blastocyst cultures: one from the cells isolated from zebrafish *fli:GFP* embryos and the other from *kdrl:GFP* embryos. The cells cultured in the wells of 96 well plate, with or without gelatin coating, were isolated for the quantification of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ at successive time-points (days 2, 4, 6 and 8). The gelatin was used at a concentration of 0.1 mg/cm^2 (1.7 µL of 2% gelatin solution per well). For each of the above mentioned condition (i.e. transgene line, gelatin coating and time-points), the blastocyst cells cultured in six replicate wells were transferred to separate Eppendorf tubes (see below, Trypsinization of cultured cells). Each cells isolate was subjected to quantification of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ using the procedure described below (Data collection).

Experiment 3: Secondary blastocyst cell culture

The blastocyst cell culture in 12.5 cm^2 flasks was maintained for 21 days with media refreshment every four days. The cells were then isolated from the flask using 2mL of 0.05% trypsin solution (Trypsinization of cultured cells), and sub-cultured in a new flask in LDF medium containing 10% FBS. These secondary cells were then passaged every seven days. At least three 12.5 cm^2 flasks were platted, for the primary culture as wells as for the secondary cultures, to allow for statistical analysis of growth rates at successive passages. During transfer a small amount of cell isolate at each passage was used to determine the total number of cells harvested and the population doubling time (see below, Statistical analysis).

At passage eight the cells were isolated from the flask and distributed at 17000 cells per well in a 96-well plate (Figure 1). These cells were maintained in media supplemented with different concentrations of FBS (5, 10, 15 and 20%) or ZEE (15, 30, 45 and 60 µg/mL), or on different concentrations of gelatin substratum (see below, Gelatin coating of wells). The effect of FBS, ZEE and gelatin substratum was analysed by calculating the total number of cells harvested per well and the viability of the isolated cells on day 7 of culture (see below, Data collection).

Vimentin staining of secondary blastocyst cell culture

To identify possible fibroblasts, the blastocyst secondary cells at passage eight were stained with anti-vimentin antibody [39]. For this purpose, mouse anti-vimentin antibody, developed by Michael Klymkowsky and maintained by The University of Lowa, Department of Biology, was obtained from the Developmental Studies Hybridoma Bank. The culture was established in chambered coverglass plate for imaging. All of the following steps were performed at room temperature.

On day 7 of culture the medium was removed and the cells were washed twice with CMF-PBS. Then the cells were fixed for 20 min in 4% paraformaldehyde (PFA) solution. The fixing solution was removed and the cells were washed twice with washing buffer (0.1% bovine serum albumin or BSA in CMF-PBS). Then the cells were treated with blocking buffer (10% normal sheep serum, 1% BSA, 0.3% triton X-100 in CMF-PBS) for 45 min. The blocking buffer was removed and the cells were incubated for 3 h with primary anti-vimentin antibody at a concentration of 3 µg/mL in CMF-PBS containing 1% normal sheep serum, 1% BSA and 0.3% triton X-100. The cells were then washed with washing buffer and incubated for 1 h in 1 µg/mL of the secondary antibody (goat anti-mouse IgG Alexa Fluor 568) to visualize the stained cells. Finally, the cells were treated with 1 µg/mL of DAPI (4',6-diamidino-2-fenylindool) to stain the nuclei.

Gelatin coating of wells

The wells of a 96 well plate were coated with different concentrations (0.03, 0.06, 0.12, 0.24 and 0.48 mg/cm^2) of gelatin. Calculated volume of 2% gelatin solution (Table 2), to get the desired concentration of substratum, was added to the wells of a 96 well plate. Each concentration was replicated in six wells, in addition six wells were used as a control without gelatin coating. The plate was allowed to air dry at room temperature for one hour. The blastocyst secondary cells at passage eight were cultured in these wells in LDF medium containing 10% FBS and 50 µg/mL ZEE. The cells were isolated and counted on day 7 of culture (see below, Data collection).

Trypsinization of cultured cells

In order to isolate the cells from 96 well plate for counting or from 12.5 cm^2 flasks for sub-culturing, the medium was aspirated and the cells were washed with CMF-PBS. The cultures were then incubated in 0.05% trypsin solution (Table 2) at 28 °C. the solution was triturated several times using a p-1000 micropipette to detach remaining cells and to dissociate the cell clumps. The detachment of cells was observed under an inverted microscope. When the majority of cells were in the suspension (approximately after 2 min), the trypsin was inactivated by adding FBS to a final concentration of 10%. The suspensions were then transferred to separate Eppendorf tubes from each well or flask and centrifuged at 300 g for 3 min. The supernatant was discarded and the cells were

washed twice with LDF medium containing 10% FBS. Finally, the cells were re-suspended in the same medium for cell counting or re-plating.

Data collection

The blastocyst explant cultures were imaged using an inverted phase contrast light microscope, and the following measurements were made at each successive time-point (day 2, 4 and 6 of culture) with the software Image-J, version, 1.46r [40], using a pre-calibrated scale: (i) Area (mm^2) of initial explants (marked as 1 in Figure 2); (ii) area (mm^2) covered by the explant aggregates (marked as 2 in Figure 2); and (iii), the area (mm^2) covered by the flattening, fibroblast-like cells spreading at the periphery of the explants (marked as 3 in Figure 2). The percentages of growth in covered area was calculated as described in the section below (Statistical analysis).

The primary *fli:GFP* and *kdrl:GFP* blastocyst cell cultures were isolated from the 96 well plates at days 2, 4, 6 and 8 using 0.05% trypsin solution (see above, Trypsinization of cultured cells). The cell isolates from each well were transferred in a 5 µL droplet on a glass slide to the confocal microscope. Each drop was imaged with two channels; one showing GFP^+ cells and the other showing a phase contrast image of all the cells in the same field. For cell counting, both of the channels were combined, and the *fli:GFP*$^+$ or *kdrl:GFP*$^+$ and non-positive cells were counted in each image. From these counts the average percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells was calculated (Statistical analysis).

The secondary embryonic cells cultured in 96-well plate in different concentrations of FBS, ZEE or gelatin substratum were isolated and counted on day 7. In total six replicate wells were cultured for each condition. The cells were isolated from the wells using 0.05% trypsin solution (see above, Trypsinization of cultured cells). The total number of cells harvested per well was calculated using a heamocytometer. The viable cells were distinguished from non-viable cells using trypan blue dye exclusion (1:1 ratio of 0.4% trypan blue in CMF-PBS).

Statistical analysis

The percentage increase in the relative area of the blastocyst explant, at each successive time-point, compared to the area on day 0, and the area covered by the emigrating fibroblast-like cells as a percentage of the total covered area (explant + emigrating cells), was calculated from the above measurements (Data collection) using the following formulas (see Figure 2 for the number references with the equations).

Percent increase in area covered by cells in the explant culture:

(1) Area increased = $Total\ area\ covered\ by\ cells\ (2+3) - size\ of\ explant\ at\ day\ 0\ (1)$

(2) Percent increase in area = $\frac{Area\ increased}{Size\ of\ explant\ at\ day\ 0\ (1)} \times 100$

Area covered by flattened, fibroblast-like cells as a percentage of total covered area:

(3) Percent area covered by the flattened cells = $\frac{Area\ covered\ by\ flattened\ cells\ (3)}{Total\ covered\ area\ (2+3)} \times 100$

The percentage of *fli:GFP*+ or *kdrl:GFP*+ cells was calculated from cell counts in the confocal images of the cell isolates of the primary blastocysts cell cultures (Data collection) using the following formula.

(4) Percentage of GFP+ cells = $\frac{Number\ of\ GFP^{+}\ cells/field}{total\ number\ of\ cells/field} \times 100$

The total number of cells harvested per culture, cell viability and population doubling level of the secondary blastocyst cell cultures was calculated from the cell counts in the heamocytometer using the following formulas.

(5) Total cells harvested = $Number\ of\ cells/\mu L\ \times total\ volume\ of\ cell\ isolate$ in μL

(6) Cells viability = $\frac{Number\ of\ viable\ cells/\mu L}{Total\ number\ of\ cells/\mu L} \times 100$

(7) Population doubling level (PDL) = $3.32 \times (logNH - logNI)$

Where NH is the total number of cells harvested after a period of time, and NI is number of cells plated.

(8) Population doubling time(PDT) = $\frac{\frac{1}{PDL}}{total\ growth\ hrs}$

Arithmetic means of six replicates were calculated for all percentages under each condition, using the software SPSS 21.0. One-way analysis of variance (ANOVA) was performed to calculate p values to compare different culture conditions as well as different time-points of the same condition. Multiple mean values that varied significantly, were further analysed for pair comparisons using post-hoc Tukey's test.

Results

Blastocyst explant culture

Images of zebrafish blastocyst explant cultures are shown in Figure 2. By day 4 of cultures the fibroblast-like (flattened, elongated) cells, emerging from the explants, spread and covered the surrounding substratum (Figure 2B, dashed area marked with 3). Nearby blastocyst explants often fused to form larger bodies, and the area covered by fibroblast-like cells increased with time (Figure 2C).

On average, two blastocyst embryos yielded 8.3 ± 0.8 explant fragments (plated per well). The explants were of different sizes and the surface area covered by individual explant ranged from 0.01 to 0.29 mm^2. Combined all the explants per well covered an area of 0.27 ± 0.01 mm^2 of the substratum. Cultures observed on subsequent days (day 2, 4 and 6)

showed a gradual decrease in the total number of explants per well (6.2 ± 0.7, 4.4 ± 0.4 and 3.2 ± 0.3 respectively), due to fusion between adjacent explants (Figure 2C). The effect of fetal bovine serum (FBS) and zebrafish embryo extract (ZEE) on the explant cultures and the area covered by the emigrating fibroblast-like cells is discussed in the following sections.

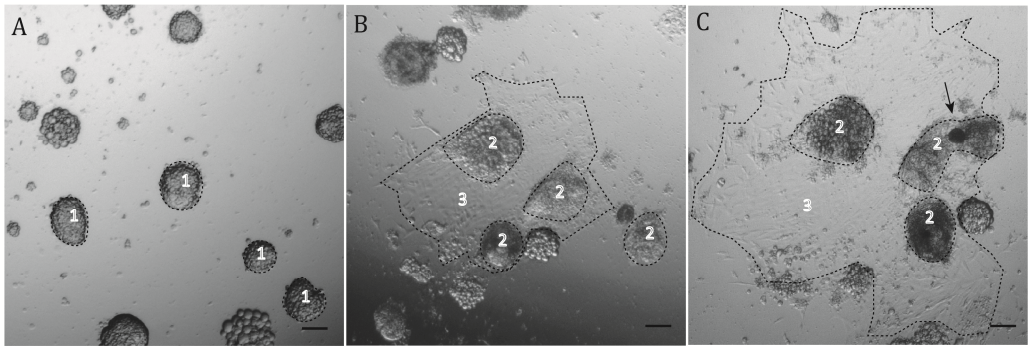

Figure 2: Culture of zebrafish blastocyst explants on day 0 (A), day 4 (B) and day 6 (C). The dotted lines represent (**1**) size of explant just after plating, (**2**) area covered by the rounded explant cells during culture, (**3**) area covered by elongated, fibroblast-like cells. In (**C**) the arrow showing the combination of two explants to form larger aggregate. Scale bar, 100 μm.

Effect of FBS on the blastocyst explant culture

FBS increases the spreading of cells around the explant

By day 6 the cells spreading around the explants covered 350-430% of the initial explant area on different FBS concentrations (Figure 3A). The increase in area covered by the cells around the explants was significantly lower in cultures without FBS compared to cultures with 5% FBS or above on day 4 and 6 of culture. Until day 2 the percent increase in covered area of explant and emigrating cells was similar in all media compositions including medium without FBS. After day 2, the covered area increased significantly faster in media supplemented with different FBS concentrations (Figure 3A). The explants cultured in medium without FBS did not show further increase in the area covered by cells after day 2. No significant differences were observed in area covered by cells cultured in different FBS concentrations.

FBS increases the percent area covered by flattened cells in explant culture

The percent area covered by the flattened, fibroblast-like cells was significantly higher in cultures supplemented with FBS (5-20%) compared to cultures without FBS (Figure 3B). The explants cultured in medium without FBS showed less extension of fibroblast-like cells, and remained similar with time. In the media with 5% or more FBS, the percent area covered by fibroblast-like cells increased significantly from day 2 till day 6. Cultures with

20% FBS had significantly higher percentage of area covered by the fibroblast-like cells on the day 4 and day 6, compared to cultures with 5% FBS. On day 2 the percent area covered by the fibroblast-like cells was similar in cultures with different FBS concentrations (5-20%).

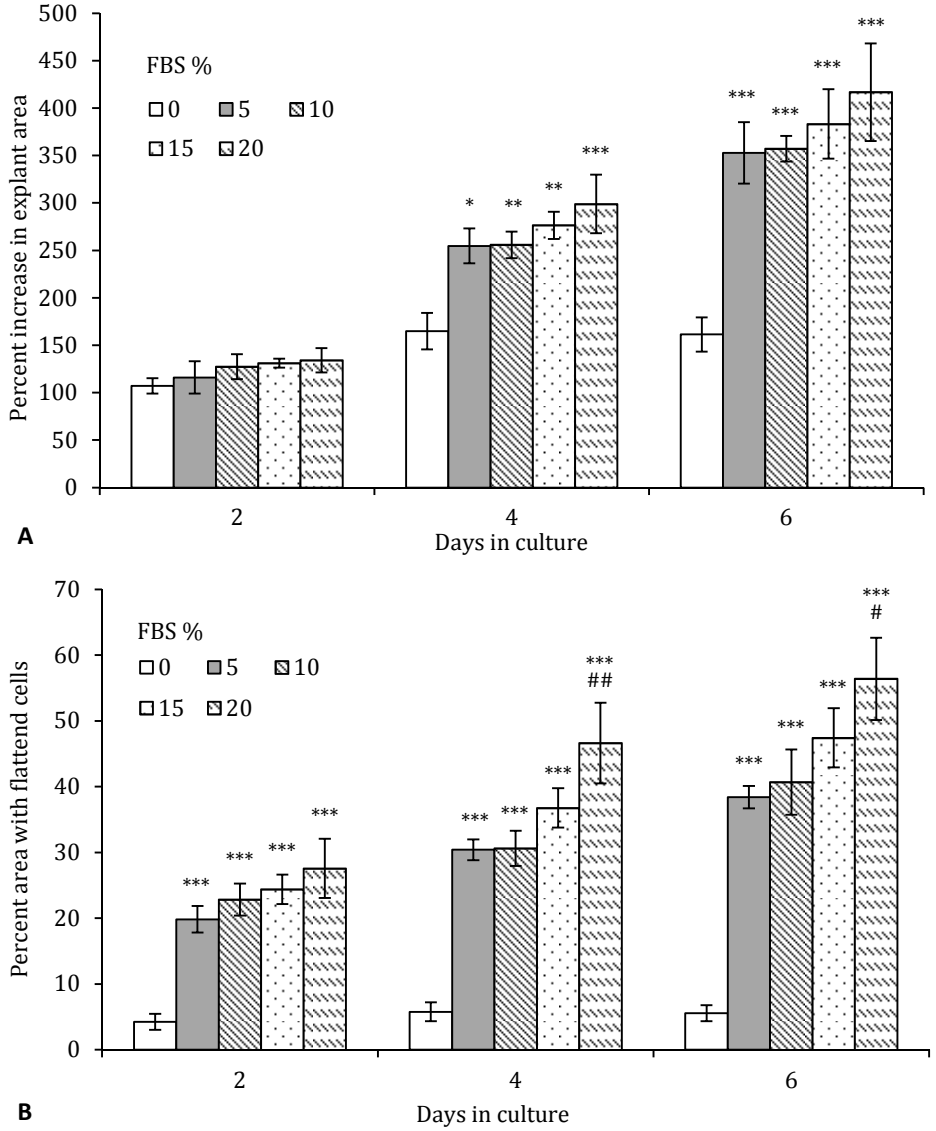

Figure 3: Blastocyst explant culture in media supplemented with different fetal bovine serum (FBS) concentrations. (**A**) Percentage increase in relative area of explant + outgrowth relative to area of initial explant (see equation 2 in Statistical analysis). (**B**) Percentage of area covered by flattening cells (area marked with number 3 in Figure 2; see equation 3 in Statistical analysis). Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to cultures with no FBS; ##, $p < 0.01$, #, $p < 0.05$ compared to cultures with 5% FBS).

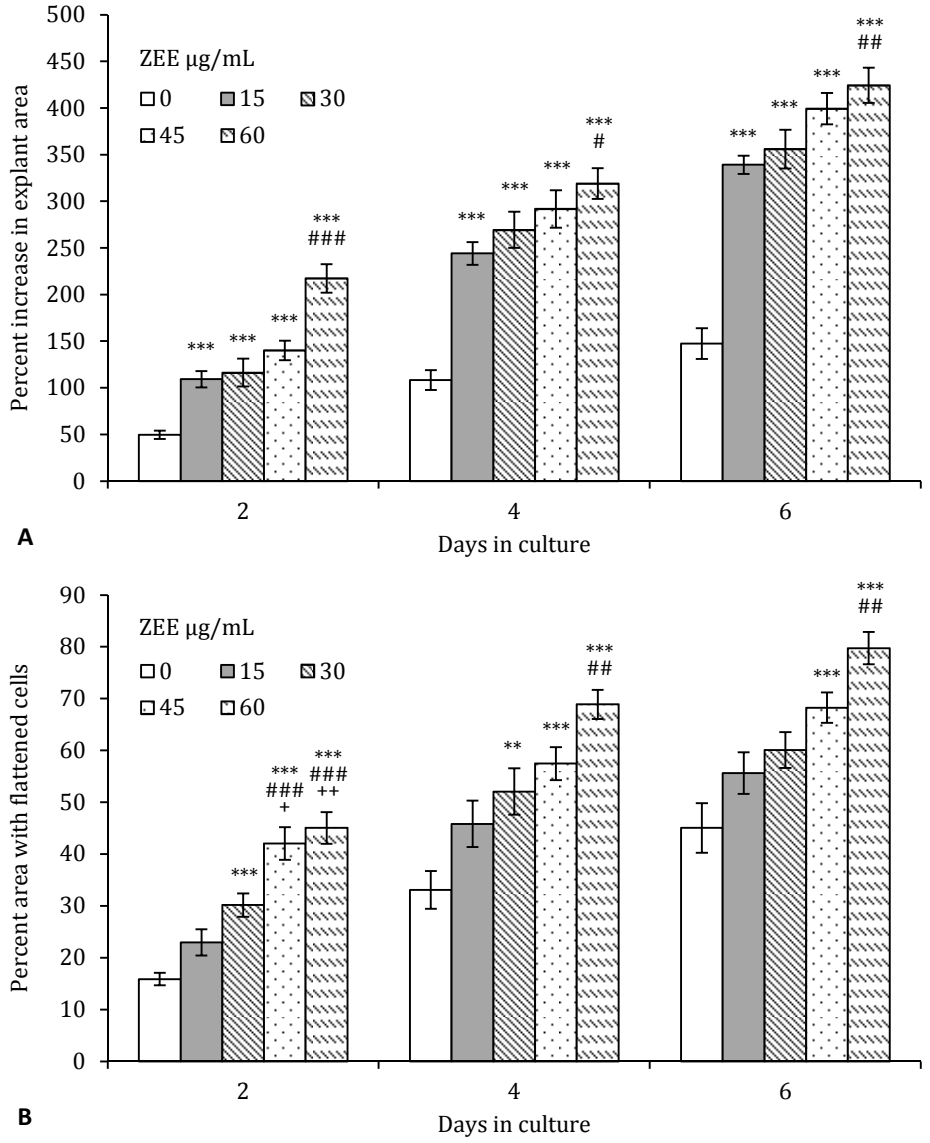

Figure 4: Effect of different zebrafish embryo extract (ZEE) concentration in medium on zebrafish blastocyst explant culture. (**A**) Percentage increase in relative area covered by cells around the explant compare to the initial area of explant (see equation 2 in Statistical analysis). (**B**) Percentage of area covered by flattening cells (area marked with number 3 in Figure 2; see also equation 3 in Statistical analysis). Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, compared to cultures with no ZEE; ###, $p < 0.001$, ##, $p < 0.01$, #, $p < 0.05$ compared to cultures with 15 µg/mL ZEE; ++, $p < 0.01$, +, $p < 0.05$ compared to cultures with 30 µg/mL ZEE).

Effect of zebrafish embryo extract on blastocyst explant culture

ZEE increases area covered by cells around blastocyst explant

Similar to FBS, ZEE also showed significant effect on the expansion in area covered by the emigrating cells from the blastocyst explants (Figure 4A). The increase in the area covered by cells around the explant was higher on day 2 in cultures with 15 μg/mL ZEE compared to cultures without ZEE in the medium. More expansion of the fibroblast-like cells around the explant was observed in cultures supplemented with 60 μg/mL compared to 15 μg/mL ZEE (Figure 4A). No significant differences between 15, 30 and 45 μg/mL ZEE concentrations was observed on the expansion of the cultures. Without ZEE in the medium the area of the explant remained similar from day 2 to day 6. In the presence of ZEE (15-60 μg/mL), significant increase was observed in the explant area from day 2 to day 6 ($p<0.001$).

Percent area covered by flattened cells is increased by ZEE

The area covered by the flattened cells, as a percentage of the total area covered by the explant (including the fibroblastic cells), was higher with 30-60 μg/mL ZEE concentration, compared to no ZEE in the medium (Figure 4B). Cultures maintained with 60 μg/mL ZEE showed higher percentage of area covered by fibroblast-like cells compared to cultures with 15 μg/mL ZEE. No significant differences were observed between cultures with zero and 15 μg/mL ZEE. The percent area covered by fibroblast-like cells increased significantly from day 2 to day 4 under all ZEE conditions ($p<0.01$). However, after day 4 to day 6 a significant increase was observed only with 60 μg/mL ZEE ($p<0.05$).

Trypsinized blastocyst primary cell culture

The trypsinized blastocyst cells formed aggregates known as EBs within the first 24 h of culture and then the elongated, fibroblast-like cells started to emigrate on the culture substratum as a monolayer from these EBs (Figure 5A-E). The primary cultures predominantly contained two types of cells: (i) flattened, elongated, fibroblast-like cells; (ii) round, EB cells. At this stage other cell types such as neuron-like (long thread-like) cells, and melanocytes (pigment cells; Figure 5F) were also visible in the EBs. The appearance of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells in the EBs was also observed in cultures (Figure 5B). By day 4 both the EBs and the proliferating fibroblast like cells contained cells expressing *fli:GFP* or *kdrl:GFP* marker (Figure 5C and G).

Expression of *fli:GFP*$^{+}$ cells in blastocyst primary cell culture

The *fli:GFP* blastocyst cell culture showed the *fli:GFP* expression in cells in culture medium without supplementation of endothelial/hematopoietic growth factors (Figure 5B-E). By day 2 of culture the *fli:GFP*$^{+}$ cells were observed on the periphery of the EBs (Figure 5B).

The percentage of *fli:GFP*$^{+}$ cells in cultures without surface coating (on tissue culture treated polystyrene), increased significantly from day 2 to day 4 ($p<0.001$) and then declined at the same rate from day 4 to day 6 ($p<0.01$; Figure 6B). The percentage of *fli:GFP*$^{+}$ cells was smallest in cells isolated from cultures on day 8. In contrast to polystyrene substratum, in cultures on gelatin-coated substratum the percentage of *fli:GFP*$^{+}$ cells remained unchanged overtime. On day 4 the percentage of *fli:GFP*$^{+}$ cells was significantly higher on polystyrene compared to gelatin substratum.

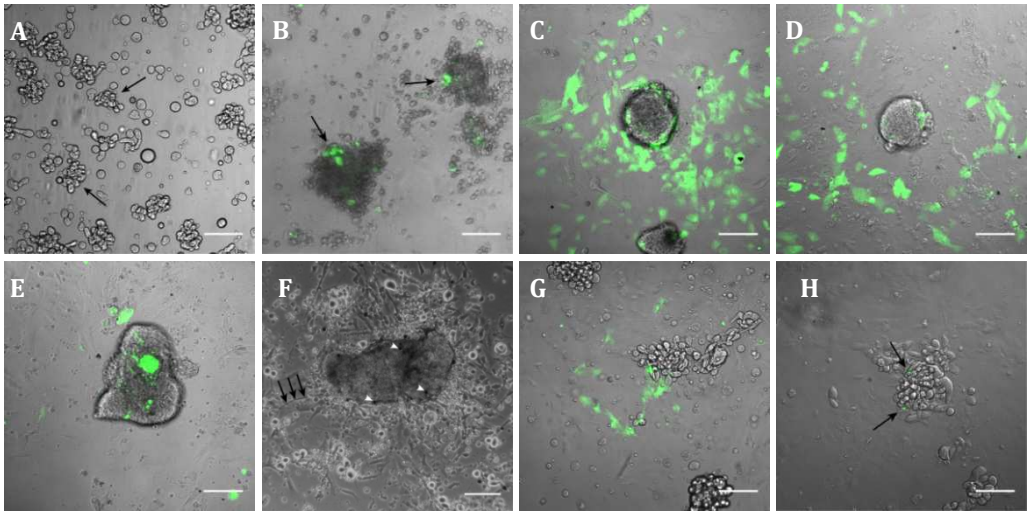

Figure 5: Culture of trypsinized blastocyst cells isolated from transgenic zebrafish embryos (B-E *fli:GFP*; G,H *kdrl:GFP*). (**A**) Image 1 h after cell plating, arrows showing the cells attaching each other to form aggregates. (**B**) Within the initial 24 h of culture the cells combine to form large aggregates called embryoid bodies (EBs). Arrows showing the appearance of *fli:GFP*$^{+}$ cells on the periphery of EBs. (**C**) More *fli:GFP*$^{+}$ cells appear and proliferate around the EBs by day 4 of culture. (**D**). By day 6 of culture the cells expressing GFP reduces. (**E**) Cells expressing *fli:GFP*$^{+}$ marker almost diminishes by day 8 of culture, while the non-GFP, fibroblast-like cell continue to grow. (**F**) phase contrast image of day 6 EB showing the extensions of fibroblast like cells (white arrow heads showing melanocytes; arrows showing neuron-like cell). (**G**) *kdrl:GFP* blastocyst cell culture at day 4 of culture with numerous *kdrl:GFP*$^{+}$ cells. (**H**) *kdrl:GFP* blastocyst cell culture at day 6 of culture. The *kdrl:GFP*$^{+}$ cells are completely disappeared on day 6 except few (arrows). Scale bar, 100 µm.

Expression of *kdrl:GFP*$^{+}$ cells in blastocyst primary cell culture

As with the *fli:GFP* cultures, the *kdrl:GFP*$^{+}$ cells were distributed at the periphery of the EB (Figure 5G). However, the overall percentage of *kdrl:GFP* $^{+}$ cells in cultures (1.8 ± 0.2%) was less than *fli:GFP*$^{+}$ cells (6.1 ± 0.4%). The percentage of *kdrl:GFP*$^{+}$ cells in blastocyst cell cultures was found to be 2.6 ± 0.3% on day 2 of culture (Figure 6C). The cells isolated from cultures had a rounded morphology and were readily counted without any need for nuclear staining (Figure 6A). No significant increase in the percentage of *kdrl:GFP*$^{+}$ cells was observed between days 2 and 4. Similar to *fli:GFP* cell cultures the percentage of *kdrl:GFP*$^{+}$ cells in the blastocyst cell cultures decreased significantly from day 4 to day 6

(p<0.001) and then remained the same until day 8. No significant differences were observed in the percentage of *kdrl:GFP*+ cells between cultures on polystyrene or gelatin.

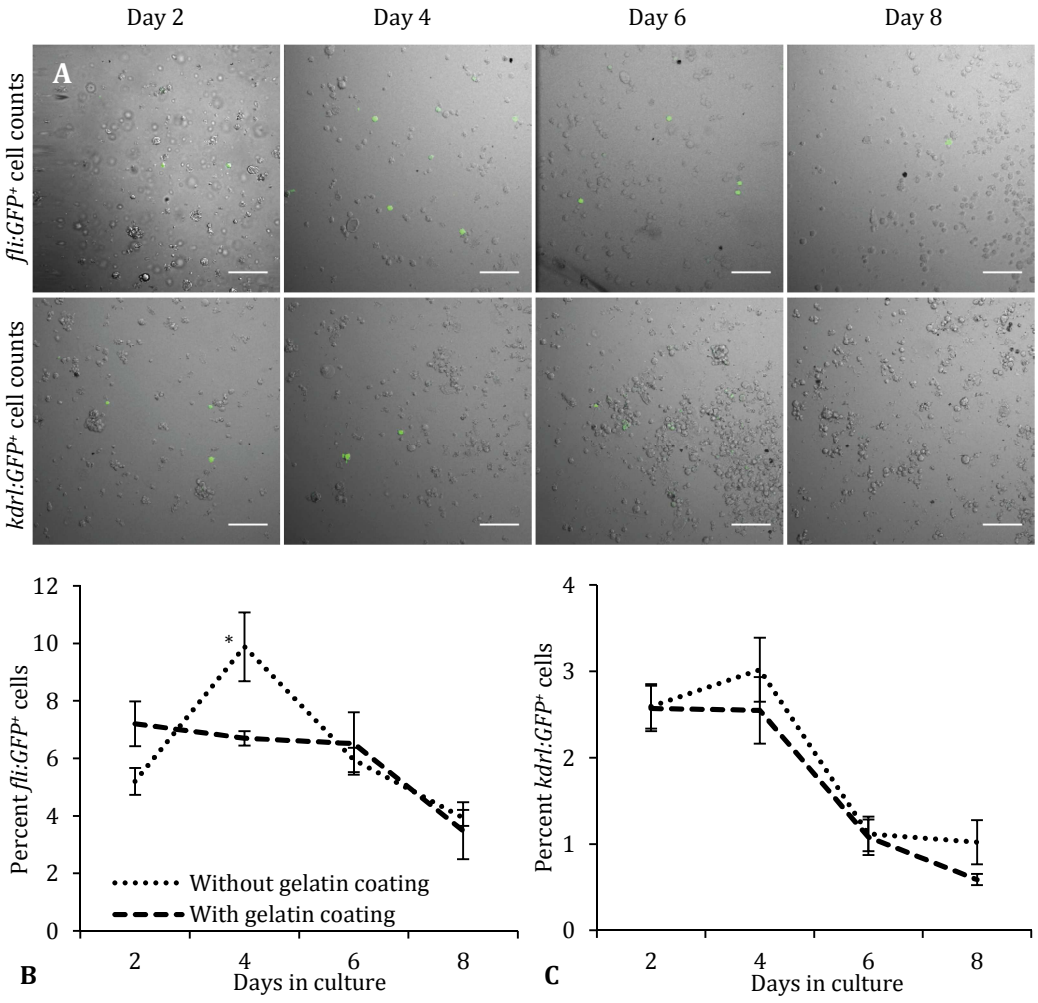

Figure 6: Percentage of *fli:GFP*+ and *kdrl:GFP*+ cells in cells isolated from blastocyst cell culture in LDF medium with 15% FBS. (**A**) Overlaid confocal and phase contrast images of cell isolates from cultures of different ages (days 2-8) for counting of *fli:GFP*+ or *kdrl:GFP*+ cells (cells illuminating green fluorescence) as a percentage of total cells (round non-fluorescence) in a microscopic field.. Scale bar, 100 μm. (**B**) Change in number over time of *fli:GFP*+ cells as a percentage of total cells sampled by trypsinization of cultures. (**C**) Change in number over time of *kdrl:GFP*+ cells as a percentage of total cells sampled by trypsinization of cultures (see equation 4 for B and C). Error bars represent standard error. (*, $p < 0.05$).

Blastocysts secondary cell culture

Subsequent sub-culturing of the primary blastocyst cells eliminated other cell types including the *fli:GFP*+ and *kdrl:GFP*+ cells, leaving a homogeneous population of fibroblast-like cells which expressed the fibroblast marker vimentin in some cells (Figure 7 B-D). The

primary cultures grow slower and took 21 days to reach sub-confluence. After passaging, the secondary cells showed a faster growth compared to primary cultures and reach to sub-confluence within seven days.

The population doubling time (PDT) was longer for primary cells then for the passaged cells (Figure 8A); however, the difference was not significant. Subsequent passaging did not have much effect on PDT. The time required for the cells to become sub-confluent was longer in primary cultures (3 weeks). The secondary cultures were sub-confluent within the first week. The viability of cells, as measured by trypan blue exclusion, was lower in primary cell culture (75.2 ± 4.2%) than in secondary culture (85 - 96%; Figure 8B). No significant differences were observed in the viability of primary and secondary cells up to six passages. However after passage seven the viability of cells was significantly higher than that of the primary cells ($p<0.01$). The fibroblast-like cells at passage 8 were used to determine the FBS, ZEE and gelatin concentrations for optimal growth of zebrafish embryonic cells in secondary culture.

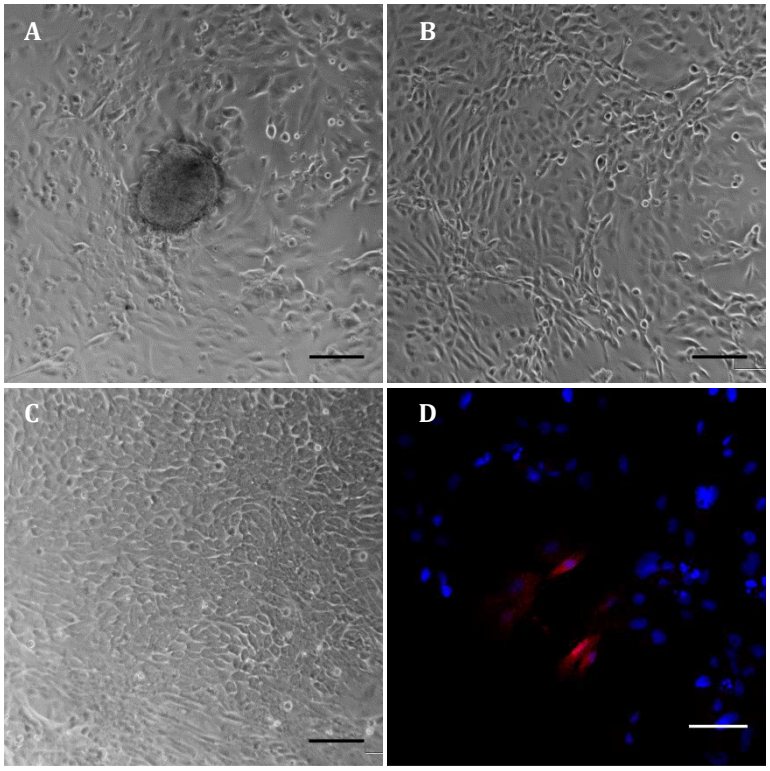

Figure 7: Zebrafish blastocyst cell culture at subsequent passages. (**A**) primary blastocyst cell culture (day 20), the flattened fibroblast-like cells grow around the periphery of EB. (**B**) Passage 1 (day 6), cells with similar elongated fibroblast-like morphology retain in culture. (**C**) passage 2 (day 6), Almost all cells have similar fibroblast-like morphology. (**D**) Cells at passage eight stained with fibroblast marker anti-vimentin (red), nuclei stained with DAPI (blue). Scale bar, A-C, 100 µm; D, 50 µm.

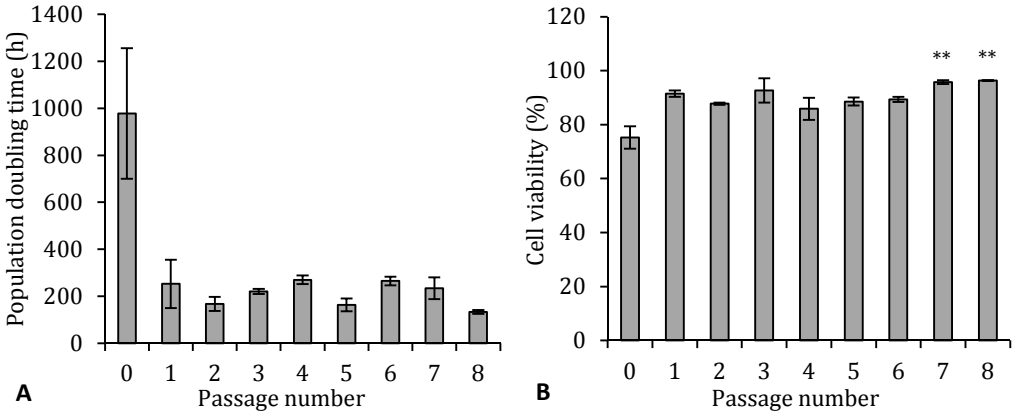

Figure 8: Growth curve of zebrafish blastocyst cells at subsequent passages. (**A**) Population doubling time (PDT) in hours based on heamocytometer counts of total number of cells harvested (see equation 7). (**B**) Viability of cells (as measured with trypan blue exclusion; see equation 5) at subsequent passages. Error bars represent standard error. (**, $p < 0.01$ compared to passage 0).

FBS and ZEE effect on secondary cell culture

With increasing concentrations of FBS, there was a significant increase in the proliferation of zebrafish embryonic secondary cell cultures. The total number of cells harvested per well, cultured for seven days in media with different FBS concentrations, was lowest in cultures with 5% FBS (Figure 9A). The number of cells harvested per well was significantly higher in 15% FBS compared to 5% FBS ($P<0.01$). Cell counts from cultures maintained with 10% FBS were intermediate between cell counts from cultures with 5 and 15% FBS. No significant difference in the number of cells harvested per well, was observed between cultures with 15 and 20% FBS supplementation.

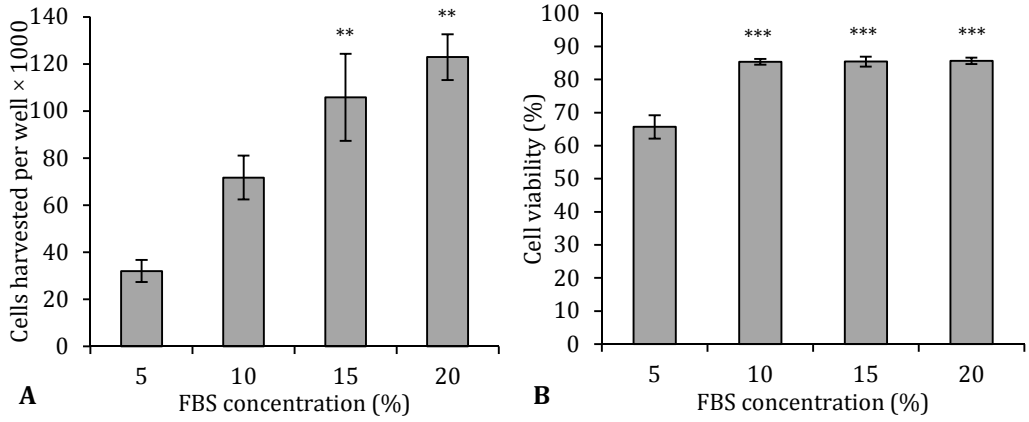

Figure 9: Effect of FBS concentration on secondary blastocyst cell culture. (**A**) Total number of cells harvested per well, from cultures maintained at different FBS concentrations. (**B**) Viability of isolated cells (trypan blue exclusion). Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$ compared to cultures maintained at 5% FBS).

The viability of cells, measured after isolation from culture by trypsinization, also varied with FBS concentration (Figure 9B). Lower cell viability was observed in cultures with 5% FBS supplementation compared to higher FBS concentrations ($P<0.001$). No significant differences were observed in the viability of cells cultured with 10, 15 and 20% FBS.

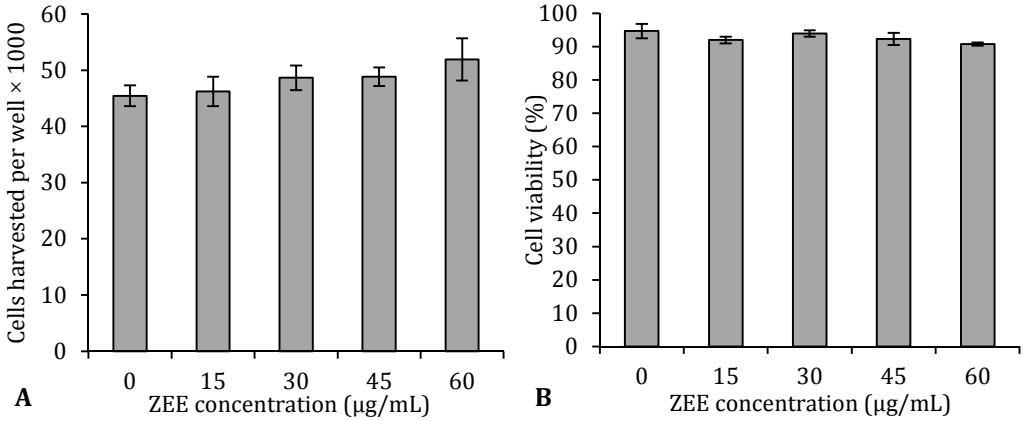

Figure 10: Total counts and viability of secondary blastocyst cell culture maintained for 7 days at different concentrations of zebrafish embryo extract. (**A**) Total number of cells harvested per well of a 96 well plate. (**B**) Viability of the isolated cells (trypan blue exclusion). Error bars represent standard error.

Different concentrations of ZEE showed no significant effect on cell counts or viability of blastocyst secondary cell culture. The total number of cells harvested per well, after seven days of culture in different ZEE concentrations, was 48213.7 ± 1128.9 on average. Cell counts were similar in all wells regardless of the ZEE concentration (range: 0-60 µg/mL; Figure 10A). The viability of the cell isolates were also similar between cultures maintained with different concentrations of ZEE (Figure 10B).

Effect of gelatin substratum on secondary cell culture

In this experiment, an increase in the concentration of gelatin substratum was correlated with a decrease in the total number of cells harvested per well (Figure 11A). Significantly higher numbers of cells were recovered from cells cultured on 0.03 mg/cm^2 of gelatin compared to higher gelatin concentrations (0.12, 0.24 and 0.48 mg/cm^2). No significant differences in total cell counts were observed between cells cultured without gelatin and with different concentrations of gelatin substratum. Higher gelatin concentrations also reduced the viability of the cells (Figure 11B). Viability of the cells cultured on polystyrene substrate ranged from 91 to 97%, which was significantly higher than the viability of cells cultured on 0.24 and 0.48mg/cm^2 gelatin substrate. The viability of cells cultured on lower gelatin concentrations (0.03 and 0.06mg/cm^2) was also higher than the viability of cells cultured on 0.48mg/cm^2 of gelatin.

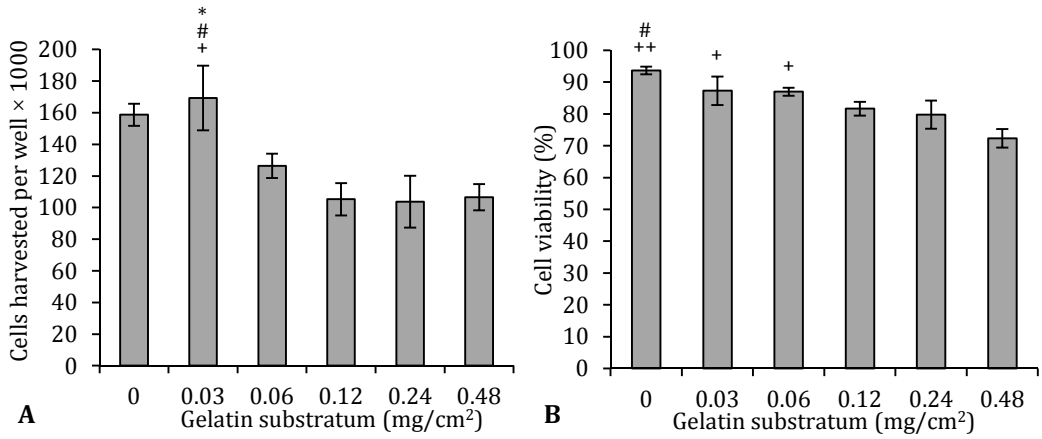

Figure 11: Effect of different concentration of gelatin substratum on growth and viability of secondary blastocyst cell culture. (**A**) Total number of cells harvested per well on day 7 of culture. (**B**) Viability of isolated cells. Error bars represent standard error. (*, $p < 0.05$ compared to cultures on 0.12 mg/cm^2 gelatin; #, $p < 0.05$ compared to 0.24 mg/cm^2 gelatin; ++, $p < 0.01$, +, $p < 0.05$ compared to 0.48 mg/cm^2 gelatin).

Discussion

Zebrafish blastocyst stage embryonic cells are usually used to develop ESC cultures, that have the potential to differentiate into a specific cell line [7]. These cells have been used to establish zebrafish embryonic fibroblast [19], embryonic neurons [10] and embryonic muscle cell lines [12]. Methods for culturing cells from zebrafish embryos are still developing. Depending on the research question, different conditions have been used for the culture of zebrafish embryonic cells (Table 1). Zebrafish embryonic cells are usually cultured on a feeder monolayer of growth-arrested cells [21, 22] or a gel substratum [10, 12, 20, 27]. In this study, we described a procedure for initiating zebrafish embryonic cell culture, using blastocyst stage embryos, without any feeder layer or gel substrate, an approach which is similar to that previously reported (Ref. [17, 19]). The media composition and culture procedures we used were modified from those used for zebrafish embryonic cell culture by Fan and Collodi [21].

Zebrafish blastocyst explant culture

Blastocyst explant cultures are usually performed to understand the early developmental and differentiation processes in zebrafish embryos [30, 41]. Historically, mouse embryonic blastocyst explants were used to develop pluripotent ESC cultures [42]. Similarly, cell lines can be developed using embryonic explants, such as mouse blastocyst cultures for the development of skeletal muscle cell line [43].

In this study, we analysed the emergence of fibroblast-like cells from zebrafish blastocyst explant cultures in media having different concentrations of FBS and ZEE. Higher

concentrations of FBS (15-20%) and ZEE (45-60µg/mL) were found to be optimum for zebrafish primary explant culture. Thus, at the concentrations indicated of FBS and ZEE, the fibroblast-like cells expanded to cover significantly more area at the periphery of the explant. We also noted that the fibroblast-like cells can be repeatedly sub-cultured. The number of blastocyst fragments (explants) per well decreased gradually overtime, because neighboring explants often fuse to form larger aggregates.

Blastocyst cell culture: *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cell differentiation

At the blastocyst stage, a zebrafish embryo consists of pluripotent cells [44] that can be induced to differentiate into a specific cell type, e.g. neurons, astrocytes [16] and myocytes [12], by the relevant *in vitro* manipulation. When the pluripotent blastocyst cells are cultured on a growth-arrested feeder cell layer, they retain their pluripotency [7]. When cultured in the absence of specific differentiation factors or feeder layer, blastocyst-derived cells differentiate spontaneously into EBs, composed of different cell types found in the early embryo [2]. The EBs formed in primary embryonic cell culture in our experiments contained different cell types including melanocytes, neuron-like cells, *fli:GFP*$^{+}$ cells, *kdrl:GFP*$^{+}$ cells and fibroblast-like cells. Similar populations of cells have been reported in EBs formed in mouse embryonic cell culture [2].

Studies have shown higher concentration of hematopoietic cells compared to other cell types in mouse EBs [45, 46]. This would be consistent with our finding of higher percentages of *fli:GFP*$^{+}$ cells compared to *kdrl:GFP*$^{+}$ cells in the blastocyst cell cultures. However, it should be remembered that *fli:GFP* include but is not specific for hematopoietic cells [23]. Culture substrata coated with different matrix-derived molecules have also been reported to increase the gene expression of specific cell types in human ESC cultures [47]. In that study, the EBs derived from human ESCs higher levels of neural and endodermal genes on different substrates (laminin and fibronectin, respectively) [47]. Zebrafish ESCs have also been reported to differentiate into fibroblast, neuron and epithelial cells on gelatin substrata [27], and into myocytes on laminin substrata [12]. Similarly, gelatin and collagen substrates have been used for differentiation of mouse ESCs into endothelial cells [48, 49]. In our experiments, in contrast to polystyrene substratum, the percentage of *fli:GFP*$^{+}$ cells was stable in cultures overtime on gelatin substratum.

Blastocyst secondary cell culture

In order to obtain a continuous cell line, zebrafish embryonic cells are cultured on a feeder layer of growth-arrested cells or on gelatin-coated culture dishes [50]. In this study, we established zebrafish embryonic cell cultures, with EB formation, on a tissue culture treated polystyrene surface without any feeder layer or gel coating. A similar strategy has previously been used for development of a fibroblast-like cell line from zebrafish embryonic cell culture [17]. The primary embryonic cells in our culture setup were slow

growing, being characterized by longer population doubling times, and lower cell viability. This may be because of the considerable amount of unattached cells observed in the primary cultures, which were washed away while refreshing the media.

Another reason for the slower growth rate of zebrafish primary cell cultures, could be the occurrence of cellular differentiation, because different cell types have different growth requirements [17]. In the present study, the most abundant fibroblast-like cells developed into a monolayer on the surface of the culture dish when passaged. The secondary cells, we obtained after passaging the primary blastocyst embryonic cells, were fast growing, mostly homogeneous and were similar in morphology to the fibroblast-like cell line derived from zebrafish embryos in ref. [17]. The mouse anti-vimentin antibody that we used to stain fibroblasts in the blastocyst secondary culture, has been previously reported to detect fibroblasts in zebrafish [39]. Similar cultures derived from zebrafish embryonic cells after several passages were referred to as a subpopulation of the primary cells that suited best or they have adapted themselves to the culture conditions [18].

Effect of FBS on blastocyst secondary cell culture

FBS is the most widely used supplement for cell cultures including zebrafish cell culture [21]. It contains growth factors, hormones, amino acids, vitamins, trace minerals as well as cell attachment, proliferating and binding factors [51]. In our study, the presence of higher than 15% FBS significantly increased the area covered by proliferating cells around the explant overtime. Similar FBS concentration (15%) have been used previously for zebrafish embryonic cell cultures [20, 21]. Secondary zebrafish embryonic cells we obtained after passaging the primary cells also showed higher growth rate with increasing FBS concentration. For these secondary cells, the use of 10% FBS in the medium was optimum, in our hands, for the growth of cultures. Similar to these findings, total cell counts of zebrafish embryonic cell lines (Z428 and ZEB2J) increased with increasing FBS concentrations in the culture media [52, 53]. Contrary to these findings, in early studies [18, 54], higher FBS concentrations were shown to inhibit the growth of zebrafish ESC culture. However, similar to our results, omitting FBS from the media significantly reduced the growth of these cells [18].

Effect of zebrafish embryo extract on blastocyst secondary cell culture

Fish embryo extract has been shown to support the attachment and growth of medaka [50, 55] and turbot [56] ESCs. Zebrafish embryo extract is particularly important for the growth of some of the zebrafish cell lines, e.g. in the case of zebrafish caudal fin cells [18]. However, this is not critical for most of the zebrafish cells cultures (Table 1). In our study, the emigrating cells around the blastocyst explants covered significantly more area in media supplemented with 60 μg ZEE protein per mL medium compared to lower ZEE concentrations. Similar ZEE concentrations have been used previously for zebrafish

embryonic cell cultures [20, 21]. Different concentrations of ZEE did not have significant effects on the growth rate and viability of the zebrafish secondary embryonic cells in our experiments. These results recommend the use of ZEE for zebrafish primary embryonic cell cultures. However, for secondary cell cultures ZEE is not important for population growth. Previous studies have shown significant mitogenic effect of medaka fish embryo extract on the zebrafish embryonic cell line ZF428 [52].

Effect of gelatin substratum on blastocyst secondary cell culture

Gelatin coating has been used as an alternative to a feeder cell layer for medaka [50], turbot [56] and zebrafish [52] embryonic cell cultures. In the current study, we cultured the zebrafish secondary embryonic cells on different concentrations of gelatin substrate. At higher gelatin concentrations lower number of cells were harvested, although the number was not significantly different compared to cells cultured without gelatin substrate. Cell viability was significantly higher in cultures without gelatin coating compared to cultures on 0.24 and 0.48 mg/cm^2 gelatin. Cells cultured on a range of gelatin substrates up to 0.12 mg/cm^2, or cultured without gelatin, had similar growth rates and viability. These results suggest that gelatin concentrations up to 0.12 mg/cm^2 may be used to coat the culture dish for the attachment and proliferation of zebrafish cells. Similar gelatin concentrations have been previously used for zebrafish cell culture [52]. A reason for lower cell growth and viability on high gelatin concentrations might because of the change in substrate after growing the cells for several passages on a polystyrene surface of the tissue culture flask without gelatin coating.

Conclusions

Zebrafish blastocyst cells differentiate *in vitro* into cell types including vimentin$^+$ fibroblastic cells, *kdrl:GFP*$^+$ cells, and *fli:GFP*$^+$ cells. However, in the absence of a selective medium, the latter two cell types decline in abundance over time in the primary cultures, and disappear with subsequent passaging. Only the fast growing fibroblast-like cells remain in the cultures. Zebrafish primary embryonic cells require a high FBS concentration (15%) in medium, while secondary fibroblast-like cell grow at a lower (10%) FBS concentrations. ZEE at a total protein concentration of 45-60 µg/mL medium is important for the maintenance of primary cell cultures. However, ZEE does not affect the growth of the zebrafish secondary cells. Gelatin can be used at low concentrations (0.03-0.12 mg/cm^2) as a substratum for zebrafish blastocyst cell culture.

References

[1] Bethesda MD. What are embryonic stem cells? In Stem Cell Information, 2015; Available from: http://stemcells.nih.gov/info/basics/pages/basics3.aspx.

[2] Keller GM. *In vitro* differentiation of embryonic stem cells. Curr Opin Cell Biol 1995; 7(6): 862-9.

[3] Evans M. Discovering pluripotency: 30 years of mouse embryonic stem cells. Nat Rev Mol Cell Biol 2011; 12(10): 680-6.

[4] Koh S, Piedrahita JA. From "ES-like" cells to induced pluripotent stem cells: a historical perspective in domestic animals. Theriogenology 2014; 81(1): 103-11.

[5] Tachibana M, Amato P, Sparman M, Gutierrez NM, Tippner-Hedges R, Ma H*, et al.* Human embryonic stem cells derived by somatic cell nuclear transfer. Cell 2013; 153(6): 1228-38.

[6] Festing S, Wilkinson R. The ethics of animal research. Talking Point on the use of animals in scientific research. EMBO Rep 2007; 8(6): 526-30.

[7] Alvarez MC, Bejar J, Chen S, Hong Y. Fish ES cells and applications to biotechnology. Mar Biotechnol (NY) 2007; 9(2): 117-27.

[8] Barut BA, Zon LI. Realizing the potential of zebrafish as a model for human disease. Physiological Genomics 2000; 2(2): 49-51.

[9] Fan L, Crodian J, Collodi P. Culture of embryonic stem cell lines from zebrafish. Methods Cell Biol 2004; 76: 151-60.

[10] Chen Z, Lee H, Henle SJ, Cheever TR, Ekker SC, Henley JR. Primary neuron culture for nerve growth and axon guidance studies in zebrafish (*Danio rerio*). PLoS One 2013; 8(3): e57539.

[11] Fan L, Crodian J, Liu X, Alestrom A, Alestrom P, Collodi P. Zebrafish embryo cells remain pluripotent and germ-line competent for multiple passages in culture. Zebrafish 2004; 1(1): 21-6.

[12] Myhre JL, Pilgrim DB. Cellular differentiation in primary cell cultures from single zebrafish embryos as a model for the study of myogenesis. Zebrafish 2010; 7(3): 255-66.

[13] Paw BH, Zon LI. Primary fibroblast cell culture. Methods Cell Biol 1999; 59: 39-43.

[14] Xing JG, El-Sweisi W, Lee LE, Collodi P, Seymour C, Mothersill C*, et al.* Development of a zebrafish spleen cell line, ZSSJ, and its growth arrest by gamma irradiation and capacity to act as feeder cells. In Vitro Cell Dev Biol Anim 2009; 45(3-4): 163-74.

[15] Sander V, Sune G, Jopling C, Morera C, Izpisua Belmonte JC. Isolation and *in vitro* culture of primary cardiomyocytes from adult zebrafish hearts. Nat Protoc 2013; 8(4): 800-9.

[16] Ghosh C, Liu Y, Ma CG, Collodi P. Cell cultures derived from early zebrafish embryos differentiate *in vitro* into neurons and astrocytes. Cytotechnology 1997; 23(1-3): 221-230.

[17] Choorapoikayil S, Overvoorde J, den Hertog J. Deriving cell lines from zebrafish embryos and tumors. Zebrafish 2013; 10(3): 316-25.

[18] Collodi P, Kamei Y, Ernst T, Miranda C, Buhler DR, Barnes DW. Culture of cells from zebrafish (*Brachydanio rerio*) embryo and adult tissues. Cell Biol Toxicol 1992; 8(1): 43-61.

[19] He S, Salas-Vidal E, Rueb S, Krens SF, Meijer AH, Snaar-Jagalska BE*, et al.* Genetic and transcriptome characterization of model zebrafish cell lines. Zebrafish 2006; 3(4): 441-53.

[20] Badakov R, Jazwinska A. Efficient transfection of primary zebrafish fibroblasts by nucleofection. Cytotechnology 2006; 51(2): 105-10.
[21] Fan L, Collodi P. Zebrafish embryonic stem cells. Methods Enzymol 2006; 418: 64-77.
[22] Ma C, Fan L, Ganassin R, Bols N, Collodi P. Production of zebrafish germ-line chimeras from embryo cell cultures. Proc Natl Acad Sci U S A 2001; 98(5): 2461-6.
[23] Lawson ND, Weinstein BM. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 2002; 248(2): 307-318.
[24] Renaud O, Herbomel P, Kissa K. Studying cell behavior in whole zebrafish embryos by confocal live imaging: application to hematopoietic stem cells. Nat Protoc 2011; 6(12): 1897-1904.
[25] Jin SW, Beis D, Mitchell T, Chen JN, Stainier DY. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. Development 2005; 132(23): 5199-209.
[26] Melet F, Motro B, Rossi DJ, Zhang L, Bernstein A. Generation of a novel Fli-1 protein by gene targeting leads to a defect in thymus development and a delay in Friend virus-induced erythroleukemia. Mol Cell Biol 1996; 16(6): 2708-18.
[27] Ho SY, Goh CW, Gan JY, Lee YS, Lam MK, Hong N*, et al.* Derivation and long-term culture of an embryonic stem cell-like line from zebrafish blastomeres under feeder-free condition. Zebrafish 2014; 11(5): 407-20.
[28] Thompson MA, Ransom DG, Pratt SJ, MacLennan H, Kieran MW, Detrich HW, 3rd*, et al.* The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. Dev Biol 1998; 197(2): 248-69.
[29] Bussmann J, Lawson N, Zon L, Schulte-Merker S, Committee ZN. Zebrafish VEGF receptors: a guideline to nomenclature. PLoS Genet 2008; 4(5): e1000064.
[30] Grinblat Y, Lane ME, Sagerstrom C, Sive H. Analysis of zebrafish development using explant culture assays. Methods Cell Biol 1999; 59: 127-56.
[31] Edgar LG, Goldstein B. Culture and manipulation of embryonic cells. Methods Cell Biol 2012; 107: 151-75.
[32] Sweet H, Amemiya S, Ransick A, Minokawa T, McClay DR, Wikramanayake A*, et al.* Blastomere isolation and transplantation. Methods Cell Biol 2004; 74: 243-71.
[33] Schmidt JV. Embryonic stem (ES) cell culture basics. Curr Protoc Toxicol 2001; Chapter 15: Unit15 1.
[34] Sun L, Bradford CS, Ghosh C, Collodi P, Barnes DW. ES-like cell cultures derived from early zebrafish embryos. Mol Mar Biol Biotechnol 1995; 4(3): 193-9.
[35] Experiments on Animal Act, 2014. The State Secretary of Health and Environmental Protection, Soestdijk. http://wetten.overheid.nl/BWBR0003081/2014-12-18
[36] Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes Text with EEA relevance, 2010. The European Parliament and the council of the European Union, Official Journal of the European Union. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:32010L0063
[37] Westerfield M. The zebrafish book: a guide for the laboratory use of zebrafish (*Danio Rerio*). 4 ed, University of Oregon Press, Eugene 2000, pp.
[38] Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995; 203(3): 253-310.
[39] Chablais F, Veit J, Rainer G, Jazwinska A. The zebrafish heart regenerates after cryoinjury-induced myocardial infarction. BMC Dev Biol 2011; 11: 21.

[40] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012; 9(7): 671-5.
[41] Sagerstrom CG, Grinbalt Y, Sive H. Anteroposterior patterning in the zebrafish, *Danio rerio*: an explant assay reveals inductive and suppressive cell interactions. Development 1996; 122(6): 1873-83.
[42] Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981; 292(5819): 154-6.
[43] Smith J, Merrick D. Embryonic skeletal muscle microexplant culture and isolation of skeletal muscle stem cells. Methods Mol Biol 2010; 633: 29-56.
[44] Kimmel CB, Warga RM. Tissue-specific cell lineages originate in the gastrula of the zebrafish. Science 1986; 231(4736): 365-8.
[45] Keller G, Kennedy M, Papayannopoulou T, Wiles MV. Hematopoietic commitment during embryonic stem cell differentiation in culture. Mol Cell Biol 1993; 13(1): 473-86.
[46] Wang L, Cerdan C, Menendez P, Bhatia M. Derivation and characterization of hematopoietic cells from human embryonic stem cells. Methods Mol Biol 2006; 331: 179-200.
[47] Laperle A, Masters KS, Palecek SP. Influence of substrate composition on human embryonic stem cell differentiation and extracellular matrix production in embryoid bodies. Biotechnol Prog 2014.
[48] Blancas AA, Lauer NE, McCloskey KE. Endothelial differentiation of embryonic stem cells. Curr Protoc Stem Cell Biol 2008; Chapter 1: Unit 1F 5.
[49] Liu Z, Lebrin F, Maring JA, van den Driesche S, van der Brink S, van Dinther M*, et al.* ENDOGLIN is dispensable for vasculogenesis, but required for vascular endothelial growth factor-induced angiogenesis. PLoS One 2014; 9(1): e86273.
[50] Hong N, Li Z, Hong Y. Fish stem cell cultures. Int J Biol Sci 2011; 7(4): 392-402.
[51] Gstraunthaler G. Alternatives to the use of fetal bovine serum: serum-free cell culture. ALTEX 2003; 20(4): 275-81.
[52] Hong N, Schartl M, Hong Y. Derivation of stable zebrafish ES-like cells in feeder-free culture. Cell Tissue Res 2014; 357(3): 623-32.
[53] Xing JG, Lee LEJ, Fan LC, Collodi P, Holt SE, Bols NC. Initiation of a zebrafish blastula cell line on rainbow trout stromal cells and subsequent development under feeder-free conditions into a cell line, ZEB2J. Zebrafish 2008; 5(1): 49-63.
[54] Ghosh C, Collodi P. Culture of cells from zebrafish (*Brachydanio rerio*) blastula-stage embryos. Cytotechnology 1994; 14(1): 21-6.
[55] Hong Y, Schartl M. Isolation and differentiation of medaka embryonic stem cells. Methods Mol Biol 2006; 329: 3-16.
[56] Holen E, Hamre K. Towards obtaining long term embryonic stem cell like cultures from a marine flatfish, Scophtalmus maximus. Fish Physiol Biochem 2003; 29(3): 245-252.