Development of an in vitro vascular network using zebrafish embryonic cells

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Chapter 4

Influence of medium composition and substratum on the growth of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cell culture

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Abstract

Embryonic stem cells (ESCs) are important tools to study lineage commitment and cellular differentiation in early embryos. The differentiation of ESCs can be directed towards a specific cell type by culturing them in appropriate conditions. In this study we analysed the effect of different media formulations and culture substrates on the quantification of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cell cultures. These cultures were derived from transgenic zebrafish (*fli:GFP* and *kdrl:GFP*) lines which express green fluorescent protein under the *fli1* and VEGFR2 promoters, respectively. The different media compositions used to culture the blastocyst cells were (i) LDF medium (a combination of Leibowitz's L-15, Dulbecco's modified Eagle's and Ham's F12 media); (ii) combined LDF and endothelial growth medium (LDF:EGM); and (iii) LDF medium supplemented with endothelial growth supplement mix (LDF:EGS). The blastocyst cells formed embryoid body aggregates within the first 24 h of culture containing *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells. The percentage of *fli:GFP*$^{+}$ cells was higher in cultures compared to *kdrl:GFP*$^{+}$ cells. This is presumably because the *kdrl:GFP* line is specific for endothelial cells, while the *fli:GFP* is expressed in multiple cell types. On day 6 of cultures, higher percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells were observed in LDF:EGS medium (31.8 ± 1.6% and 3.7 ± 0.4%, respectively) compared to LDF medium (21.6 ± 1.9% and 2.1 ± 0.2%, respectively). In LDF:EGM medium the percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells was comparable to that in LDF:EGS medium; however, the total cell yield was significantly less in LDF:EGM cultures. The percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells was higher in cultures on collagen type-I substratum compared to gelatin substratum. Recombinant zebrafish vascular endothelial growth factor protein was also found to increase the concentration of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures. Both *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells were no longer detectable in cultures after 14 days and eight days, respectively. Possible reasons for this may be the down regulation of the transgenes, change in endothelial identity and occurrence of cell death.

Introduction

Embryonic stem cells (ESCs) are cells, derived from blastocyst embryos, that have not started to differentiate yet [1]. By specific *in vitro* manipulation these ESCs can maintain their growth and pluripotency (the ability of cells to differentiated into multiple cell types) almost indefinitely [2]. ESCs are important tools for regenerative medicine [3], genome manipulation in animals [4], development of transgenic animals [5] and toxicity testing [6]. The ESCs have the ability to differentiated into the cells of three germ layers (ectoderm, endoderm and mesoderm), and further into a specific cell type by manipulating culture conditions [1]. Examples of differentiated cell types derived from ESCs *in vitro* include human cardiomyocytes [7], human neural progenitor cells [8], mouse hematopoietic progenitor cells [9], alveolar epithelial cells [10] and endothelial progenitor cells [11].

Research into endothelial cells is fundamental for understanding important processes regulated by these cells e.g. tissue homeostasis, blood cell activation and coagulation [12]. Endothelial cell culture can be used for important applications such as tissue regeneration. In one study, endothelial cells derived from ESCs modified into organ specific endothelial cells and helped regeneration of liver sinusoidal vessels in mice, after transplantation [13]. Similarly vascular networks cultured in 3D hydrogel matrix using endothelial cells derived from human pluripotent ESCs, were able to incorporate with the microvasculature of mouse and sustain blood flow after implantation [14].

Culturing endothelial cells alone is difficult to maintain, to overcome this problem co-culturing techniques have been developed, in which endothelial cells are cultured in the presence of supporting cells including fibroblasts, mural cells, pericytes and mesenchymal stem cells [15]. These endothelial co-culture techniques may be used to engineer vascularized organ culture. In one example, human umbilical vein endothelial cells (HUVECs) co-cultured with human mesenchymal stem cells, in a combination of endothelial growth medium (EGM) and osteogenic medium, formed an *in vitro* vascularized bone model [16]. It has been suggested that such vascularized organ cultures may one day be used for tissue transplantation [17].

Heamangioblasts, the common progenitors of endothelial and hematopoietic lineages, differentiate from the mesoderm during the early development of embryos [18]. The differentiation of heamangioblasts is initiated by various factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and bone morphogenic protein 4 (BMP-4) [19]. Haemangioblasts can be generated *in vitro* by treating ESCs with these various differentiation factors just mentioned, as well as others used in the differentiating media (Table 1; [20, 21]).

In vitro differentiation of ESCs into vascular cells have potential applications in studying vascular development, proposing vascular regenerative therapy, culturing vascularized

organs and developing endothelial cell line [22]. In order to induce differentiation the ESCs are usually cultured in differentiation media in a suspension culture to form embryoid bodies (EBs); on a feeder cell layer of stromal cell line; or on an artificial matrix e.g. collagen type-IV [23, 24].

Table 1: Culture substrates and media composition used for *in vitro* differentiation of ESCs into endothelial lineage.

Culture components	Units	Sources of ESCs						
		Mouse	Human	Human	Mouse	Mouse	Human	Mouse
Substrate		Col-I	MEF	Fbn	Col-IV	Gelatin	PEC	Gelatin
IMDM				-	-	-	-	-
Lv-EGM		-	-		-	-	-	-
α-MEM		-	-	-		-	-	-
SP-34 medium		-	-	-	-		-	-
DMEM/F12		-	-	-	-	-		-
DMEM (high glucose)		-	-	-	-	-	-	
Methyl cellulose	%	1	-	-	-	-	-	-
FBS	%	15	-	-	5	-	-	20
Knockout serum replacement	%	-	-	-	-	-	20	-
BIT 9500	%	-	20	-	-	-	-	-
Insulin	µg/mL	10	-	-	-	-	-	-
Penicillin	U/mL	50	50	-	-	-	-	-
Streptomycin	µg/mL	50	50	-	-	-	-	-
PS	%	-	-	-	1	1	1	100X
Monothioglycerol	µM	450	450	-	-	-	-	-
VEGF	ng/mL	50	50	-	-	20	10	50
FGF2	ng/mL	100	50	3	-	10	5	-
BMP4	ng/mL	-	50	-	-	5	10	-
hEGF	ng/mL	-	-	10	-	-	-	-
ActivinA	ng/mL	-	-	-	-	5	-	-
Erythropoietin	U/mL	2	-	-	-	-	-	-
Interleukin 6	ng/mL	10	-	-	-	-	-	-
BHT	µg/mL	-	-	-	-	200	-	-
L-glutamin		-	2mM	-	1%	1%	1%	2mM
NE amino acids		-	0.1mM	-	-	-	1%	100X
Hydrocortisone	µg/mL	-	-	1	-	-	-	-
Heparin	µg/mL	-	-	10	-	-	-	-
Ascorbic acid	mM	-	-	-	-	0.5	-	-
β-mercaptoethanol		-	-	-	50µM	0.1%	1%	50µM
ESC qualified nucleotides		-	-	-	-	-	-	100X
References		[25, 26]	[27]	[28]	[20]	[13]	[13]	[29]

Abbreviations: α-MEM, minimum essential medium (sigma); BHT, Bovine holo-transferrin; BMP, bone morphogenic protein; Col-I, collagen type-I; Col-IV, collagen type-IV; DMEM, Dulbecco's modified Eagle's medium; ESC, embryonic stem cell; F12, Ham's F12 medium; Fbn, fibronectin; FBS, fetal bovine serum, BIT, combined bovine serum albumin, insulin and transferrin (Stem Cell Technologies); FGF, fibroblast growth factor; hEGF, human epidermal growth factor; IMDM, Iscove's modified Dulbecco's medium; Lv-EGM, large-vessel endothelial growth medium; MEF, mouse embryonic fibroblasts; NE, non-essential amino acids; PEC, primary endothelial cells; PS, penicillin streptomycin mix; SP-34, Stem Pro-34 medium (Invitrogen); VEGF, vascular endothelial growth factor; grey boxes indicate the base medium; -, not added.

Mouse ESCs cultured on collagen type-IV substrate have been shown to differentiate along the mesodermal lineage with higher efficiency compared to EB culture [30]. In living embryos the mesodermal cells differentiate into endothelial and hematopoietic progenitor cells [12]. In *in vitro* studies, collagen type-IV has been successfully used for differentiation of endothelial cells from mouse ESCs [20]. As an alternative to collagen type-IV, gelatin has also been used as a substratum for mouse ESCs to induce endothelial differentiation [20]. The differentiated endothelial cells are identified using specific antibodies to stain $CD31^+$, $CD34^+$, or VE-cadherin$^+$ cells [31]. These cells are then enriched by florescence cells sorting or by other means e.g. magnetic beads, which isolates the stained cells from the rest of the cells [32]. Using the same strategy, vascular progenitor cells have been derived from mouse ESCs using *flk1* marker [33].

Various ESC cultures have been utilized to develop endothelial cell cultures using differentiating media (Table 1). ESCs from the mouse and other mammals are usually used for these studies [21]. However, it is desirable to develop alternative models in order to reduce the use of mammals in research. Zebrafish can be an excellent model for various cell culture applications because there is no need to sacrifice the mother to get embryos, as would be the case in mice. Also, the zebrafish embryo model provides easy and large-scale availability of embryos for cell isolation and comparatively simple conditions required for cell culture [34].

Beside the general advantages of zebrafish for cell culture applications, there are transgenic zebrafish lines that express green fluorescence protein (GFP) in a specific cell type. Two of the transgenic zebrafish reporter lines: (i) *Kdrl:GFP*, which expresses GFP under the promoter of VEGF receptor (VEGFR2), also known as *flk-1* (fetal liver kinase 1) or *KDR-like* (kinase insert domain receptor like) gene i.e. specifically expressed in endothelial cells [35]; and (ii) *fli:GFP*, which expresses GFP under the promoter of friend leukemia virus integration site 1, i.e. expressed in endothelial, hematopoietic and neural crest cells [36]. Being a relatively new research model, *in vitro* studies on zebrafish hematopoietic and endothelial cells are rare, except for a recently developed zebrafish embryonic stromal trunk cell line that was reported to support proliferation and differentiation of zebrafish hematopoietic stem cells [37].

In this chapter, we examine the development of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish blastocyst cell cultures. We analyse the yield of these cells under different culture conditions. We used different media compositions to test their potential to generate *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in blastocyst cell culture. The three media compositions used were: (i) LDF (combined Lebovitz-15, Dulbecco's modified Eagle's medium and Ham's F-12 medium) medium; (ii) 1:1 mixture of LDF and EGM (endothelial growth medium) medium; and (iii) LDF medium supplemented with endothelial growth supplement. Furthermore, we analyse the effect of different substrates (gelatin and collagen type-I),

and different concentrations of recombinant zebrafish vascular endothelial growth factor ($VEGF_{165}$), on the percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in culture.

Materials and methods

Embryo collection

All the animal experiments were performed according to the Netherland Experiments on Animals Act [38], based on the guidelines on the protection of experimental animals, laid by the Council of European Union [39]. Adult zebrafish were maintained as previously described [40]. Two different transgenic zebrafish lines *fli:GFP* and *kdrl:GFP* were used. To obtain embryos, adult male and female fish, at a proportion of 1 to 1, were transferred to small breeding tanks in the evening. The zebrafish usually laid eggs when the light comes on. The eggs were collected at the bottom of the tank, separated from adults using a cotton mesh.

Embryos were transferred to a temperature controlled room (28 °C) and were distributed in 9cm 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. These embryos were allowed to develop to the high blastula stage of Kimmel et al. [41] (approximately 3 h after fertilization) at 28 °C.

Sterilization of embryos

The embryos were transferred to a flow cabinet at room temperature for sterilization and cell isolation. For sterilization, the embryos, with the chorion intact, were immersed in 70% ethanol for 10 sec and then in two changes of 0.05% sodium hypochlorite (available chlorine 10-15%, Sigma, catalog 425044) for 4 min each. The sterilization was done according to the procedure described in Ref. [42]. After each immersion in ethanol or sodium hypochlorite, the embryos were rinsed with LDF medium (see below, Media combinations and composition). Finally the embryos were left in 0.5 mL of LDF medium for dechorionation. Before dechorionating, the embryos were observed under a dissecting microscope and any dead or abnormal embryos (with cloudiness in the perivitelline fluid) were removed.

Blastocyst cells isolation and culture

The following steps were all conducted at room temperature. The embryos were dechorionated in LDF medium using sterile No. 5 watchmaker's forceps and then transferred to Eppendorf tubes. The LDF medium was gently triturated with a P-200 Gilson micropipette (Gilson, B.V., Europe: Den Haag) to remove the yolk. The solution was then centrifuged at 300 g for 1 min and the supernatant was discarded. The blastocysts were washed once with CMF-PBS (calcium magnesium free phosphate buffered saline;

Invitrogen catalog 14190) and then dissociated with 1 mL of 0.25% trypsin solution (Invitrogen catalog 15090) containing 1 mM EDTA (ethylenediaminetetraacetic acid). The trypsin solution was gently triturated with a p-1000 Gilson pipette for 2min. The trypsin was inactivated with 0.1 mL FBS (Fetal bovine serum; Invitrogen, 10500) and the cells were isolated by centrifugation at 300 g for 3 min. The cells were washed three times with LDF medium with 10% FBS and re-suspended in 200 µL LDF medium containing 20% FBS. The cells were counted using a heamocytometer and distributed at 17,000 cells per well in a 96-well microtiter plates.

For each of the culture conditions (i.e. media compositions, substrates and vascular endothelial growth factor concentrations, described below) the blastocyst cells were cultured in 6 wells of the 96-well plate. Separate cultures were established for *fli:GFP* and *kdrl:GFP* blastocyst cells. For data collection at each time point (i.e. day 2, 4, 6 and 8), cultures were established in separate 96-well plates. The medium was refreshed on day 4 for cultures that were maintained till days 6 and 8. All the cultures were maintained at 28 °C in a humidified incubator in 0.5% CO_2.

Media combinations and composition

Different media combinations were used to analyse the effect of media composition on the quantification of GFP^+ cells in the cultures. All the media combinations contained LDF and EGM. LDF is a combination of different nutrient media commonly used for zebrafish cell culture [42-46], whereas EGM is usually used to culture human umbilical vein endothelial cells [47, 48]. EGM has also been used for human pluripotent stem cells to induce vascular endothelial cell differentiation [14]. In our preliminary experiments, zebrafish blastocyst cells did not grow well in EGM. Therefore, it was always subsequently used in combination with LDF medium. Three different media combinations (Table 2) were prepared to culture the zebrafish blastocyst cells. After allowing 1 h to the cells distributed in 96-well plate (as described above), each of the medium combination was added to separate wells. The cultures were maintained until data collection (see below)

Culture substrates

In this experiment different substrates namely gelatin from porcine skin (Sigma, Cat. No. G1890); and collagen type-I rat protein (Invitrogen, Cat. No. A1048301) were used to culture the *fli:GFP* or *kdrl:GFP* blastocyst cells. In addition, cultures were established on polystyrene surface of the tissue culture plate, without any extra substrate coating, for comparison. The percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells was evaluated in cell isolates from cultures established on these substrates on subsequent days. Gelatin was used at a concentration of 0.1 mg/cm^2 to coat the culture wells. Each well of the 96-well plate was coated with 1.7 µL of 2% gelatin solution and allowed to air dry for 1 h before the cells were plated.

Table 2. Media compositions used to optimize culture conditions for the growth of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cell culture.

Media composition (supplier, catalog number)	Final concentrations
LDF medium	
Lebovitz L-15 (Invitrogen, 11415) : DMEM (Invitrogen, 11966) : Ham's F-12 medium (Invitrogen, 21765)	55 : 32.5 : 12.5
HEPES	15 mM
Antibiotic/antimycotic mix (Invitrogen, 15240)	1%
$NaHCO_3$	0.015%
Fetal bovine serum (Invitrogen, 10500)	15%
Zebrafish embryo extract	50 µg/mL
Basic fibroblast growth factor (Invitrogen, PHG0024)	10 ng/mL
Recombinant zebrafish $VEGF_{165}$ (R&D systems, 1247-ZV)	10 ng/mL
LDF:EGM medium	
LDF medium	50%
Endothelial growth medium 2 (Promocell, C-22011)	50%
Recombinant zebrafish $VEGF_{165}$	5 ng/mL
FBS	7.5%
ZEE	25 µg/mL
LDF:EGS medium	
LDF medium	95.8%
Endothelial growth supplement mix (Promocell, C-39216)	4.1%
Recombinant zebrafish $VEGF_{165}$	10 ng/mL

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; HEPES, (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid); VEGF, vascular endothelial growth factor.

Collagen type-I was used at a concentration of 3 mg/mL. To coat the wells, the collagen type-I solution was neutralized using 7.5% sodium bicarbonate, and then added at 5 µL per well in a 96-well plate. The plate was incubated at 37 °C for 1 h to promote gel formation. The wells were then rinsed with 1X CMF-PBS before adding the cells. The blastocyst cells distributed in the pre-coated wells (with gelatin or Collagen type-I) were allowed to attach to the substrate for 1 h. The LDF:EGS medium was then added at 250 µL per well. The cultures were subjected to cell isolation and data collection at subsequent time points (see below).

Vascular endothelial growth factor

In this experiment the effect of recombinant zebrafish $VEGF_{165}$ was evaluated on the percentage of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells in blastocyst cell culture. The LDF:EGS medium was used as the basal medium for this experiment. This medium was further supplemented with different concentrations (0, 10, 20 and 40 ng/mL) of zebrafish $VEGF_{165}$. Media with different concentrations of VEGF was added to separate wells of the 96-well plate pre-distributed with blastocyst cells derived from *fli:GFP* or *kdrl:GFP* embryos. The cultures were isolated from the wells at days 2, 4, 6 and 8 and counted for GFP^{+} and GFP^{-} as described below.

Isolation of cultured cells for data collection

The percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells, total number of cells harvested per well and cell viability was calculated in cell isolates from the cultures at subsequent time-points. To isolate the cells, the medium was aspirated from each well. The wells were then rinsed with 200 µL of 1X CMF-PBS. Then 250 µL of 0.05% trypsin solution containing 1mM EDTA was added to each well. The solution was triturated in the wells several times and then the plate was incubated for 2 min at 28 °C. The degree of detachment was monitored under an inverted microscope. When most cells were rounded up, the trypsin was inactivated by adding 25 µL of FBS, and the cell suspension from each well was transferred to separate 1.5 mL Eppendorf tubes. The suspension was centrifuged at 300 g for 3 min and the supernatant discarded. The cell pellet was washed twice with LDF medium (without growth factors) and re-suspended in 15-20 µL of the same medium. These cells were used for further analysis.

Cell counts and data collection

For the cultures maintained in different media compositions (i.e. LDF, LDF:EGM and LDF:EGS media), the total number of cells harvested per well and the cell viability was calculated in addition to the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells. For other conditions (i.e. different substrates and VEGF concentrations) only the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells was calculated in cell isolates. The total number of cells harvested per well was estimated by counting the cells in a heamocytometer. Viability of the cells was determined by using trypan blue exclusion dye (0.4% trypan blue in CMF-PBS) to differentiate viable and non-viable cells.

The percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in the living cell suspensions was calculated using a confocal microscope. A 5 µL drop from each of the cell isolates was transferred to a cover glass slide. The slide was placed under the confocal microscope and the cells were allowed to settle for 30 s. An image was then captured from the center of each drop. Each image was taken in duplex, one fluorescence image showing GFP$^+$ cells and one phase contrast image showing all the cells. For both *fli:GFP* and *kdrl:GFP* cultures, number of GFP$^+$ and GFP$^-$ cells per microscopic field (image) were then counted in image J software. From these counts the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells was calculated for each sample.

Statistical analysis

Number of EBs formed, total number of cells harvested, percentage of viable cells and the percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells per well, collected from 6 replicate wells for each culture condition, were analysed for means and standard errors using SPSS software version 21.0. One way ANOVA was performed to calculate the probability values to analyse differences between different culture conditions, as well as differences at different

time-points at the same culture condition. Pair-wise comparison was performed using Post-Hoc Tukey's test.

Results

Effect of medium composition on zebrafish blastocyst cell culture

Embryoid body formation

The zebrafish blastocyst cells formed EB aggregates within the first 24 hours of culture, containing differentiated *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells. Later these EB aggregates become mature and the percentage of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells within it varied by maintaining the cultures in different media (following sections). The number of EBs formed was higher in LDF:EGM and LDF:EGS media compared to LDF medium (Figure 1A). The highest number of EBs per well were formed in LDF:EGS medium (12.5 ± 0.9) followed by LDF:EGM (10.1 ± 0.6) and LDF (7.1 ± 0.5) media. The average size per EB was smaller in LDF:EGM and LDF:EGS media compared to LDF medium (Figure 1B).

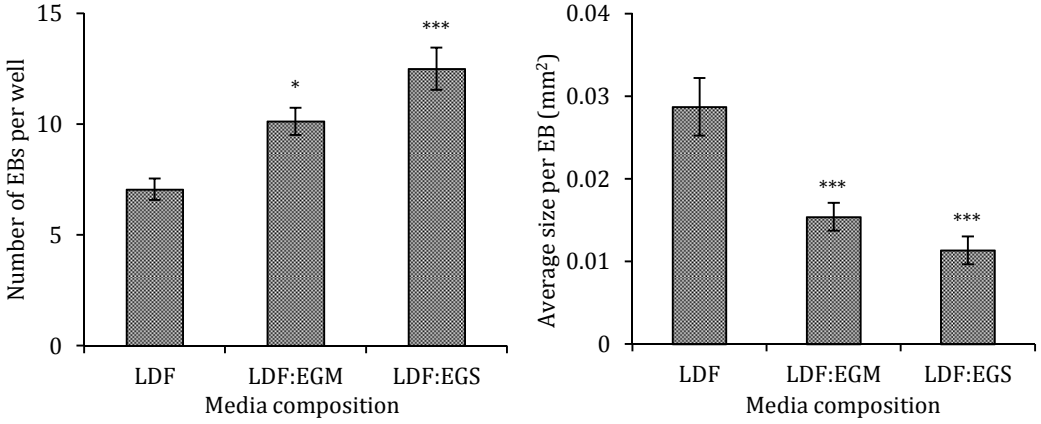

Figure 1: Formation of embryoid bodies (EBs) from zebrafish blastocyst cells cultured in different media. Data obtained at day 4 of culture. (**A**) Total number of EBs per well. (**B**) Average area (mm^2) covered by one EB formed in different media. Error bars represent standard error. (***, $p < 0.001$, *, $p < 0.05$ compared to LDF medium).

Quantification of *fli:GFP*$^{+}$ cells per medium

Different media compositions used to culture zebrafish blastocyst cells showed significant differences in the percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ in cultures (Figure 2). The percentage of *fli:GFP*$^{+}$ cells in cultures on LDF:EGM was significantly higher than cultures on LDF medium at each of the time-points (Figure 2C). No significant differences in the percentage of *fli:GFP*$^{+}$ cells were observed between LDF:EGM and LDF:EGS media except on day 6, where a higher percentage of *fli:GFP*$^{+}$ cells were found in LDF:EGM medium.

Compared to LDF medium, cells cultured in LDF:EGS medium showed significantly higher percentage of *fli:GFP*$^+$ cells on days 4 and 6. However, on days 2 and 8 the differences were not significant.

Quantification of *fli:GFP*$^+$ with duration of culture

In LDF medium the percentage of *fli:GFP*$^+$ cells increased gradually from day 2 to day 8 ($p<0.05$), with no significant increase until day 6. In LDF:EGM and LDF:EGS media the percentage of *fli:GFP*$^+$ cells increase significantly from day 2 to day 4 ($p<0.05$ and 0.01, respectively), with no significant variations after day 4 till day 8. The highest percentage of *fli:GFP*$^+$ cells (39.2 ± 1.1%) was found in cells cultured in LDF:EGM medium on day 6.

In a preliminary experiment blastocyst cell cultures maintained for 14 days in LDF:EGS medium contained 21.9 ± 2.3% of *fli:GFP*$^+$ cells. However, when these cells were sub-cultured, the number of *fli:GFP*$^+$ cells could be maintained in the secondary cultures.

Quantification of *kdrl:GFP*$^+$ cells per medium

Similar to *fli:GFP*$^+$ cells, a higher percentage of *kdrl:GFP*$^+$ cells was found in cultures maintained on LDF:EGS and LDF:EGM media, compared to LDF medium (Figure 2D). However, the percentage of *kdrl:GFP*$^+$ cells in culture was much less than *fli:GFP*$^+$ cells. The percentage of *kdrl:GFP*$^+$ cells was similar in different media composition until day 4 of culture. After day 4 the cultures maintained on LDF medium contained significantly lower percentage of *kdrl:GFP*$^+$ cells compared to cultures on LDF:EGS medium. The percentage of *kdrl:GFP*$^+$ cells in cultures maintained with LDF:EGS medium was slightly higher compared to LDF:EGM medium; however the differences were not significant.

Quantification of *kdrl:GFP*$^+$ cells with duration of culture

The quantification of *kdrl:GFP*$^+$ cells at subsequent time points showed a slight increase in percent *kdrl:GFP*$^+$ cells from day 2 to day 4 and then a decrease after day 4. In LDF medium the percentage of *kdrl:GFP*$^+$ cells dropped significantly from day 4 to day 6 ($p<0.01$) and then continued to decline until day 8. Similarly, in the LDF:EGM and LDF:EGS media, the percentage of *kdrl:GFP*$^+$ cells decreased significantly from day 4 to day 8 ($p<0.05$). The highest percentage of *kdrl:GFP*$^+$ cells was found in cultures maintained on LDF:EGS medium on day 4 of culture (4.2 ± 0.3%). In general, *kdrl:GFP*$^+$ cells could be observed in all different culture conditions for a maximum of 10 days.

Total number of cells harvested per medium

The total number of cells harvested from cultures grown in different media compositions were the lowest in LDF:EGM cultures (Figure 3A). On day 2 of culture, the total number of cells isolated from cultures maintained on LDF:EGS medium was higher than cultures on LDF and LDF:EGM medium. After day 2, the total number of cells declined until day 6 in all media compositions; it then increased again.

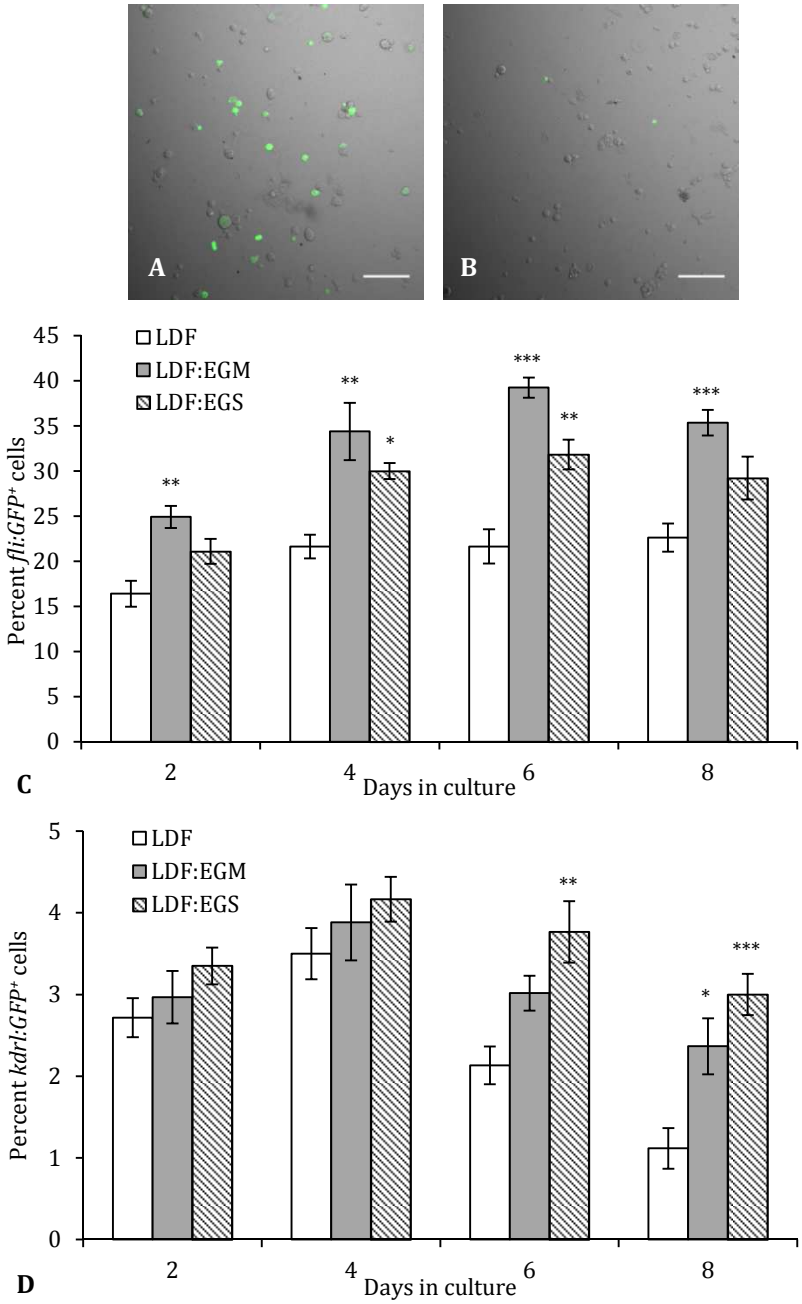

Figure 2: Percent quantification of *fli:GFP*⁺ and *kdrl:GFP*⁺ cells in blastocyst cell cultures maintained on different media. (**A and B**) Overlaid confocal and phase contrast images of cells isolated from *fli:GFP* (**A**) and *kdrl:GFP* (**B**) blastocyst cell culture. Cells in green shows *fli:GFP*⁺ or *kdrl:GFP*⁺ cells. Scale bar, 100 µm. (**C**) Quantification of *fli:GFP*⁺ cells in cells isolated from cultures, on subsequent days. (**D**) Quantification of *kdrl:GFP*⁺ cells in different media composition. All the media were supplemented with 15% FBS, 10 ng/mL FGF and 10 ng/mL zebrafish $VEGF_{165}$. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to LDF medium).

On day 4 the cell counts in LDF and LDF:EGS media were similar and both higher than LDF:EGM medium. No significant differences were found in cell counts isolated from cultures on day 6. After day 6 the cell counts in LDF medium increased significantly compared to LDF:EGM and LDF:EGS media. No significant differences were observed in the cell viability of cultures maintained in different media composition except day 6 (Figure 3B), where the cell viability was significantly higher in LDF:EGS medium.

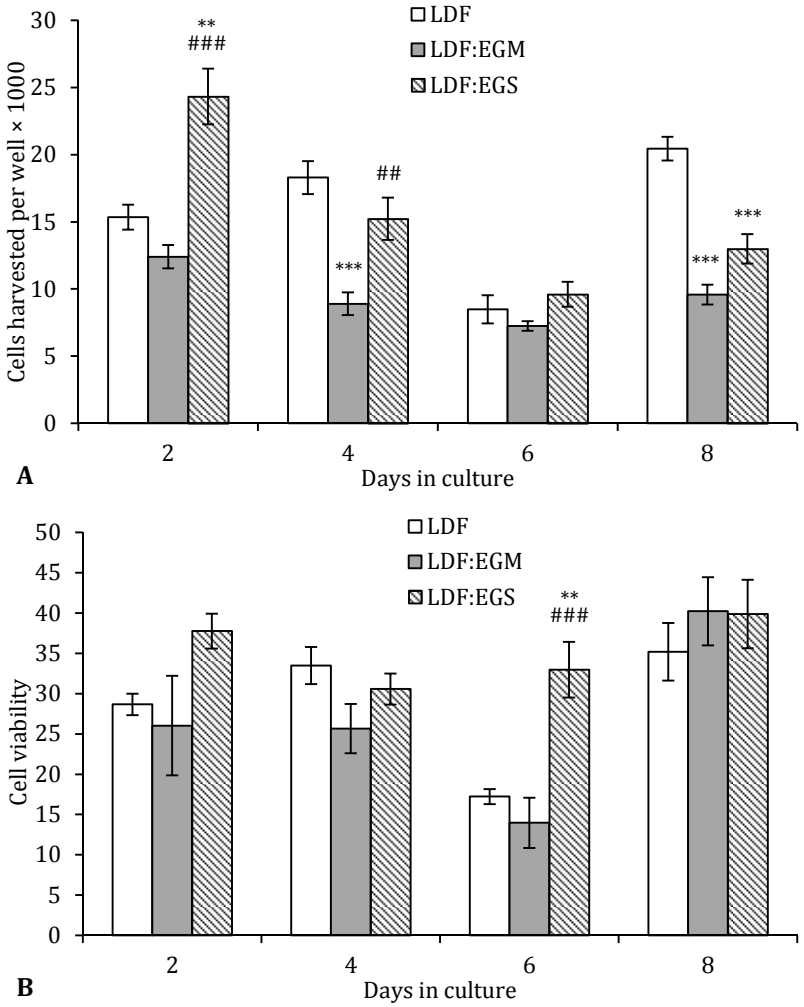

Figure 3: Total number and viability of isolated cells from cultures maintained on different media composition. (**A**) Total number of cells harvested per well at subsequent time-points. (**B**) Viability of isolated cells. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$ compared to LDF medium; ###, $p < 0.001$, ##, $p < 0.01$ compared to LDF:EGM medium).

Effect of substratum on blastocyst cell culture

Percentage of *fli:GFP*$^{+}$ cells in cultures on different substrate

The confocal images of cultures showed spreading out of *fli:GFP*$^{+}$ cells from EBs on collagen type-I and polystyrene substrate (Figure 4). However, on gelatin substratum the *fli:GFP*$^{+}$ cells remained on the periphery of the EBs. The percentage of *fli:GFP*$^{+}$ cells in cultures differed significantly between gelatin and collagen type-I substrates, such that collagen type-I was more favorable. Compared to the uncoated polystyrene surface, the percentage of *fli:GFP*$^{+}$ cells was slightly lower on gelatin substratum and slightly higher on collagen type-I substratum (Figure 5A). The percentage of *fli:GFP*$^{+}$ cells in cells isolated on day 8 of the cultures was significantly higher on collagen type-I (30.6 ± 2.0%) compared to gelatin (20.9 ± 1.6%) substratum (Figure 5A).

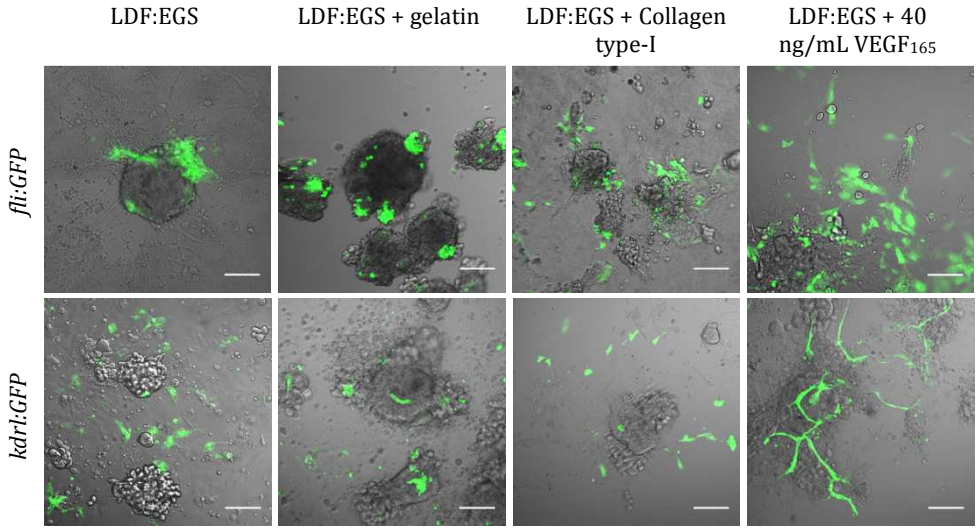

Figure 4: Induction of *fli:GFP*$^{+}$ cells and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cells cultured in different conditions. The morphological differences between *fli:GFP* and *kdrl:GFP* cells can be observed. The *fli:GFP*$^{+}$ cells develop in the form of a layer around the EBs in cultures. The *kdrl:GFP*$^{+}$ cells are more longitudinal and develop in the form of elongated structures around the EBs. In the presence of $VEGF_{165}$ the *kdrl:GFP*$^{+}$ cells form elongated cord-like structures. images taken on day 6 of cultures. Scale bar 100 µm.

Percentage of *kdrl:GFP*$^{+}$ cells in cultures on different substrate

Similar to *fli:GFP*, the *kdrl:GFP* blastocyst cells cultured on different substrate also showed differences in the percentage of *kdrl:GFP*$^{+}$ cells between gelatin and collagen type-I (Figure 5B). Migration of *kdrl:GFP*$^{+}$ cells from the EBs was observed on collagen type-I and polystyrene substrate, while on gelatin they remained mainly inside the EBs (Figure 4). The results showed significantly higher percentage of *kdrl:GFP*$^{+}$ cells in cultures maintained on collagen type-I substratum compared to gelatin substratum at different

time-point (day 2, day 4 and day 6; Figure 5B). No significant differences in the percentage of *kdrl:GFP*$^{+}$ cells was observed between cultures maintained on collagen type-I and polystyrene substratum. Compared to gelatin the percentage of *kdrl:GFP*$^{+}$ cells was higher in cultures on polystyrene substratum; however, the differences were not significant except on day 6 (Figure 5B).

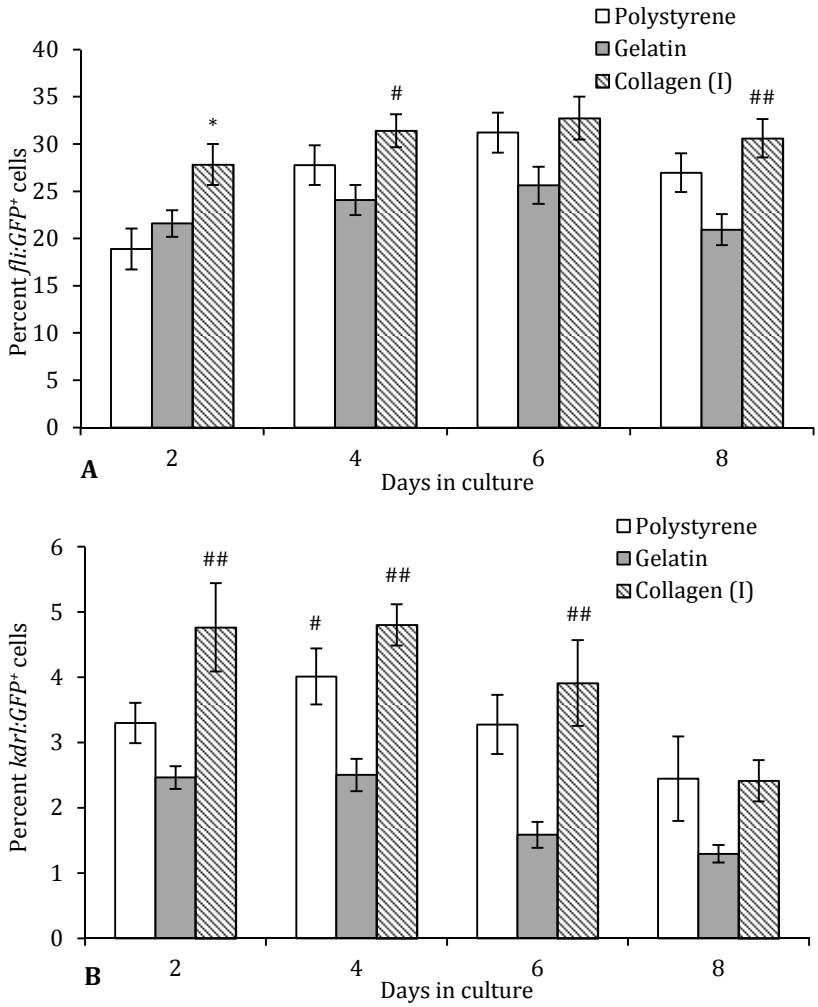

Figure 5: Percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures maintained on different substrates. (**A**) Percentage of *fli:*GFP^{+} cells in cells isolated from cultures on different substrates on subsequent days. (**B**) Percentage of *kdrl:GFP*$^{+}$ cells found in cultures on different substrates. Error bars represent standard error. (*, $p < 0.05$ compared to polystyrene surface; ##, $p < 0.01$, #, $p < 0.05$ compared to gelatin substratum).

Effect of zebrafish vascular endothelial growth factor

VEGF increases the percentage of *fli:GFP*$^+$ in culture

Zebrafish $VEGF_{165}$ protein showed significant effect on the percentage of *fli:GFP*$^+$ cells in the blastocyst cell cultures (Figure 6A). Cultures without $VEGF_{165}$ contained significantly less percentage of *fli:GFP*$^+$ cells on all time-points compared to cultures with $VEGF_{165}$. On day 2 no significant differences were observed in the percentage of *fli:GFP*$^+$ cells in cultures grown in media with different $VEGF_{165}$ concentrations (10, 20 and 40 ng/mL). On days 4, 6 and 8 the cultures maintained on 40 ng/mL $VEGF_{165}$ contained significantly higher percentage of *fli:GFP*$^+$ cells compared to cultures with 10 ng/mL $VEGF_{165}$. The only significant difference in the percentage of *fli:GFP*$^+$ cells between 10 and 20 ng/mL $VEGF_{165}$ was observed on day 8. No significant differences were observed between cultures maintained with 20 and 40 ng/mL $VEGF_{165}$ (Figure 6A).

In the absence of $VEGF_{165}$ the percentage of *fli:GFP*$^+$ cells increased from day 2 to 4 ($p<0.05$) and then decreased until day 8 ($p<0.01$). At 10 and 20 ng/mL $VEGF_{165}$ the percent *fli:GFP*$^+$ cells increased significantly from day 2 to 4 ($p<0.001$) and then remained similar until day 8 of culture. The percentage of *fli:GFP*$^+$ cells in cultures with 40 ng/mL $VEGF_{165}$ continued to increase until day 6 ($p<0.001$ for day 2 to 4; $P<0.05$ for day 4 to 6).

VEGF increases the percentage of *kdrl:GFP*$^+$ cells in culture

The *kdrl:GFP*$^+$ cells formed vascular cord-like structures in the presence of $VEGF_{165}$ in the culture medium (Figure 4). On day 2 the percentage of *kdrl:GFP*$^+$ cells was similar in cultures maintained with or without $VEGF_{165}$ in the medium (Figure 6B). An increase was observed in the percentage of *kdrl:GFP*$^+$ cells from day 2 to day 4 at all $VEGF_{165}$ concentrations. On day 4, cultures at 40 ng/mL $VEGF_{165}$ contained a significantly higher percentage of *kdrl:GFP*$^+$ cells compared to cultures without $VEGF_{165}$. No differences were observed in cultures with 10 and 20 and 40 ng/mL $VEGF_{165}$ in the medium on day 4 of culture.

After day 4 the percentage of *kdrl:GFP*$^+$ cells started to decrease in cultures. This decrease was significant in cultures without $VEGF_{165}$ from day 4 to day 6 ($p<0.01$). Similarly, the cultures with 10 ng/mL $VEGF_{165}$ in the medium showed a gradual decrease in the percentage of *kdrl:GFP*$^+$ cells from day 4 to day 8 ($p<0.05$). Cultures maintained with 20 and 40 ng/mL $VEGF_{165}$ showed a similar percentage of *kdrl:GFP*$^+$ cells after day 4 till day 8. On days 6 and 8 cultures with different concentrations of $VEGF_{165}$ showed significantly higher percentage of *kdrl:GFP*$^+$ cells compared to cultures without $VEGF_{165}$. Furthermore, the percentage of *kdrl:GFP*$^+$ cells was higher in cultures with 40 ng/mL $VEGF_{165}$ compared to cultures with 10 ng/mL $VEGF_{165}$ on days 6 and 8.

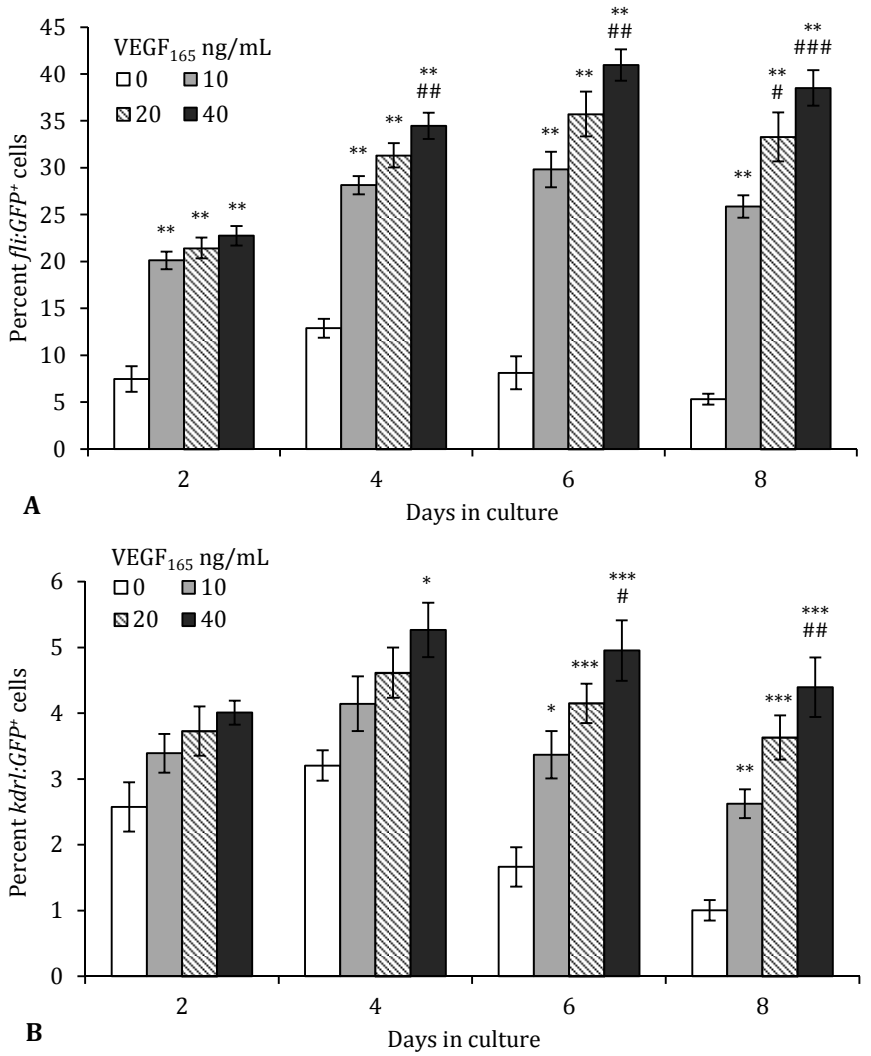

Figure 6: Effect of different concentrations of zebrafish $VEGF_{165}$ on the percentage of *fli:GFP*⁺ and *kdrl:GFP*⁺ cells in blastocyst cell culture. (**A**) Percentage of *fli:GFP*⁺ cells in cultures with different $VEGF_{165}$ concentrations at subsequent time points. (**B**) Percentage of *kdrl:GFP*⁺ cells in cultures with different $VEGF_{165}$ concentrations. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to culture without $VEGF_{165}$; ###, $p < 0.001$, ##, $p < 0.01$, #, $p < 0.05$ compared to culture with 10 ng/mL $VEGF_{165}$).

Discussion

Relationship of culture conditions, EB formation and *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cell quantification in culture

We have investigated different culture conditions for zebrafish blastocyst cells, with the objective to generate differentiated *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in relatively high numbers. The results showed important information for a controlled differentiation process in zebrafish blastocyst cell cultures. Studies have shown that without any anti-differentiation factors or feeder cells mouse and human ESCs undergo cellular differentiation (similar in some respects to embryogenesis) and form EBs [49]. In this study, we found that medium composition affects the development of EBs and the percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish blastocyst cell cultures.

Study on mouse ESC culture have shown that EBs developed in adherent cultures contained a higher number of total cells and a lower percentage of hematopoietic and endothelial cells, compared to the EBs grown in suspension cultures [50]. In our study, zebrafish blastocyst cells cultured in LDF medium developed a few, large-sized EBs, while cells cultured in LDF:EGM and LDF:EGS media developed more numerous, but smaller EBs with a higher percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells. These results suggest a direct relation between the number of EBs and the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells.

Previous studies have shown that the relative less cell-to-cell interaction, in the adherent cultures compared to 3D or suspension cultures, may favor the growth or differentiation of cell types other than hematopoietic and endothelial cells [50]. This conclusion is supported by our hanging-drop experiments in which there is by definition no outgrowth, but there is a high percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells (see Chapter 5). In our experiments described in this chapter, the supplementation of the LDF medium with endothelial growth supplement resulted in higher percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in cultures. This could be because the supplementation leads to increased cell-cell-interactions in the plated blastocyst cells as they are aggregating into EBs.

Differences between *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in culture

The blastocyst cell cultures initiated from *fli:GFP* embryos in our experiments expressed GFP in a higher percentage of cells compare to *kdrl:GFP*. This might be explained by the lineage-specificity of GFP expression in *kdrl:GFP* transgenic lines (endothelial cells only [51]), compared to the *fli:GFP* line (endothelial, lymphatic, hematopoietic and neural crest cells [36]). The *kdrl:GFP*$^+$ cells almost vanished by 8 days of culture, a similar phenomenon was recorded in a recent study using *kdrl:GFP* blastocyst cell culture for screening angiogenic compounds [52].

The *fli:GFP* signals by contrast, could be observed for up to 14 days. However, they also could not be maintained after that. This can be considered in the light of previous studies, describing *fli:GFP* expression persists in zebrafish embryos during early developmental stages [36]. Similarly, studies on mouse embryos and ESCs have shown significant reduction in the expression levels of *flk1/kdr* gene at later life stages [53]. These reports suggest as the cells matures in our zebrafish blastocyst cell cultures, they down regulate the transgenes, as these genes are expressed mainly in early stages of differentiation. However, other studies have reported that the expression of *fli1* and *kdrl* genes persists throughout vascular development in zebrafish [51, 54]. One possible explanation can be changes in gene expression and properties of endothelial cells, in our study, particularly *in vitro*, as previously described for other endothelial cells [55].

Studies have shown that primary endothelial cells have a short life span, which is usually overcome by immortalizing these cells using genetic manipulation [56]. Thus, an alternative explanation for the loss of *fli:GFP* and *kdrl:GFP* signals may be the occurrence of cell death in our primary blastocyst cell culture. This phenomena was observed as high number of floating cells in culture and long population doubling time of the primary blastocyst cell culture, discussed in chapter 3. A previous study also reported the loss of *kdrl:GFP* signal in zebrafish blastocyst cell culture due to apoptosis [57]. Another reason for this may be the development of fast growing fibroblast-like cells in our cultures, which depleted other cell types in subsequent passages (Chapter 3).

Media composition and endothelial growth supplementation

LDF is a commonly-used medium for zebrafish ESC culture [42-46]. It is also sometimes referred to as limiting dilution factor (LDF) medium, used to retain the homogeneity of a cell culture [43, 58]. The zebrafish primary blastocyst cells may have more complex nutrient requirements for their growth and attachment therefore supplements including FBS, fish embryo extract, fish serum and bFGF are usually added to the medium [59]. A nutrient rich medium is required to culture the primary blastocyst cells as the initial cell death is high in these cells because of bleaching the embryos [60]. The blastocyst cells are pluripotent in nature, therefore specific differentiation can be promoted by changing the culture conditions [34]. In some experiments additional supplementation or a substrate coating may be required to induce specific differentiation in these pluripotent ESCs. Examples include sonic hedgehog protein for myocyte differentiation [61], and poly-D-lysine coating for neurons and astrocytes differentiation [45].

Endothelial growth medium (EGM) is usually used to culture human umbilical vein endothelial cells (HUVECs; [47, 48]), as well as for the differentiation of human pluripotent stem cells into vascular endothelial cells [14]. The complete EGM is a combination of endothelial basal medium and endothelial growth supplement (EGS) mix. The EGS is composed of growth factors including human epidermal growth factor, bFGF,

insulin-like growth factor (IGF-1) and human VEGF. These components are usually used in differentiation media to induce endothelial differentiation in mouse [13, 25] and human [13, 27, 28] ESCs. Other components of EGS are heparin and hydrocortisone, which have been used for endothelial differentiation in human ESCs [28]. Similarly, ascorbic acid (also a component of EGS) have also been used in endothelial differentiation medium for mouse ESC culture [13].

In our experiments, the LDF medium increased the total cell counts after day 6 of the cultures. However, the percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells was significantly lower in this medium compared to LDF:EGM and LDF:EGS media. Previous studies on zebrafish blastocyst cell culture have shown the necessity of a feeder layer of growth arrested stromal cells in combination with LDF medium, to obtain a pluripotent zebrafish ESC culture [42, 44, 62]. Without the support of a feeder layer the blastocyst cells differentiate into EBs (that contain various cell types), and adherent fibroblast-like cells [59]. In further passages only the adherent cell type that suit best or adapt itself to the medium remains in culture [43, 58]. These studies suggest the suitability of LDF medium for growth and differentiation of cells other than *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells. However, we found that the addition of EGS and $VEGF_{165}$ to the LDF medium, increased the percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells compared to cultures in basic LDF medium.

When combined with the EGM, the LDF:EGM (1:1) medium increased the percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures. However, the total cells counts were significantly less in LDF:EGM medium compared to LDF medium. One of the reasons for this can be that EGM is an optimized medium for *human* endothelial cell lines [47, 48], and not for primary zebrafish cells. Therefore under an optimum medium condition (which is LDF for zebrafish cells) the cells grow at a normal rate. However, when combined with a non-specific medium (EGM in this case), the nutrient balance in the media changes and this then causes the slower growth rate of cultures in our study. On the other hand, EGM contains constituents to support endothelial cells, that may stimulate zebrafish blastocyst cells to differentiate into hematopoietic and endothelial lineages. This is why we obtained higher percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures with LDF:EGM compared to LDF medium.

LDF medium supplemented with endothelial growth supplement (EGS) significantly increased the percentages of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in our experiments as expected. As noted above, LDF is defined as a standard medium in many zebrafish cell culture procedures [42-46]. By using the LDF:EGS medium for our experiments, the full strength of LDF medium was ensured for the optimum growth of the cell cultures, which we quantified by total cell counts. The EGS contains the necessary factors required for the growth of endothelial and hematopoietic cells as discussed above. Therefore, the combination of LDF medium and EGS, represents a medium that can support cell growth

and viability, while at the same time induces maximum differentiation of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in cultures.

Culture substrate for endothelial differentiation

Extracellular matrix (ECM) is an important component of tissues *in vivo*, which directly interact with cells by cell receptors and support their growth and differentiation [63]. Different tissues possess ECM of differing composition and physical properties (stiffness, elasticity etc.), that influences the behavior and differentiation of cells in that tissue [63]. The same principle applies to cells *in vitro*. Different ECM substrates have been identified to direct differentiation of ESCs towards different cell lineages, as is reviewed in Ref. [64]. Some ECM substrates including collagen type-I [25, 26], collagen type-IV [20], and gelatin [13, 29], have been used to stimulate endothelial differentiation in mouse ESCs. Fibronectin has been used to promote the differentiation of human ESCs along endothelial lineage [28].

In our experiments the blastocyst cells cultured on collagen type-I substratum contained higher percentage of *fli:GFP*$^{+}$ or *kdrl:GFP*$^{+}$ cells, compared to gelatin substratum. The percentages of these cells were slightly higher on collagen type-I compared to polystyrene substratum. The only significant difference in percentage of *fli:GFP*$^{+}$ cells between collagen type-I and polystyrene substrate was observed on day 2 of culture. A similar phenomena was observed in *kdrl:GFP* cultures. Where the percentage of *kdrl:GFP*$^{+}$ cells was higher on collagen type-I substratum compared to gelatin; however, the differences were not significant compared to polystyrene.

This may suggests that collagen type-I is not necessary for the differentiation of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish. However, based on our results collagen type-I is favorable compared to gelatin substratum for the differentiation of these cells. Previous research on zebrafish ECM have shown the production of fibronectin and laminin in early developing embryos, and the synthesis of collagen at later stages [65]. Similarly, other studies describe the role of collagen type-I in formation of tubular blood vessels from endothelial cells at advance developmental stages [66]. This may explain why collagen type-I did not show significant effect on the percentage of *fli:GFP*$^{+}$ and *kdrl:GFP*$^{+}$ cells in zebrafish blastocyst cell culture in our study.

Zebrafish vascular endothelial growth factor

VEGF is a known factor for the differentiation and growth of endothelial cells in early embryogenesis, as well as for the development of vascular networks in embryos and adult tissues [67]. VEGF has also been identified to increase endothelial differentiation in human ESC culture [68]. In our experiments, on day 6 of cultures the percentages of *fli:GFP*$^{+}$ cells was 5.0 fold higher, and *kdrl:GFP*$^{+}$ cells 2.9 fold higher, in cultures with 40 ng/mL $VEGF_{165}$ compared to cultures without $VEGF_{165}$ in medium. These results are

comparable with a previous study on human ESCs where VEGF at 50 ng/mL has been reported to increase endothelial differentiation by 4.7 folds [68].

Conclusions

The growth of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish blastocyst cell culture can be increased by manipulating culture conditions. Supplementation of cell culture medium with EGS (used as medium supplementation for human endothelial cell cultures) increases the percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish blastocyst cell culture. Collagen type-I substratum should be preferred over gelatin for the differentiation of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish blastocyst cell culture. However, compared to polystyrene substratum the effect of collagen is not significant for this purpose. Recombinant zebrafish $VEGF_{165}$ also increases the percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in culture.

References

[1] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS*, et al.* Embryonic stem cell lines derived from human blastocysts. Science 1998; 282(5391): 1145-7.

[2] Chen J, Zhou L, Pan SY. A brief review of recent advances in stem cell biology. Neural Regen Res 2014; 9(7): 684-7.

[3] Schroeder IS. Stem cells: are we ready for therapy? Methods Mol Biol 2014; 1213: 3-21.

[4] Rossant J, Bernelot-Moens C, Nagy A. Genome manipulation in embryonic stem cells. Philos Trans R Soc Lond B Biol Sci 1993; 339(1288): 207-15.

[5] Pirity M, Hadjantonakis AK, Nagy A. Embryonic stem cells, creating transgenic animals. Methods Cell Biol 1998; 57: 279-93.

[6] Yu GY, Cao T, Ouyang HW, Peng SQ, Deng XL, Li SL*, et al.* Development of human embryonic stem cell model for toxicity evaluation. Beijing Da Xue Xue Bao 2013; 45(1): 9-11.

[7] Lam AT, Chen AK, Li J, Birch WR, Reuveny S, Oh SK. Conjoint propagation and differentiation of human embryonic stem cells to cardiomyocytes in a defined microcarrier spinner culture. Stem Cell Res Ther 2014; 5(5): 110.

[8] Liao MC, Diaconu M, Monecke S, Collombat P, Timaeus C, Kuhlmann T*, et al.* Embryonic stem cell-derived neural progenitors as non-tumorigenic source for dopaminergic neurons. World J Stem Cells 2014; 6(2): 248-55.

[9] Thompson HL, McLelland BT, Manilay JO. Indirect immune recognition of mouse embryonic stem cell-derived hematopoietic progenitors *in vitro*. Exp Hematol 2014; 42(5): 347-359 e5.

[10] Sun H, Quan Y, Yan Q, Peng X, Mao Z, Wetsel RA*, et al.* Isolation and characterization of alveolar epithelial type II cells derived from mouse embryonic stem cells. Tissue Eng Part C Methods 2014; 20(6): 464-72.

[11] Nishikawa S. Embryonic stem cells as a source of hematopoietic and vascular endothelial cells *in vitro*. J Allergy Clin Immunol 1997; 100(6 Pt 2): S102-4.
[12] Risau W. Differentiation of endothelium. FASEB J 1995; 9(10): 926-33.
[13] Nolan DJ, Ginsberg M, Israely E, Palikuqi B, Poulos MG, James D*, et al.* Molecular signatures of tissue-specific microvascular endothelial cell heterogeneity in organ maintenance and regeneration. Dev Cell 2013; 26(2): 204-19.
[14] Kusuma S, Shen YI, Hanjaya-Putra D, Mali P, Cheng L, Gerecht S. Self-organized vascular networks from human pluripotent stem cells in a synthetic matrix. Proc Natl Acad Sci U S A 2013; 110(31): 12601-6.
[15] Costa-Almeida R, Granja PL, Soares R, Guerreiro SG. Cellular strategies to promote vascularisation in tissue engineering applications. Eur Cell Mater 2014; 28: 51-66; discussion 66-7.
[16] Correia C, Grayson WL, Park M, Hutton D, Zhou B, Guo XE*, et al. In vitro* model of vascularized bone: synergizing vascular development and osteogenesis. PLoS One 2011; 6(12): e28352.
[17] Rivron NC, Liu JJ, Rouwkema J, de Boer J, van Blitterswijk CA. Engineering vascularised tissues *in vitro*. Eur Cell Mater 2008; 15: 27-40.
[18] Marcelo KL, Goldie LC, Hirschi KK. Regulation of endothelial cell differentiation and specification. Circ Res 2013; 112(9): 1272-87.
[19] Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. Nature 2005; 438(7070): 937-45.
[20] Blancas AA, Lauer NE, McCloskey KE. Endothelial differentiation of embryonic stem cells. Curr Protoc Stem Cell Biol 2008; Chapter 1: Unit 1F 5.
[21] Bai H, Wang ZZ. Directing human embryonic stem cells to generate vascular progenitor cells. Gene Ther 2008; 15(2): 89-95.
[22] Descamps B, Emanueli C. Vascular differentiation from embryonic stem cells: novel technologies and therapeutic promises. Vascul Pharmacol 2012; 56(5-6): 267-79.
[23] Nishikawa S, Jakt LM, Era T. Embryonic stem-cell culture as a tool for developmental cell biology. Nat Rev Mol Cell Biol 2007; 8(6): 502-7.
[24] Noghero A, Bussolino F, Gualandris A. Role of the microenvironment in the specification of endothelial progenitors derived from embryonic stem cells. Microvasc Res 2010; 79(3): 178-83.
[25] Feraud O, Prandini MH, Vittet D. Vasculogenesis and angiogenesis from *in vitro* differentiation of mouse embryonic stem cells. Methods Enzymol 2003; 365: 214-28.
[26] Feraud O, Cao Y, Vittet D. Embryonic stem cell-derived embryoid bodies development in collagen gels recapitulates sprouting angiogenesis. Lab Invest 2001; 81(12): 1669-81.
[27] Wang ZZ, Au P, Chen T, Shao Y, Daheron LM, Bai H*, et al.* Endothelial cells derived from human embryonic stem cells form durable blood vessels *in vivo*. Nat Biotechnol 2007; 25(3): 317-8.
[28] Kane NM, Meloni M, Spencer HL, Craig MA, Strehl R, Milligan G*, et al.* Derivation of endothelial cells from human embryonic stem cells by directed differentiation: analysis of microRNA and angiogenesis *in vitro* and *in vivo*. Arterioscler Thromb Vasc Biol 2010; 30(7): 1389-97.
[29] Li J, Stuhlmann H. *In vitro* imaging of angiogenesis using embryonic stem cell-derived endothelial cells. Stem Cells Dev 2012; 21(2): 331-42.
[30] Tada S, Era T, Furusawa C, Sakurai H, Nishikawa S, Kinoshita M*, et al.* Characterization of mesendoderm: a diverging point of the definitive endoderm and

mesoderm in embryonic stem cell differentiation culture. Development 2005; 132(19): 4363-4374.
[31] Wilson HK, Canfield SG, Shusta EV, Palecek SP. Concise review: tissue-specific microvascular endothelial cells derived from human pluripotent stem cells. Stem Cells 2014; 32(12): 3037-45.
[32] Yoder MC. Differentiation of pluripotent stem cells into endothelial cells. Curr Opin Hematol 2015; 22(3): 252-7.
[33] Yamashita J, Itoh H, Hirashima M, Ogawa M, Nishikawa S, Yurugi T*, et al.* Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. Nature 2000; 408(6808): 92-96.
[34] Alvarez MC, Bejar J, Chen S, Hong Y. Fish ES cells and applications to biotechnology. Mar Biotechnol (NY) 2007; 9(2): 117-27.
[35] 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.
[36] Lawson ND, Weinstein BM. *In vivo* imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 2002; 248(2): 307-318.
[37] Campbell C, Su T, Lau RP, Shah A, Laurie PC, Avalos B*, et al.* Zebrafish embryonic stromal trunk (ZEST) cells support hematopoietic stem and progenitor cell (HSPC) proliferation, survival, and differentiation. Exp Hematol 2015.
[38] Experiments on Animal Act, 2014. The State Secretary of Health and Environmental Protection, Soestdijk. http://wetten.overheid.nl/BWBR0003081/2014-12-18
[39] 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
[40] Westerfield M. The zebrafish book: a guide for the laboratory use of zebrafish (*Danio Rerio*). 4 ed, University of Oregon Press, Eugene 2000, pp.
[41] Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995; 203(3): 253-310.
[42] Fan L, Collodi P. Zebrafish embryonic stem cells. Methods Enzymol 2006; 418: 64-77.
[43] 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.
[44] 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.
[45] 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.
[46] 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.
[47] Allen P, Melero-Martin J, Bischoff J. Type I collagen, fibrin and PuraMatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks. J Tissue Eng Regen Med 2011; 5(4): e74-86.

[48] Arnaoutova I, Kleinman HK. *In vitro* angiogenesis: endothelial cell tube formation on gelled basement membrane extract. Nat Protoc 2010; 5(4): 628-35.
[49] Kurosawa H. Methods for inducing embryoid body formation: *in vitro* differentiation system of embryonic stem cells. J Biosci Bioeng 2007; 103(5): 389-98.
[50] Dang SM, Kyba M, Perlingeiro R, Daley GQ, Zandstra PW. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. Biotechnol Bioeng 2002; 78(4): 442-53.
[51] 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.
[52] Zhao H, Huang H, Lin S. Chemical approaches to angiogenesis in development and regeneration. Methods Cell Biol 2016; 134: 369-76.
[53] Kabrun N, Buhring HJ, Choi K, Ullrich A, Risau W, Keller G. Flk-1 expression defines a population of early embryonic hematopoietic precursors. Development 1997; 124(10): 2039-48.
[54] 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.
[55] Jackson CJ, Nguyen M. Human microvascular endothelial cells differ from macrovascular endothelial cells in their expression of matrix metalloproteinases. Int J Biochem Cell Biol 1997; 29(10): 1167-77.
[56] Nisato RE, Harrison JA, Buser R, Orci L, Rinsch C, Montesano R*, et al.* Generation and characterization of telomerase-transfected human lymphatic endothelial cells with an extended life span. Am J Pathol 2004; 165(1): 11-24.
[57] Huang HG, Lindgren A, Wu XR, Liu NA, Lin SO. High-throughput screening for bioactive molecules using primary cell culture of transgenic zebrafish embryos. Cell Rep 2012; 2(3): 695-704.
[58] Choorapoikayil S, Overvoorde J, den Hertog J. Deriving cell lines from zebrafish embryos and tumors. Zebrafish 2013; 10(3): 316-25.
[59] Helmrich A, Barnes D. Zebrafish embryonal cell culture. Methods Cell Biol 1999; 59: 29-37.
[60] 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.
[61] Norris W, Neyt C, Ingham PW, Currie PD. Slow muscle induction by Hedgehog signalling *in vitro*. J Cell Sci 2000; 113 (Pt 15): 2695-703.
[62] Fan L, Crodian J, Collodi P. Culture of embryonic stem cell lines from zebrafish. Methods Cell Biol 2004; 76: 151-60.
[63] Gattazzo F, Urciuolo A, Bonaldo P. Extracellular matrix: a dynamic microenvironment for stem cell niche. Biochim Biophys Acta 2014; 1840(8): 2506-19.
[64] Wang H, Luo X, Leighton J. Extracellular matrix and integrins in embryonic stem cell differentiation. Biochem Insights 2015; 8(Suppl 2): 15-21.
[65] Jessen JR. Recent advances in the study of zebrafish extracellular matrix proteins. Dev Biol 2015; 401(1): 110-21.
[66] Whelan MC, Senger DR. Collagen I initiates endothelial cell morphogenesis by inducing actin polymerization through suppression of cyclic AMP and protein kinase A. J Biol Chem 2003; 278(1): 327-34.

[67] Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling - in control of vascular function. Nat Rev Mol Cell Biol 2006; 7(5): 359-71.
[68] Nourse MB, Halpin DE, Scatena M, Mortisen DJ, Tulloch NL, Hauch KD*, et al.* VEGF induces differentiation of functional endothelium from human embryonic stem cells: implications for tissue engineering. Arterioscler Thromb Vasc Biol 2010; 30(1): 80-9.