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The growth of endothelial-like cells in zebrafish embryoid body culture

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ABSTRACT

There is increasing interest in the possibility of culturing organ-like tissues (organoids) *in vitro* for biomedical applications. The ability to culture organoids would be greatly enhanced by having a functional circulation *in vitro*. The endothelial cell is the most important cell type in this context. Endothelial cells can be derived from pluripotent embryonic blastocyst cells in aggregates called embryoid bodies. Here, we examine the yield of endothelial-like cells in embryoid bodies (EBs) developed from transgenic zebrafish *fli:GFP* and *kdrl:GFP* blastocyst embryos. The isolated blastocyst cells developed into EBs within the first 24 h of culture and contained *fli:GFP*$^+$ (putative endothelial, hematopoietic and other cell types); or *kdrl:GFP*$^+$ (endothelial) cells. The addition of endothelial growth supplements to the media and culture on collagen type-I substratum increased the percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in culture. We found that EBs developed in hanging-drop cultures possessed a higher percentage of *fli:GFP*$^+$ (45.0 ± 3.1%) and *kdrl:GFP*$^+$ cells (8.7 ± 0.7%) than those developed on conventional substrata (34.5 ± 1.4% or 5.2 ± 0.4%, respectively). The transcriptome analysis showed a higher expression of VEGF and TGFβ genes in EB cultures compared to the adherent cultures. When transferred to conventional culture, the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells declined significantly over subsequent days in the EBs. The *fli:GFP*$^+$ cells formed a monolayer around the embryoid bodies, while the *kdrl:GFP*$^+$ cells formed vascular network-like structures in the embryoid bodies. Differences were observed in the spreading of *fli:GFP*$^+$ cells, and network formation of *kdrl:GFP*$^+$ cells on different substrates. The *fli:GFP*$^+$ cells could be maintained in primary culture and sub-cultures. By contrast, *kdrl:GFP*$^+$ cells were almost completely absent at 8d of primary culture. Our culture model allows real-time observation of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in culture. The results obtained from this study will be important for the development of vascular and endothelial cell culture using embryonic cells.

1. Introduction

Embryonic stem (ES) cells are cells derived from early embryos that have not started to differentiate. By specific *in vitro* manipulation, these ES cells can maintain their growth and pluripotency (the ability to differentiate into multiple cell types) almost indefinitely [1]. ES cells are important tools for regenerative medicine [2], genome manipulation in animals [3], development of transgenic animals [4] and toxicity testing [5]. Pluripotent ES cells can differentiate into specific cell types according to the culture conditions. Examples of differentiated cell types derived *in vitro* from ES cells include human cardiomyocytes [6], human neural progenitor cells [7], mouse hematopoietic progenitor cells [8], and alveolar epithelial cells [9]. One of the important cell types derived from embryonic stem cell culture is endothelial progenitor cells which form the epithelial lining of the cardiovascular system [10].

Research into endothelial cells is fundamental for understanding important processes regulated by these cells e.g. tissue homeostasis, blood cell activation and coagulation [11]. Endothelial cell culture can be used for important applications such as tissue regeneration. In one study, endothelial cells derived from ES cells were transplanted into host mice and developed into organ-specific endothelial cells which contributed to the regeneration of liver sinusoidal vessels [12].

Similarly, vascular networks cultured in a 3D hydrogel matrix using endothelial cells derived from human pluripotent embryonic stem cells were able to become incorporated into the microvasculature of mouse and sustain blood flow after implantation [13]. It is difficult to maintain cultures of pure endothelial cells. 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 [14]. These endothelial co-culture techniques, in combination with organoid (un-vascularized

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Table 1
Culture substrates and medium composition used for the *in vitro* differentiation of ES cells into the endothelial lineage.[34]

Differentiating media	Units	Sources of ES cells							
		Mouse	Human	Human	Mouse	Mouse	Human	Mouse	Human
Substrate		Col-I	MEF	Fbn	Col-IV	Gelatin	PEC	Gelatin	Gelatin
IMDM	-			-	-	-	-	-	
Lv-EGM	-	-	-		-	-	-	-	-
α-MEM	-	-	-	-		-	-	-	-
SP-34 medium	-	-	-	-	-		-	-	-
DMEM/F12	-	-	-	-	-	-		-	-
DMEM (high glucose)	-	-	-	-	-	-	-		-
Methyl cellulose	%	1	-	-	-	-	-	-	-
FBS	%	15	-	-	5	-	-	20	20
Knockout serum replacement	%	-	-	-	-	-	20	-	-
BIT 9500	%	-	20	-	-	-	-	-	-
Insulin	μg/mL	10	-	-	-	-	-	-	-
Penicillin	U/mL	50	50	-	-	-	-	-	-
Streptomycin	μg/mL	50	50	-	-	-	-	-	-
Penicillin/streptomycin	%	-	-	-	1	1	1	100x	-
Monothioglycerol	μM	450	450	-	-	-	-	-	-
VEGF	ng/mL	50	50	-	-	20	10	50	20
FGF2	ng/mL	100	50	3	-	10	5	-	10
BMP4	ng/mL	-	50	-	-	5	10	-	25
hEGF	ng/mL	-	-	10	-	-	-	-	-
ActivinA	ng/mL	-	-	-	-	5	-	-	-
Erythropoietin	U/mL	2	-	-	-	-	-	-	-
Thrombopoietin	ng/mL	-	-	-	-	-	-	-	25
Stem cell factor	ng/mL	-	-	-	-	-	-	-	20
Flt 3 Ligand	ng/mL	-	-	-	-	-	-	-	100
Interleukin 6	ng/mL	10	-	-	-	-	-	-	-
Bovine holo-transferrin	μg/mL	-	-	-	-	200	-	-	-
L-glutamin	-	-	2mM	-	1%	1%	1%	2mM	2mM
NE amino acids	-	-	0.1mM	-	-	-	1%	100x	1%
Hydrocortisone	μg/mL	-	-	1	-	-	-	-	-
Heparin	μg/mL	-	-	10	-	-	-	-	-
Ascorbic acid	mM	-	-	-	-	0.5	-	-	-
β-mercaptoethanol	-	-	-	-	50μM	0.1%	1%	50μM	0.1mM
ES cell qualified nucleotides	-	-	-	-	-	-	-	100x	-
References		**(29, 30)**	**(31)**	**(32)**	**(21)**	**(12)**	**(12)**	**(33)**	**(34)**

Abbreviations: α-MEM, minimum essential medium (sigma); BIT, combined bovine serum albumin, insulin and transferring (Stem cell Technologies); BMP, bone morphogenic protein; Col, collagen gel; DMEM, Dulbecco's modified eagles medium; F12, Ham's F12 medium; Fbn, fibronectin; FBS, fetal bovine serum; FGF, fibroblast growth factor; hEGF, human epidermal growth factor; IMDM=Iscove's modified Dulbecco's medium; Lv-EGM, large-vessel endothelial growth media; MEF, mouse embryonic fibroblasts; NE, non-essential; PEC, primary endothelial cells; SP-34, Stem Pro-34 medium (Invitrogen); VEGF, vascular endothelial growth factor; grey boxes indicate the base medium; -, not added.

organ-like) culture [15], may be used to engineer vascularized organ cultures [16]. 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 [17]. It has been suggested that such vascularized organ cultures may one day be used for tissue transplantation [18].

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

The *in vitro* differentiation of embryonic stem cells into vascular cells has potential applications in vasculogenesis and angiogenesis research, vascular regenerative therapy, vascularized organ culture and development of endothelial cell lines [22]. In order to induce differentiation, three methods have been used: (i) culture of ES cells in differentiation media in suspension culture to form embryoid bodies (ii) culture on a feeder cell layer of a stromal cell line (iii) culture on an artificial matrix, e.g. collagen-IV [23,24].

Mouse ES cells cultured on the collagen-IV substrate have been shown to differentiate along the pathway of the mesodermal lineage with higher efficiency than the embryoid body (EB) cultures [25]. As mesodermal cells differentiate into endothelial and hematopoietic progenitor cells in embryos [11], collagen-IV can be used for the differentiation of endothelial cells from ES cells [21]. As an alternative to collagen-IV, gelatin has also been used as a substratum for mouse ES cells to induce endothelial differentiation [21]. The differentiated endothelial cells are identified using specific antibodies (reviewed in Ref. [26]) and are enriched by cells sorting or isolation [27]. Using the same strategy, vascular progenitor cells have been derived from mouse embryonic stem cells using *flk1* marker [28]. Various ES cell cultures have been used to develop endothelial cell cultures using differentiating media (Table 1).

ES cells from the mouse and other mammals are usually used for endothelial differentiation assays (Table 1). However, it is desirable to develop alternative models in order to reduce the use of mammals in research. Zebrafish can be a model of choice for various cell culture applications for several reasons as follows. There is no need to sacrifice the mother to get embryos, as would be the case in mice. The zebrafish model provides easy and large-scale availability of embryos for cell isolation and comparatively simple conditions required for cell culture [35]. In addition to these general advantages of zebrafish for cell culture applications, are transgenic zebrafish lines that express fluorescent reporters in a specific cell type. This expression allows the *in vivo* and *in vitro* observation and tracking of a particular cell-type in zebrafish models. Two of these transgenic zebrafish reporter lines are: (i) *Kdrl:GFP*, which expresses green fluorescence protein (GFP) under the promoter of vascular endothelial growth factor receptor (VEGFR2), also known as Flk-1 (fetal liver kinase 1) or KDR (kinase insert domain receptor) gene and which is expressed in endothelial cells [36]; and (ii) *fli:GFP*, which expresses GFP under the promoter of friend leukemia virus integration site 1, and is expressed in endothelial, lymphatic, hematopoietic, some yolk sac and neural crest cells [37].

The zebrafish is a relatively recent research model, and *in vitro* studies on zebrafish hematopoietic and endothelial cells are few, except for a recently developed zebrafish embryonic stromal trunk cell line that was reported to support proliferation and differentiation of zebrafish hematopoietic stem cells [38]. In a recent study, we reported the establishment of an *in vitro* vascular network using zebrafish embryonic cells [39]. One of the advantages of the *in vitro* manipulation of zebrafish cells is the availability of a large number of primary embryonic cells, and this eliminates the necessity of developing cell lines (with their associated drawbacks) [40]. In view of the advantages mentioned above, zebrafish *in vivo* and *in vitro* models of vasculogenesis and angiogenesis would be of great importance for the initial screening of new compounds and the testing of new protocols that could be further implemented in higher organisms.

In the current study, we examine the development of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in the EBs derived from zebrafish blastocyst cell cultures. We have analyzed different strategies for the improved yield of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in zebrafish blastocyst cell cultures. Different media compositions, culture substrates and culture conditions (suspension *vs* adherent) were used to analyze the potential of blastocyst cells to generate *fli:GFP*$^+$ and *kdrl:GFP*$^+$ progeny in cultures. Furthermore, the spreading of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells on culture substrata was observed and measured in growing live cultures.

2. Materials and methods

2.1. Embryo collection

All the animal experiments were performed according to the Netherland Experiments on Animals Act, based on the guidelines on the protection of experimental animals, laid by the Council of Europe (1986), Directive 86/609/EC. Adult zebrafish were maintained in 5-L tanks having continuously circulating egg water ("Instant Ocean" sea salt 60 μg/ml demi water), on 14 h light: 10 h dark cycle. The temperature of the water and air was controlled at 26 °C and 23 °C, respectively. Two different transgenic zebrafish lines *fli:GFP* and *kdrl:GFP* were used. To obtain embryos, adult male and female fish, at a ratio of 1:1, were transferred to small breeding tanks in the evening. The zebrafish usually laid eggs in the morning with the first light, and 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. These embryos were allowed to develop to the *high blastula* stage of Kimmel et al. [41] (approximately 3 h after fertilization) at 28 °C.

2.2. Sterilization of embryos

The embryos were transferred to a laminar 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 s and then in two changes of 0.05% sodium hypochlorite in water (the undiluted stock solution had an available chlorine level of 10–15%, Sigma; Zwijndrecht; Cat. No. 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 basic LDF medium (Table 2). Finally, the embryos were left in 0.5 ml LDF medium for dechorionation. Before dechorionating, the embryos were observed under a dissecting microscope and any dead or abnormal embryos (with cloudiness in the peri-vitelline fluid) were removed.

2.3. Culturing blastocyst cells

2.3.1. Cell isolation from blastocyst embryos

The following steps were all conducted at room temperature. The embryos were dechorionated in the LDF medium using sterile No. 5 watchmakers' 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; Landsmeer; Cat. No. 14190) and

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

Medium composition (supplier, catalog number)	Final concentration
Basic LDF medium	
Lebovitz L-15 medium (Invitrogen; Landsmeer; Cat. No. 11415); Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Landsmeer; Cat. No. 11966); Ham's F-12 medium (Invitrogen; Landsmeer; Cat. No. 21765)	55 : 32.5 : 12.5
HEPES (4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid)	15 mM
Antibiotic/antimycotic mix (Invitrogen; Landsmeer; Cat. No. 15240)	1%
$NaHCO_3$	0.015%
Fetal bovine serum (FBS)	15%
Zebrafish embryo extract (ZEE)	50 μg/ml
Supplemented LDF medium	
Basic LDF medium	99.8%
Basic fibroblast growth factor (recombinant human protein; Invitrogen; Landsmeer; Cat. No. PHG0024)	10 ng/ml
Recombinant zebrafish $VEGF_{165}$ (R&D systems; bio-techne Ltd; Abingdon; Cat. No. 1247-ZV)	10 ng/ml
LDF:EGM medium	
Supplemented LDF medium	50%
Endothelial growth medium-2 (Promocell; bio-connect B·V.; Huissen; Cat. No. C-22011)	50%
Recombinant zebrafish $VEGF_{165}$	5 ng/ml
FBS	6.5%
ZEE	25 μg/ml
LDF:EGS medium	
Basic LDF medium	95.8%
Endothelial growth supplement mix (Promocell; bio-connect B·V.; Huissen; Cat. No. C-39216)	4.1%
Recombinant zebrafish $VEGF_{165}$	10 ng/ml

then dissociated with 1 ml of 0.25% trypsin solution (Invitrogen; Landsmeer; Cat. No. 15090) containing 1 mM EDTA (ethylene diamine tetraacetic acid). The trypsin solution was gently triturated with a p-1000 Gilson pipette for 2 min. The trypsin was inactivated with 0.1 ml FBS (fetal bovine serum; Invitrogen; Landsmeer; Cat. No. 10500) and the cells were isolated by centrifugation at 300*g* for 3 min. The cells were washed three times with LDF medium containing 15% FBS and resuspended in 200 μl of the same medium. The cells were counted using a hemocytometer and were plated at 17,000 cells per well in 96-well plates to test different media compositions and culture substrates, or 1000 cells per hanging drop (suspension) culture to develop into embryoid bodies (see below).

2.3.2. Optimization of medium composition

To analyze the effect of medium composition on the quantification of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in the cultures, different medium combinations were used (Table 2). All the medium combinations contained LDF or EGM as major components. LDF is a combination of different nutrient media commonly used for zebrafish cell culture [42–45], whereas EGM is usually used to culture human umbilical vein endothelial cells [46,47]. EGM has also been used for the differentiation of human pluripotent stem cells into vascular endothelial cells [13]. In our preliminary experiments, zebrafish blastocyst cells did not grow well in EGM. Therefore, EGM was always subsequently used in combination with the LDF medium. In total, four different medium combinations were prepared to culture the zebrafish blastocyst cells (Table 2).

2.3.3. Culture conditions

All cultures described here were carried out in a forced draft, humidified incubator at 28 °C in 0.5% CO_2. When cultures were maintained longer than four days, the medium was refreshed on day 4.

2.3.4. Substrates

In this experiment different substrates, namely gelatin from porcine skin (Sigma; Zwijndrecht; Cat. No. G1890); and collagen type-I rat protein (Invitrogen; Landsmeer; Cat. No. A1048301), were used to culture the *fli:GFP* or *kdrl:GFP* blastocyst cells. The percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells were evaluated on these substrates on subsequent days. Gelatin was used at a concentration of 0.1 mg/cm^2. 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. Collagen type-I was used at a concentration of 3 mg/ml. To coat the wells with collagen type-I, the solution was neutralized using 7.5% sodium bicarbonate, and then plated 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 were cultured in LDF:EGS medium on these substrates.

2.3.5. Endothelial growth factors

In this experiment, the effect of recombinant zebrafish vascular endothelial growth factor (isoform $VEGF_{165}$) was evaluated on the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in cultures. The blastocyst cells were cultured in LDF:EGS medium supplemented with different concentrations (0, 10, 20 and 40 ng/ml) of zebrafish $VEGF_{165}$ to find the optimum VEGF concentration for the growth of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in culture. The cells were recovered from the 96-well plates at days 2, 4, 6 and 8 of culture and the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells determined (see below).

2.3.6. Cell isolation from culture wells

To quantify the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells, the cells were isolated separately from each well of the 96-well plate. For cell isolation, the medium was aspirated from each well. The cells were then washed twice with 200 μl of 1x CMF-PBS. Then 250 μl of 0.05% trypsin solution containing 1 mM EDTA was added to the wells. The solution was briefly triturated in the wells and incubated for 2 min at 28 °C. The degree of detachment was monitored under an inverted microscope. When most cells had rounded-up, the trypsin was inactivated by adding 25 μl of FBS, and the cell suspension from each well was transferred to a separate 1.5 ml Eppendorf tube. The suspension was centrifuged at 300 g for 3 min and the supernatant was discarded. The cell pellet was washed twice with the basic LDF medium and re-suspended in 20 μl of the same medium. The cells in the suspension were then counted under a confocal microscope (see below), to quantify the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$.

2.3.7. Quantification of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells

For each of the above-mentioned culture conditions (i.e. medium composition, substrata and VEGF concentration) and for each time-point, the zebrafish blastocyst cells were cultured in six replicate wells of a 96-well plate. Cultures were established in separate 96-well plates for isolation at different time points (i.e. day 2, 4, 6 and 8). To quantify the *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in the cultures, three drops of 5 μl of each cell suspension (isolated from the wells at different time-points)

were transferred to the confocal microscope on a cover glass slide. The cells were allowed to settle (for 30 s) and then the cell population of each 5 μl drop was imaged in duplicate, one with 488 nm wavelength excitation light to visualize the *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells, and one with phase-contrast showing all the cells. Multiple images were taken from each droplet if necessary to capture all the cells. For both *fli:GFP* and *kdrl:GFP* cultures, the number of GFP$^+$ and GFP$^-$ cells in the microscopic fields (images) per sample were then counted in ImageJ software version 1.46r [48]. From these counts, the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells was calculated for each culture well.

2.4. Culturing embryoid bodies

2.4.1. Hanging-drop (HD) cultures

The blastocyst cells isolated from *fli:GFP* or *kdrl:GFP* embryos according to the above-mentioned protocols, were re-suspended (at a final concentration of 50 cells/μL) in EB induction medium (LDF medium supplemented with 4.1% EGS, 20% FBS, 50 μg/ml ZEE, 40 ng/ml zebrafish $VEGF_{165}$ and 10 ng/ml bFGF). The cell suspension was distributed in 20 μl droplets (containing 1000 blastocyst cells each) onto the inside of the lids of 60 mm Petri dishes with no pre-treatment of the substratum. The lids with the droplets were inverted and replaced on the Petri dishes to initiate the hanging drop (HD) cultures. To diminish evaporation from the droplets, enough CMF-PBS was added to cover the area of each Petri dish (approximately 5 ml). The cultures were maintained in the incubator for four days to allow the formation of embryoid bodies (EBs). These EBs were then used to evaluate the *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells population overtime. On day 4 the EBs were transferred to the adherent culture conditions (see below) for further development.

2.4.2. Isolation of EBs

To isolate the EBs, the Petri dish lid was carefully inverted and held at a 45° angle. The droplets containing the EBs were shaken down to one side of the lid by gently tapping the lid. The EBs were transferred to a 1.5 ml Eppendorf tube using a p-1000 micropipette and were allowed to settle to the bottom of the tube by gravity. The medium was removed and the EBs were washed with the basic LDF medium. Finally, the EBs were re-suspended at 20 EB per 250 μl of EB maturation medium (LDF medium supplemented with 4.1% EGS, 15% FBS, 50 μg/ml ZEE, 40 ng/ml zebrafish $VEGF_{165}$ and 10 ng/ml bFGF), to be re-plated in 96-well plates.

2.4.3. Quantification of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in EBs

The *fli:GFP* or *kdrl:GFP* EBs isolated from the HD cultures were sub-cultured in 96-well plates, without extra substrate coating. The EBs were distributed in 96-well plate at 20 EBs in 250 μl medium (as re-suspended after isolation; see above) per well. For each transgenic line, a total of four 96-well plates were seeded with the EBs to analyze the cell counts at four consecutive time-points (days 2, 4, 6 and 8; one plate for one time-point). For each time-point six replicate wells of the 96-well plate were seeded with the EBs. The percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells were also calculated in the EBs on day 0 (the time of harvesting of the EBs from the HD culture, that is, day 4 of HD culture).

For cell counts on day 0, six replicates of 20 EBs per Eppendorf tube were dissociated into single cells using 250 μl of 0.05% trypsin solution. For each time point, cells were isolated from the culture wells as described above (Cell isolation from culture wells) and the contents of each well were transferred to a separate Eppendorf tube. The percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells was determined in these cultures according to the above procedure (Quantification of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells).

2.4.4. Quantification of *fli:GFP*$^+$ cells in EBs secondary cultures

For this experiment, the *fli:GFP* EBs were cultured in EB maturation medium for 8 days in a 24-well plate. A total of three wells (replicates) were cultured with 100 EBs per well. On day 8, the cells were then isolated from each well using 1 ml of 0.05% trypsin. The cells from each well were transferred to one well of a new 24-well plate (passage 1). The procedure was repeated on day four and the cells were sub-cultured in a fresh 24-well plate (passage 2). During the transfer, a small volume of cell suspension from each replicate was used to determine the percentage of *fli:GFP*$^+$ cells (as described above). The passage 2 cells were cultured for another four days in the same medium and used again to calculate the percentage of *fli:GFP*$^+$ cells in each well.

2.4.5. Culture of fli:GFP EBs on different substrates

In order to observe the development of *fli:GFP*$^+$ cells in culture, the EBs were cultured on three different substrates: (i) gelatin (Sigma; Zwijndrecht; Cat. No. G1890); (ii) collagen type-I (Invitrogen; Landsmeer; Cat. No. A1048301); (iii) fibrin, made with bovine fibrinogen (Sigma; Zwijndrecht; Cat. No. F8630). In order to image the cultures with a confocal microscope, a CS16-chambered cover glass plate (Grace Bio-Labs; bio-connect B·V.; Huissen; Cat. No. 112358) was used for these experiments. Gelatin was used at a concentration of 0.1 mg/cm^2 for coating the culture well. The well with gelatin-coating was allowed to air-dry before adding the EBs. The collagen type-I solution was prepared at 3 mg/ml and neutralized with 0.0125 ml/ml of 7.5% sodium bicarbonate. The collagen type-I gel solution was added at 5 μl per well to coat the well and allowed to polymerize at 37 °C for 30 min.

The fibrin gel was prepared by mixing fibrinogen solution (at a final concentration of 2.5 mg/ml) with 3 Units/ml of thrombin solution (Sigma; Zwijndrecht; Cat. No. T4648). The well was coated with 5 μl of the mixture. The plate was incubated at 37 °C for 30 min. All the wells coated with different substrate molecules were rinsed with the basic LDF medium before adding the EBs. The EBs re-suspended in the EB maturation medium was distributed at 20 EBs per well for each substrate condition.

2.4.6. Culture of kdrl:GFP EBs on different substrates

The *kdrl:GFP* EBs were transferred to different gel substrates namely collagen type-I, Geltrex™ and combined collage type-I + Geltrex™. A single well of the CS16-chambered coverglass plate was coated with each of the gel substrates. The collagen type-I gel mixture was prepared as above (3 mg/ml) and 5 μl of the mixture was used to coat the well. Similarly, another well was coated with 5 μl of Geltrex™ (Invitrogen; Landsmeer; Cat. No. A1413201). Geltrex™ has, according to the manufacturer's documentation, as its major components, laminin, collagen type-IV, entactin and heparin sulfate proteoglycans and has a total protein concentration of 12–18 mg/ml. A combination of collagen type-I and Geltrex™ with a final concentration of 1.5 mg/ml and 6–9 mg/ml, respectively, were also used as a substratum (5 μl per well). After coating the wells with the gel mixtures, the plate was incubated at 37 °C for 30 min. The wells were then rinsed with the LDF medium. Finally, 20 *kdrl:GFP* EBs per 250 μl of EB maturation medium was added to each well.

2.4.7. Culture of kdrl:GFP EBs in a 3D gel matrix

In this experiment, the *kdrl:GFP* EBs were embedded and cultured in a 3D gel matrix composed of collagen type-I, Geltrex™ and fibrin (2.5, 6–9 and 2 mg/ml, respectively). Before embedding EBs in the gel, a well of the CS16-chambered cover glass plate was coated with 5 μl of the gel mixture. The plate was incubated at 37 °C for 30 min. To make a 3D culture, the calculated volumes of the gel constituents were mixed with the EBs isolated from the hanging drop cultures. Ten microliters of the gel mixture containing 20 EBs were then plated in the pre-coated well. The gel with the EBs was allowed to polymerize for 30 min at 28 °C. Finally, 250 μl of EB maturation medium was added to the well. The cultures were maintained at 28 °C and were imaged on consecutive days under the confocal microscope.

2.5. Image analysis

Selected embryoid bodies showing colonies of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells were imaged on consecutive days using a confocal microscope (Axio observer inverted microscope A1). The *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells were visualized with 488 nm wavelength excitation light for imaging. Image-J software, version 1.46r [48] was used to reconstruct the images for further analysis. The EB cultures were observed from day one until day 12 to analyze changes in the following measurements of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in culture. For all the measurements, a pre-calibrated scale was used.

2.6. Measurement of the area covered by *fli:GFP*$^+$ cells per EB

The area covered by *fli:GFP*$^+$ cells around the EB, at each time-point, on different substrates, was measured. From these measurements, the percentage change in area covered by *fli:GFP*$^+$ cells at each time-point, compared to day one, was calculated for each individual EB.

2.7. Measurement of *kdrl:GFP*$^+$ cell networks in EBs

The *kdrl:GFP*$^+$ cell network formed in the EBs was measured from the confocal images at consecutive days. The parameters for these measurements were: (i) lengths of individual *kdrl:GFP*$^+$ branches per EB; (ii) average width of the branches; (iii) number of branches per EB and (iv) total length of the *kdrl:GFP*$^+$ cell network per EB.

Calculation of connectedness of the kdrl:GFP$^+$ *cell network.*

The connectedness of the *kdrl:GFP*$^+$ cell networks formed per EB was calculated using the following formula:

$$\text{Network connectedness} = \frac{\textit{Number of endpoints}}{\textit{number of junctions}}$$

Theoretically, for a well-connected network, the value obtained should be close to zero [49].

2.8. Statistical analysis

The percentages of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells per well, recorded in 6 replicates (wells) for each condition at each time-point, were analyzed for means and standard errors using SPSS software version 21.0. Area covered by *fli:GFP*$^+$ cells, and measurements of *kdrl:GFP*$^+$ cell networks, were analyzed for mean and standard error per EB using SPSS software. One-way ANOVA was performed to calculate the probability values in order to analyze variation between different conditions. Pair-wise comparisons of conditions having more than two groups were evaluated by the Post-Hoc Tukey's test. The comparisons showing p values of 0.05 or less were considered significantly different.

2.9. Transcriptome analysis of cell cultures

2.9.1. Preparation of cell cultures for RNA isolation

A total number of 14 RNA samples were extracted from the blastocyst cells and EB cultures at successive time points (day 0, day 2, day 4 and day 6). Two replicates were established for both culture types for each time point. The gene expression in the blastocyst embryos (3.5 h post-fertilization) was considered as day 0 time-points for both culture types. The blastocyst embryos were used to isolate embryonic cells, which were then used to establish EB and blastocyst cell cultures (according to the above-mentioned procedures). The blastocyst cell cultures from day 0 and the EB cultures from day 4 (after isolation from HD culture) were maintained in 48-well plates. All the cultures were maintained in LDF:EGS medium supplemented with 40 ng/ml $VEGF_{165}$.

2.9.2. RNA isolation from culture

For RNA isolation at day 0, a total number of 20 blastocyst embryos were dechorionated, washed with cell culture medium and then mixed with 500 μl of Trizole, in a 1.5 ml Eppendorf tube. For RNA isolation at subsequent days of cultures, the medium was removed from 20 EBs or one well of 48-well plate, and 500 μl Trizole was added. The cells/EBs suspended in Trizole were mixed well using a 1 ml syringe with a 21-gauge needle, in order to mechanically disrupt the cell membrane. The solutions were left at room temperature for 5 min. Then, 100 μl of chloroform was added to each tube and shacked vigorously. The tubes were left at room temperature for 2–3 min. The tubes were then centrifuged at 12,000 g for 15 min at 4 °C. The aqueous phase containing RNA was transferred to a new tube. The RNA was precipitated using 250 μl of isopropanol and then centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was removed, and the RNA pellet was washed once with 75% ethanol. The ethanol was discarded after centrifugation and the pellet was allowed to air dry for 20–30 min. The RNA pellet was resuspended in 40 μl of RNAse free water, incubated in a heat block at 56 °C for 10–15 min, and stored at −70 °C until transcriptome analysis.

2.9.3. RNA-seq and bioinformatics analysis

All 14 RNA samples were sent to BaseClear (Leiden, Netherlands) for RNA sequencing. The RNA samples were thoroughly quality checked before transcriptome analysis. For each of the 14 samples, single-end sequence reads were generated using the Illumina HiSeq 2500 system. FASTQ sequence files were generated using bcl2fastq2 version 2.18 (Illumina's software). To get clean data, initial data quality was assessed by passing the Illumina Chastity filtering. Then, those reads containing PhiX control signal were removed by an in-house filtering script (offered by BaseClear). Finally, reads containing (partial) adapters were clipped (up to minimum read length of 50bp). After clipping, we got clean data. Then with Tophat 2 [50], we aligned RNA-Seq reads to the zebrafish genome (downloaded from Ensembl [51]). After alignment, we assembled the aligned RNA reads and calculated the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) value for the transcripts with Cufflinks package [52]. Finally, we did reads normalization and got the relative genes expression with R package cummeRbund version 2.28.0.

3. Results

3.1. Medium composition effects the development of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in blastocyst cell culture

3.1.1. Effect of medium composition on quantification of *fli:GFP*$^+$ cells

The blastocyst cells isolated from *fli:GFP* embryos showed expression of the *fli:GFP* marker in cells in culture medium with or without endothelial growth supplements. Blastocyst cells formed embryoid bodies within the first 24 h, and these cultures contained 2.2 ± 0.4% *fli:GFP*$^+$ cells. Initially, the *fli:GFP* expression was observed only on the periphery of the embryoid bodies (Fig. 1A). In the subsequent days, spreading of the *fli:GFP*$^+$ cells were observed around the EBs (Fig. 1B). After day 4 in basic LDF medium, the population *fli:GFP*$^+$ cells declined significantly (Figs. 1C and 2A). However, in the LDF medium with added endothelial growth medium or supplements, the population of *fli:GFP*$^+$ cells could be maintained up to eight days (Fig. 1D, E, F and Fig. 2A).

The *fli:GFP*$^+$ cells were flattened and did not form the networks seen with *kdrl:GFP*$^+$ (see below). Different medium compositions used to culture zebrafish blastocyst cells showed significant differences in the percentage of *fli:GFP*$^+$ cells in cultures (Fig. 2A). The percentage of *fli:GFP*$^+$ cells were significantly higher in supplemented LDF, LDF:EGM and LDF:EGS media compared to the basic LDF medium ($p < 0.001$). When different supplemented LDF media were compared, the percentage of *fli:GFP*$^+$ cells were significantly higher in cultures maintained on LDF:EGM and LDF:EGS media compared to the cultures maintained on supplemented LDF medium at each time-point (Fig. 2A). No significant differences in the percentage of *fli:GFP*$^+$ cells were observed between LDF:EGM and LDF:EGS medium except on day 6, when a higher percentage of *fli:GFP*$^+$ cells were found in LDF:EGM medium

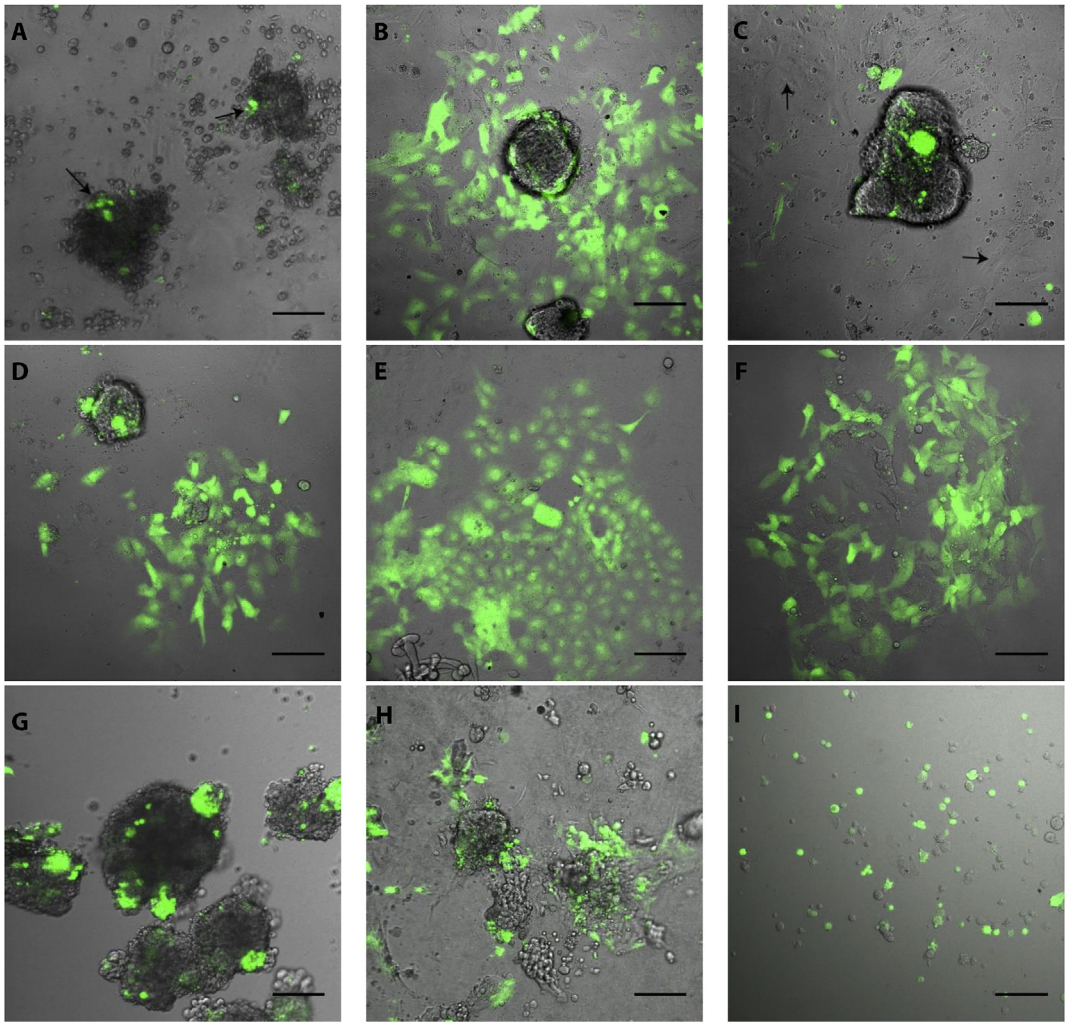

Fig. 1. Formation of *fli:GFP*$^+$ cell colonies in zebrafish blastocyst cell culture under different conditions. (**A**) Within the initial 24 h of culture, the cells combine to form aggregates called embryoid bodies. Arrows showing the appearance of *fli:GFP*$^+$ cells on the periphery of embryoid bodies. (**B**) More *fli:GFP*$^+$ cells appear and proliferate around the embryoid bodies by day 4 of culture. (**C**) By day 6 in basic LDF medium (with no additional supplementation) the *fli:GFP*$^+$ cells diminish in culture, arrows showing elongated, non-GFP, fibroblast-like cells continue to proliferate around the EBs. More *fli:GFP*$^+$ cells were observed on day 6 in cultures maintained with (**D**) supplemented LDF,(**E**) LDF:EGM and (**F**) LDF:EGS media compared to the basic LDF medium. (**G**) The blastocyst cells cultured on gelatin-coated wells, showing the *fli:GFP*$^+$ cells on the periphery of embryoid bodies. (**H**) On collagen type-I substratum comparatively smaller embryoid bodies are formed with the outgrowths of *fli:GFP*$^+$ cells. (**I**) Counting of *fli:GFP*$^+$ cells in the cells isolated from the blastocyst cell cultures. All the images are overlaid confocal image showing *fli:GFP*$^+$ cells, and a phase-contrast image. Scale bar = 100 μm.

($p < 0.01$). Although the percentage of *fli:GFP*$^+$ cells was higher in cultures maintained on LDF:EGM medium, a lower number of total cells (9584 ± 733) was harvested per well for this medium on day 8 of culture, compared to the LDF:EGS (12,987 ± 1092) and supplemented LDF medium (20,457 ± 880).

3.1.2. Percentage of kdrl:GFP$^+$ *cells in cultures maintained with different medium compositions*

The percentage of *kdrl:GFP*$^+$ cells was similar in different medium compositions until day 4 of culture (Fig. 2B). After day 4 the blastocyst cells cultured in the basic and supplemented LDF media contained a significantly lower percentage of *kdrl:GFP*$^+$ cells compared to the LDF:EGS and LDF:EGM media. The percentage of *kdrl:GFP*$^+$ cells in cultures maintained with LDF:EGS medium was slightly higher compared to the LDF:EGM medium; however the differences were not significant. 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 the basic and supplemented LDF media 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. A decrease in the percentage of *kdrl:GFP*$^+$ cells was also observed in LDF:EGM and LDF:EGS media between day 4 and day 8 ($p < 0.05$). The highest percentage of *kdrl:GFP*$^+$ cells (4.2 ± 0.3%) was found on day 4 in cultures maintained in LDF:EGS medium.

3.2. Effect of culture substrate on the quantification of fli:GFP$^+$ *and kdrl:GFP*$^+$ *cells in blastocyst cell culture*

3.2.1. Effect of substratum on the percentage of fli:GFP$^+$ *cells*

A higher percentage of *fli:GFP*$^+$ cells was found in cultures on collagen type-I compared to gelatin substratum (Fig. 3A). The cells were also cultured without any substrate coating on the polystyrene surface of the tissue culture plate for comparison. All the cultures were maintained in LDF:EGS medium. Compared to the uncoated wells, the percentage of *fli:GFP*$^+$ cells was slightly lower in gelatin-coated wells, and higher in collagen type-I coated wells. The images showed more flattened morphology of *fli:GFP*$^+$ cells on collagen type-I and polystyrene substrates, compared to gelatin substratum where the *fli:GFP*$^+$ cells remained on the periphery of the embryoid bodies (Fig. 1). The percentage of *fli:GFP*$^+$ cells in samples isolated on day 8 of the cultures was

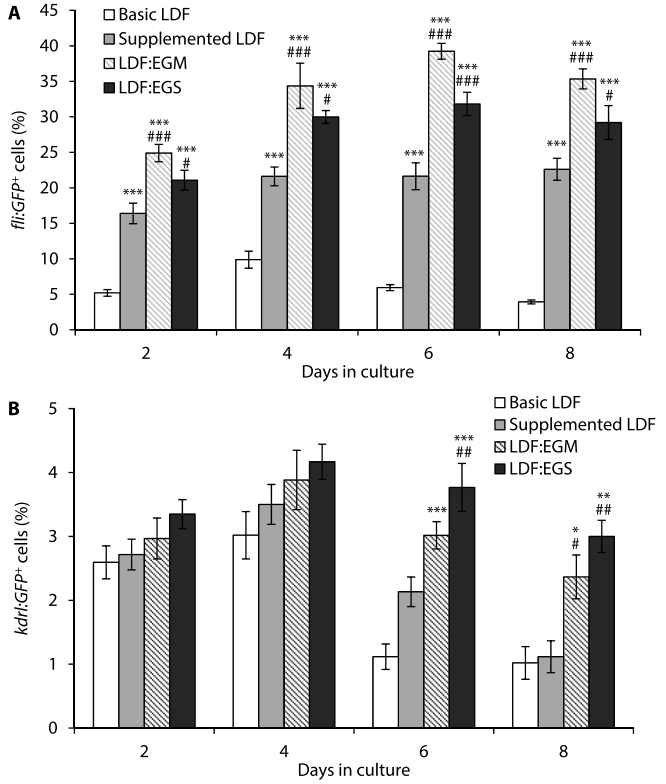

Fig. 2. Percent quantification of (A) *fli:GFP*$^+$ cells and (B) *kdrl:GFP*$^+$ cells in blastocyst cell cultures over time. (**A**) The graph shows a significant increase in the percentage of *fli:GFP*$^+$ cells with the addition of endothelial growth supplements to the medium, compared to the basic LDF medium. (**B**) The blastocyst cells cultured in LDF:EGS medium maintained a higher percentage of *kdrl:GFP*$^+$ cells until day 8 of culture. In other media compositions the percentage of *kdrl:GFP*$^+$ cells dropped significantly after day 4 of culture. The number of observations was six per medium per time-point. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to basic LDF medium; ###, $p < 0.001$, ##, $p < 0.01$, #, $p < 0.05$ compared to supplemented LDF medium).

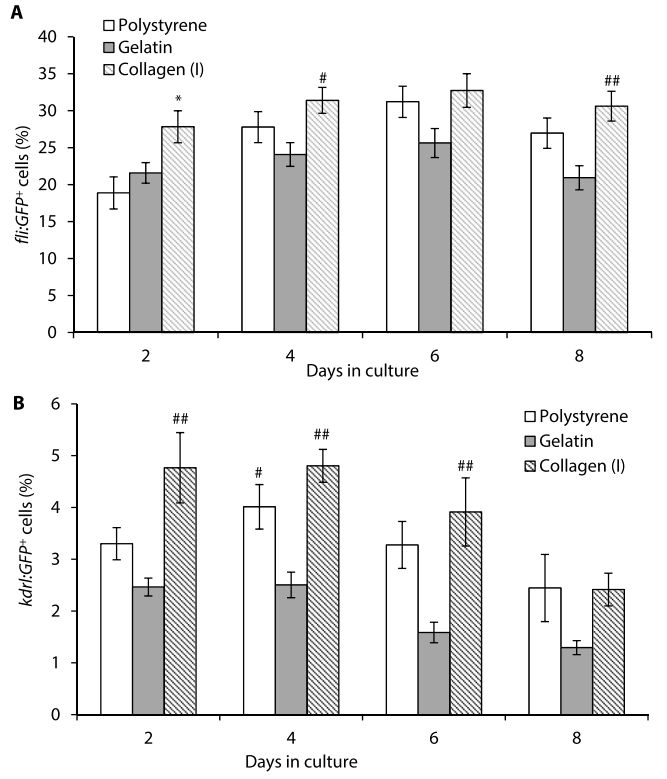

Fig. 3. Percentage of (A) *fli:GFP*$^+$ cells and (B) *kdrl:GFP*$^+$ cells in cultures maintained on different substrates. (**A**) The graph shows a higher percentage of *fli:GFP*$^+$ cells on collagen type-I compared to gelatin substratum on days 4 and 8. (**B**) Similarly, a higher percentage of *kdrl:GFP*$^+$ was observed on collagen type-I compared to gelatin substratum on days 2, 4 and 8. The number of observations was six per substrate per time-point. Error bars represent standard error. (*, $p < 0.05$ compared to polystyrene; ##, $p < 0.01$, #, $p < 0.05$ compared to gelatin).

significantly higher on collagen type-I (30.6 ± 2.0%) compared to gelatin (20.9 ± 1.6%; $p < 0.01$) substratum (Fig. 3A).

3.2.2. Effect of substratum on the percentage of kdrl:GFP$^+$ cells

The blastocyst cells cultured on different substrates showed a higher percentage of *kdrl:GFP*$^+$ cells on collagen type-I substratum compared to gelatin substratum at different time-points (Fig. 3B). The percentages of *kdrl:GFP*$^+$ cells obtained from these substrates were compared with cultures on tissue culture-treated polystyrene surface without coating. However, the differences between collagen type-I and the polystyrene substrate were not significant. The percentage of *kdrl:GFP*$^+$ cells dropped significantly from day 4 to day 8 on all the three substrates.

3.3. VEGF effects the quantification of fli:GFP$^+$ and kdrl:GFP$^+$ cells in blastocyst cell culture

3.3.1. Effect of VEGF on the percentage of fli:GFP$^+$ cells

The recombinant zebrafish vascular endothelial growth factor ($VEGF_{165}$) protein showed a significant effect on the percentage of *fli:GFP*$^+$ cells in cultures (Fig. 4A). A higher percentage of *fli:GFP*$^+$ cells was observed in cultures with 10–40 ng/ml $VEGF_{165}$ in the medium compared to cultures maintained on medium without VEGF, at each of the time point ($p < 0.001$). On day 2 of the cultures, 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). However, after day 4 the cells cultured on medium with 40 ng/ml $VEGF_{165}$ contained a significantly higher percentage of *fli:GFP*$^+$ cells compared to 10 ng/ml $VEGF_{165}$ ($p < 0.01$ for day 4 and day 6; $p < 0.001$ for day 8). No significant differences in the percentage *fli:GFP*$^+$ cells was observed in blastocyst cells cultured on 10 and 20 ng/ml $VEGF_{165}$, at different time points except on day 8 ($p < 0.05$). Similarly, no significant differences were observed between 20 and 40 ng/ml $VEGF_{165}$ cultures at different time points (Fig. 4A).

3.3.2. Percentage of kdrl:GFP$^+$ cells in media with different VEGF concentrations

No significant differences in the percentage of *kdrl:GFP*$^+$ cells were observed with different $VEGF_{165}$ concentrations on day 2 of culture. From day 2–4 the percentage of *kdrl:GFP*$^+$ cells increased in all cultures; however, the increase was greater in cultures with 40 ng/ml $VEGF_{165}$ compared to cultures without $VEGF_{165}$ in the medium (Fig. 4B). In the subsequent days the percentage of *kdrl:GFP*$^+$ cells decreased significantly in cultures without $VEGF_{165}$ ($P < 0.01$). However, in cultures with 20 and 40 ng/ml $VEGF_{165}$ a higher percentage of *kdrl:GFP*$^+$ cells was maintained until day 8. This resulted in a higher percentage of *kdrl:GFP*$^+$ cells in cultures with $VEGF_{165}$ compared to cultures without $VEGF_{165}$ on days 6 and 8.

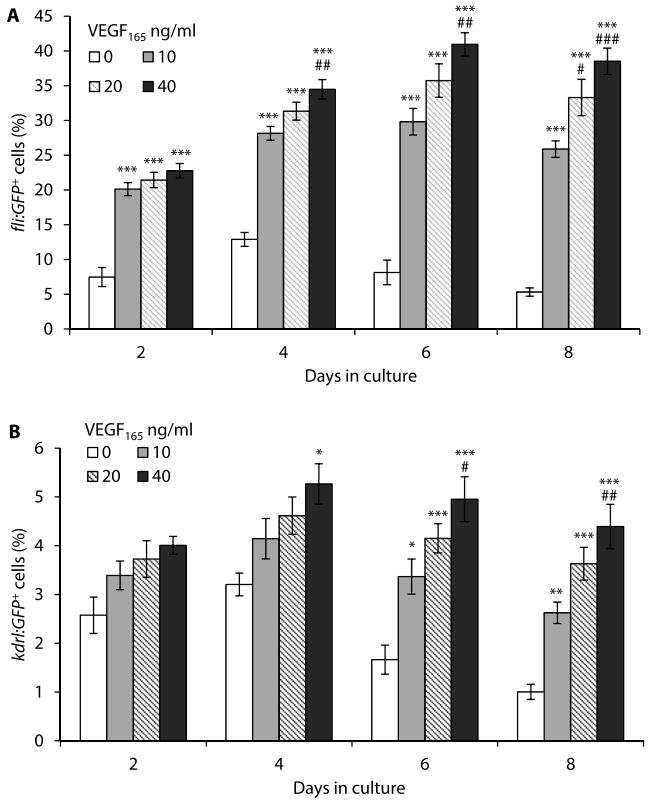

Fig. 4. Percentage of (A) *fli:GFP*$^+$ cells and (B) *kdrl:GFP*$^+$ cells in cultures maintained on increasing concentrations of zebrafish VEGF$_{165}$. (**A**) A significant increase in the percentage of *fli:GFP*$^+$ cells is shown in cultures maintained on medium with (10, 20 or 40 ng/ml) VEGF$_{165}$ compared to cultures on medium without (0 ng/ml) VEGF$_{165}$ at different time-points. (**B**) The blastocyst cells cultured in the presence of VEGF$_{165}$ maintained a significantly higher percentage of *kdrl:GFP*$^+$ cells compared to cultures without VEGF$_{165}$ overtime. The number of observations was six per VEGF concentration per time-point. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to cultures without VEGF$_{165}$ in the medium; ###, $p < 0.001$, ##, $p < 0.01$, #, $p < 0.05$ compared to medium with 10 ng/ml VEGF$_{165}$).

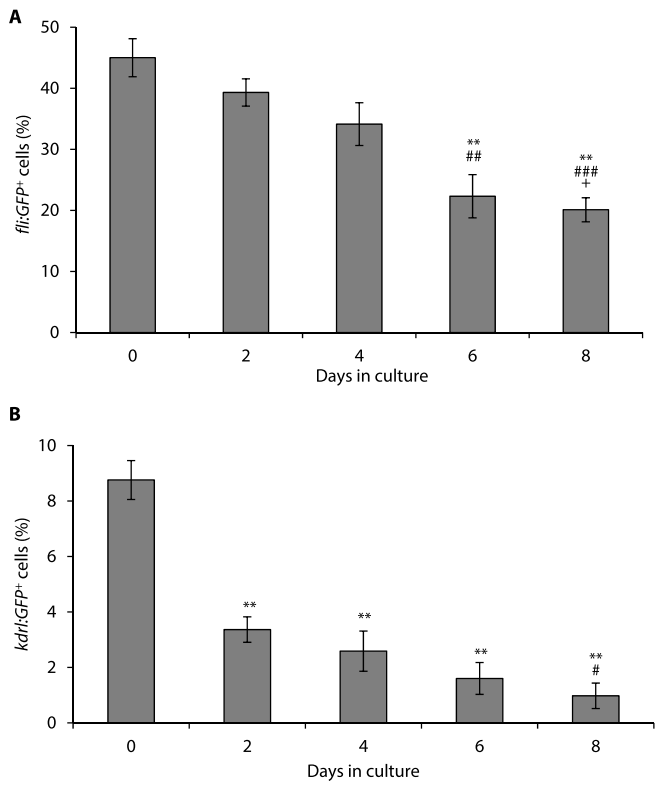

Fig. 5. Changes in the percentage of (A) *fli:GFP*$^+$ cells and (B) *kdrl:GFP*$^+$ cells over time in EB cultures. Day zero for EBs is the day four of hanging drop cultures. The EB cells were isolated from cultures at each time-point (days 2, 4, 6 and 8) to quantify the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells. (**A**) A decrease in the percentage of *fli:GFP*$^+$ cells can be observed at each time-point. (**B**) An abrupt decrease from day 0 to day 2 and then a gradual decrease after day 2 can be seen in the percentage of *kdrl:GFP*$^+$ cells. The number of observations was six per time-point. Error bars represent standard error. (**, $p < 0.001$ compared to day 0; ###, $p < 0.001$, ##, $p < 0.01$, #, $p < 0.05$ compared to day 2; +, $p < 0.05$ compared to day 4).

3.4. Quantification of fli:GFP$^+$ *and kdrl:GFP*$^+$ *cells in EBs developed in hanging drop cultures*

3.4.1. Percentage of fli:GFP$^+$ *cells in EBs*

The *fli:GFP* EBs contained a high percentage of *fli:GFP*$^+$ cells on day 0 (45.0 ± 3.1%), i.e. directly after isolation from the hanging drop cultures. When transferred to a conventional 96-well plate, the percentage of *fli:GFP*$^+$ cells dropped gradually with time (Fig. 5A). No significant decrease in the percentage of *fli:GFP*$^+$ cells was observed, on day 2 and day 4 after transferring to the 96-well plate. However, compared to day 2, a significant decrease in the percentage of *fli:GFP*$^+$ cells was observed on day 6 ($p < 0.01$) and day 8 ($p < 0.001$). The percentage of *fli:GFP*$^+$ cells in cultures on days 6 and 8 was less than half of the percentage found in EBs on day 0 ($p < 0.001$).

3.4.2. Percentage of kdrl:GFP$^+$ *cells in EBs*

Similar to the results obtained for *fli:GFP*$^+$ cells, a higher percentage of *kdrl:GFP*$^+$ cells was found in EBs on day 0 (8.7 ± 0.7%; Fig. 5B), i.e. directly after isolating from the hanging drop cultures, compared to the following days. These EBs, when transferred to a 96-well plate on conventional substratum, showed a significant decrease in the percentage of *kdrl:GFP*$^+$ cells on day 2 (3.4 ± 0.5%; $p < 0.001$). This was followed by a gradual decrease at each time-point until day 8 (Fig. 5B). The percentage of *kdrl:GFP*$^+$ cells in EB cultures on day 8 was significantly less than the percentage value on day 2 ($p < 0.05$).

3.4.3. Percentage of fli:GFP$^+$ *cells in EB secondary culture*

In this experiment, the *fli:GFP* EB cultures maintained for eight days in a 24-well plate were isolated by trypsinization and sub-cultured on a new plate. The sub-cultured (passage 1) cells were maintained in the medium for four days (Fig. 6A) and then isolated and counted for the percentage of *fli:GFP*$^+$ cells. The passage 1 EB cultures contained 15.1 ± 1.9% *fli:GFP*$^+$ cells. These cultures were then re-plated for another passage (passage 2) and maintained for another four days. The percentage of *fli:GFP*$^+$ cells in passage 2 cultures was 13.1 ± 0.8%. Although the percentage of *fli:GFP*$^+$ cells was more or less stable in sub-cultures, the intensity of the GFP signal from the cells greatly reduced in the second passage (Fig. 6B).

3.5. Development of fli:GFP$^+$ *and kdrl:GFP*$^+$ *cells in EB culture*

3.5.1. Increase in the area covered by fli:GFP$^+$ *cells in EB culture on 2D substrates*

The *fli:GFP*$^+$ cells propagated in the form of a monolayer around the EBs (Fig. 6D–F). The area covered by the *fli:GFP*$^+$ cells emerging from the EBs on different substrates (i.e. collagen type-I, gelatin and fibrin) was measured on every second day (day 2, 4, 6, 8, 10 and 12) from the confocal images. The percentage increase in surface area covered by *fli:GFP*$^+$ cells, compared to the same value on day 1, was calculated at each time-point for individual EBs. On collagen type-I substratum, a

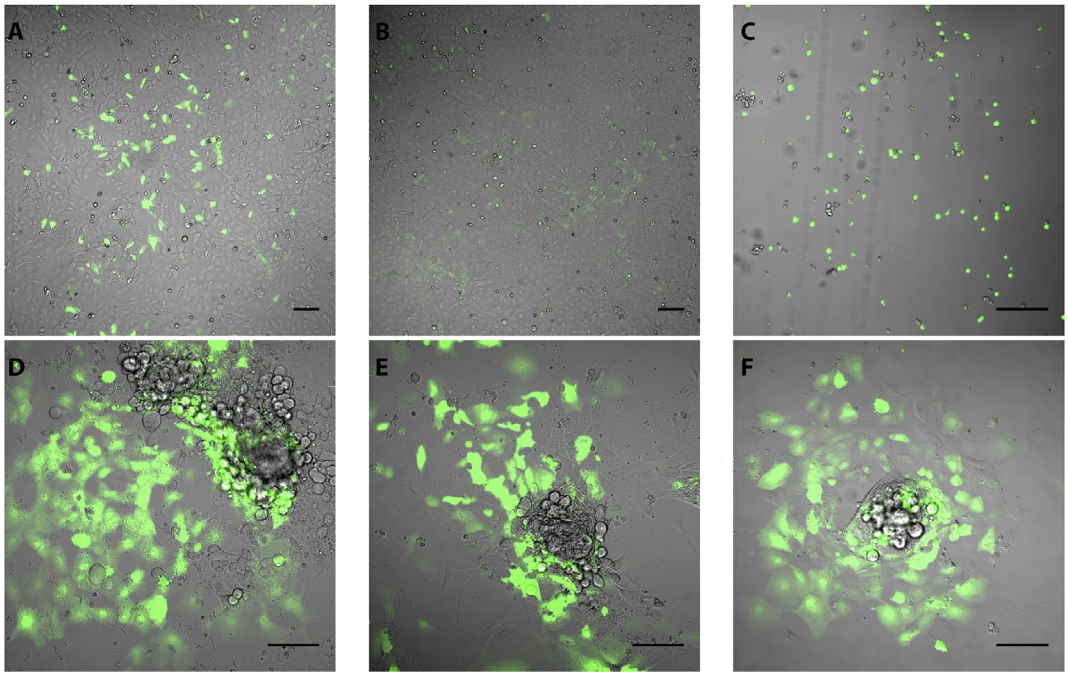

Fig. 6. Confocal images showing *fli:GFP*$^+$ cells in EB cultures. The EB cells not expressing GFP are shown in phase contrast overlaid. (**A**) Secondary EB culture (passage 1) on day 4 showing *fli:GFP* expression in multiple cells. (**B**) Secondary EB culture (passage 2) the intensity of the signal is visibly lower than the passage 1 cells. (**C**) Drop of cell suspension used for the counting of *fli:GFP*$^+$ cells in the cell-isolates from the EB cultures. (**D**) Spreading of *fli:GFP*$^+$ cells in EB culture on collagen type-I substratum on day 6. (**E**) EB cultured on gelatin substratum on day 6. (**F**) EB cultured on fibrin substratum on day 6. Scale bar = 100 μm.

significant increase in area covered by *fli:GFP*$^+$ cells was observed between day 6 and day 8 ($p < 0.05$). On gelatin substratum, the area covered by *fli:GFP*$^+$ cells slowly increased from day 2 to day 6 ($p < 0.01$). On fibrin substratum, the area covered by *fli:GFP*$^+$ cells increased significantly from day 2 to day 4 ($p < 0.001$). After day 8, no further increase in the area covered by *fli:GFP*$^+$ cells was observed on any of the three substrates.

Differences in percent increase in area covered by *fli:GFP*$^+$ cells were observed between different substrates from day 2 to day 6 (Fig. 7). During these days of cultures, the *fli:GFP*$^+$ cells covered significantly more area per EB on fibrin substratum compared to gelatin and collagen type-I. Due to the gradual increase in the percent area covered by *fli:GFP*$^+$ on collagen type-I and gelatin substrate, no significant differences were observed between the three substrates after day 8 of culture.

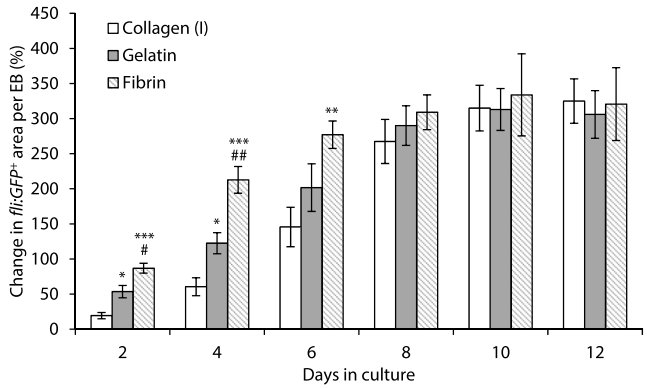

Fig. 7. Percentage increase compared to day 1, in the surface area covered by *fli:GFP*$^+$ cells per EB on different culture substrates. The number of observations was 13 for collagen type-I, 12 for gelatin and nine for fibrin per time-point. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to collagen type-I; ##, $p < 0.001$, #, $p < 0.01$ compared to gelatin).

3.5.2. Network-formation by kdrl:GFP$^+$ cells in 2D and 3D cultures

Unlike the *fli:GFP*$^+$ cells, which showed a fibroblastic morphology in culture, the *kdrl:GFP* EBs showed cell-cell extensions of *kdrl:GFP*$^+$ cells, forming cord or network-like structures on 2D substrates (i.e. collagen type-I, Geltrex™, and combined collagen type-I + Geltrex™), and in 3D gel matrix (composed of collagen type-I, Geltrex™ and fibrin) (Fig. 8). Analysis of total length of the *kdrl:GFP*$^+$ cell network per EB and length of individual *kdrl:GFP*$^+$ branches per EB showed significant differences between 2D and 3D cultures (Fig. 9). In general, network formation by *kdrl:GFP*$^+$ cells in EBs was enhanced in 3D culture, including fibrin as a component, compared to the 2D cultures on other substrates. No significant differences were observed in the total length of the *kdrl:GFP*$^+$ cell network per EB on different 2D substrates except between collage type-I + Geltrex™ and collagen type-I substrate on day 6 ($p < 0.05$).

The total network length per EB was significantly higher in 3D culture containing fibrin as a component, compared to all the three 2D substrates (with no fibrin added) on day 4 and day 6 (Fig. 9A). The network length reduced significantly on 2D collagen type-I substratum from day 2 to day 6 ($p < 0.05$). On 2D Geltrex™ and 2D collage type-I + Geltrex™ substrates, the network length remained similar at subsequent time-points. In 3D culture, a significant reduction in the total network length per EB was observed from day 4 to day 12 ($p < 0.001$). On day 12, the total network length in all the 2D and 3D cultures was similar. The individual *kdrl:GFP*$^+$ branch length per EB was also higher in 3D culture compared to 2D substrates at different time-points (Fig. 9B). Other parameters i.e. the number of *kdrl:GFP*$^+$ branches per EB and average branch width remained similar between the 2D and 3D cultures (data not shown).

3.5.3. Connectedness of the kdrl:GFP$^+$ network

The connectedness values of *kdrl:GFP*$^+$ cell networks on different substrates are given in Fig. 10. On 2D collagen type-I + Geltrex™ and in 3D collagen type-I + Geltrex™ + fibrin gel matrix, the network formed

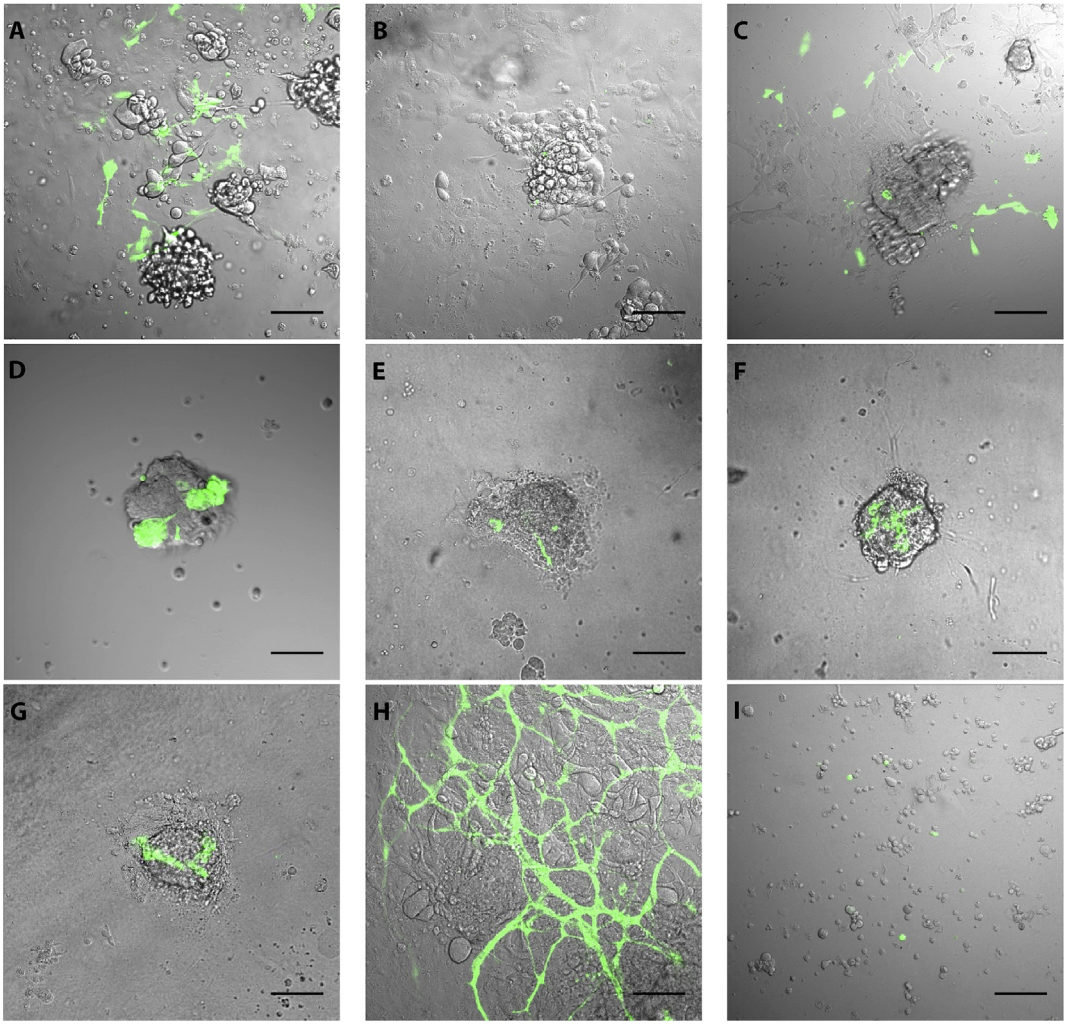

Fig. 8. Development of EBs from zebrafish *kdrl:GFP* blastocyst cells. (**A**) The blastocyst cells cultured on plastic on day 4 contain *kdrl:GFP*$^+$ cells. (**B**) By day 6 the number of *kdrl:GFP*$^+$ cells diminishes on plastic. (**C**) On collagen type-I substratum the *kdrl:GFP*$^+$ cells can still be observed on day 6 of culture (**D**) *kdrl:GFP* embryoid body on day 4 of hanging drop culture. (**E**) When transferred to adherent culture the *kdrl:GFP*$^+$ cells make cell-to-cell extensions. The image on day 4 of the adherent culture maintained on 2D collagen type-I substratum. (**F**) On 2D Geltrex™ susbtratum the *kdrl:GFP*$^+$ cell network form inside the EB. (**G**) EB on 2D collagen type I + Geltrex™ substratum on day 6 of culture. (**H**) More extensive network formation in 3D collagen type-I + Geltrex™ + fibrin gel matrix. The image was taken from the junction of three adjacent EBs. (**I**) Quantification of *kdrl:GFP*$^+$ cells in a drop of cell suspension isolated from culture. Scale bar = 100 μm.

by the *kdrl:GFP*$^+$ cells was more connected compared to collagen type-I substratum. In 3D gel matrix, the connectedness values remained constant over the 12 days of culture. While on 2D collagen type-I substratum the *kdrl:GFP*$^+$ cell network was well connected on day 1 of culture with lower endpoints divided by junctions value. However, the connectedness of the network on this substratum was lost with the duration of culture.

3.6. Transcriptome profiling of blastocyst cell and EB culture

Transcriptome analysis of the blastocyst cell and EB cultures revealed the differential expression of certain genes in both cultures (Fig. 11). The blastocyst cell culture represented an adherent culture while the EBs were cultured in suspension (HD) until day 4. The FPKM values of the transcripts showed that certain endothelial differentiation markers were expressed at a higher level, on day 6 of culture, in EB cultures compared to the blastocyst cell cultures. These markers included both the VEGF variants (vegfab and vegfaa), and several genes of the TGFβ family (tgfbrap1, tgfbr2a, tgfbr1b, tgfbr1a, tgfb2, tgfb1b and tgfb1a). On the preceding days of cultures, the FPKM values of these genes were similar in both culture types. One endothelial differentiation marker 'tgfbi' showed higher FPKM values in blastocyst cell cultures compared to the EB cultures on all time-points. The marker 'tgfbrap1' showed a higher expression in early blastocyst embryos compared to the cultured cells.

Among the endothelial maturation markers, the FPKM values of the Notch receptors (notch1a and notch1b) were higher in EB cultures compared to the blastocyst cell cultures on all time-points (Fig. 11). However, the Notch ligands (jag1a, jag1b and jag2a) showed little variation in their FPKM values among the two culture types. The pluripotency marker pou5f3 expressed at a high level in early blastocyst embryos. No expression of pou5f3 was detected in blastocyst cell cultures at all time-points. In EB cultures, however, a small expression of pou5f3 was observed in one replicate on day 2 of culture. The proliferation marker eef1a1l1 showed higher FPKM values in the adherent blastocyst cell culture compared to the EB cultures, in which the FPKM values gradually diminished with culture duration.

4. Discussion

We have investigated different culture conditions for zebrafish blastocyst cells, with the objective of generating differentiated endothelial-like *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in relatively high numbers. We have shown that the endothelial differentiation process in zebrafish

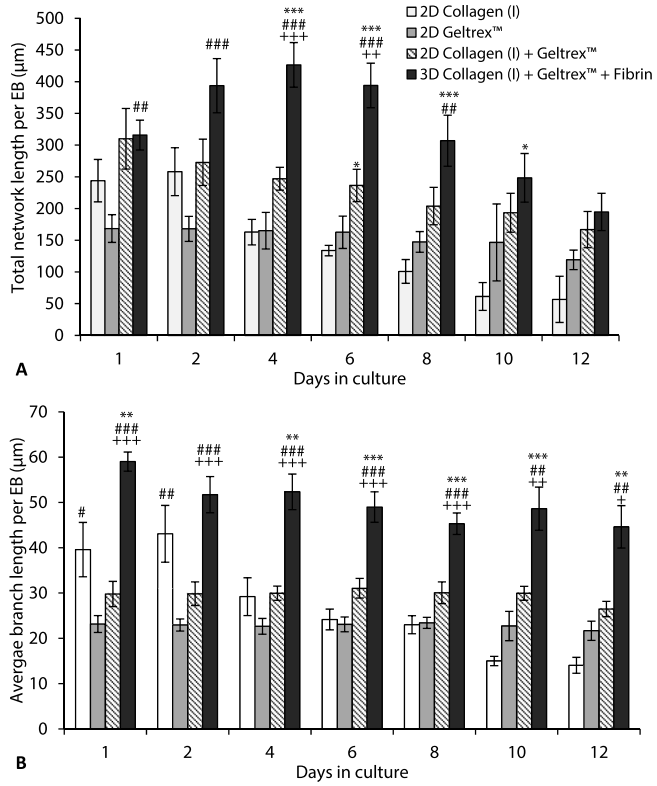

Fig. 9. Changes with time in the parameters of *kdrl:GFP*$^+$ cell networks on different substrates. (**A**) Total length of *kdrl:GFP*$^+$ cell network per EB on different 2D substrates and in 3D gel matrix. (**B**) Average branch length per EB of *kdrl:GFP*$^+$ cell network on different 2D substrates and in 3D gel matrix. The graphs shows higher length of *kdrl:GFP*$^+$ cell network in 3D culture compared to 2D culture. Number of observations: 14 for 2D collagen type-I, 12 for 2D Geltrex™, 11 for 2D collagen type-I + Geltrex™ and 11 for 3D collagen type-I + Geltrex™ + Fibrin. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to 2D collagen (I); ###, $p < 0.001$, ##, $p < 0.01$, #, $p < 0.05$ compared to 2D Geltrex™; +++, $p < 0.001$, ++, $p < 0.01$, +, $p < 0.05$ compared to 2D collagen (I) + Geltrex™).

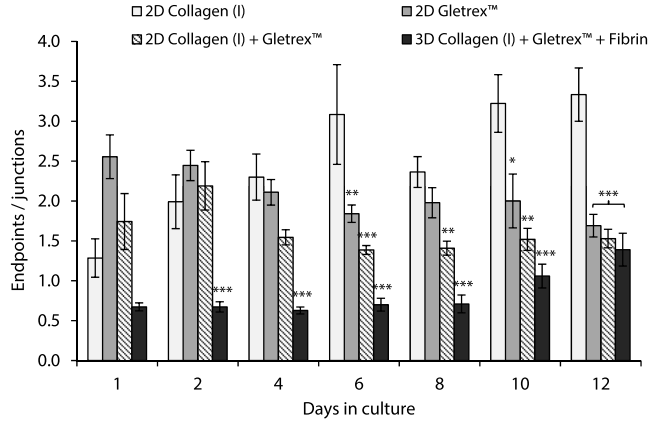

Fig. 10. Connectedness of *kdrl:GFP*$^+$ cell network on different substrates. Values nearest to zero on the vertical axis show a well-connected network. On collagen type-I substratum the network connectedness deteriorate with time, while on 2D collagen type-I + Geltrex™ substrate and in 3D collagen type-I + Geltrex™ + fibrin matrix comparatively well-connected network is maintained until the end of culture. Error bars represent standard error. (***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$ compared to collagen type-I substratum).

blastocyst cell cultures is critically influenced by the culture conditions. We also found that the ability to form network-like cell connections is largely confined to the *kdrl:GFP*$^+$ cells and 3D culture. Here we discuss the important factors which influence the growth and differentiation of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in culture.

4.1. *fli:GFP*$^+$ versus *kdrl:GFP*$^+$ cells

The blastocyst cell cultures initiated from *fli:GFP* embryos in our experiments expressed GFP in a higher percentage of cells compare to the *kdrl:GFP*$^+$ cellscells. This might be explained by the lineage-specificity of GFP expression in *kdrl:GFP* transgenic lines (endothelial cells only) [53], compared to the *fli:GFP* line (endothelial, lymphatic, hematopoietic, some yolk sac and neural crest cells) [37]. The differences in morphology and growth pattern of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells, in our study, also showed the specificity of *kdrl:GFP* marker for endothelial cells. In contrast to the fibroblast-like morphology of *fli:GFP*$^+$ cells, the endothelial-like morphology of *kdrl:GFP*$^+$ cells is manifested by the network formation of these cells. In some cases the network or cord-like structures could also be observed in *fli:GFP*$^+$ blastocyst and EB cultures; however, it was difficult to differentiate it from the other fibroblastic GFP$^+$ cells in these cultures.

The *kdrl:GFP*$^+$ cells disappeared after a maximum of eight days under all of the culture conditions tested in blastocyst cell culture. One of the reasons for this could be apoptosis, as reported in a recent study using *kdrl:GFP* blastocyst cells for screening angiogenic and anti-angiogenic compounds [54]. However, in contrast to Ref. [54], in our EB cultures the *kdrl:GFP*$^+$ cell networks could be observed up to 12 days in culture. Similarly, the *fli:GFP* signals could be observed for up to 12 days of primary cultures and in secondary cultures for up to three passages. Thus, under appropriate conditions the *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells can be maintained for longer duration *in vitro*.

Another reason for the disappearance of the *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells in cultures can be considered in the light of previous *in vivo* studies, showing that GFP expression in *fli:GFP* embryos persist at least up to 7 days post fertilization (dpf) [37]. Similarly, studies on mouse embryos and embryonic stem cells have shown a significant reduction in the expression levels of *flk1/kdr* gene at advanced developmental stages [55]. Based on those reports, the results of the current study suggest that as the endothelial cells mature in our zebrafish blastocyst cell cultures, they down-regulate the GFP expressing transgenes.

4.2. EB versus adherent culture

Research on mouse embryonic stem cell culture has shown that EBs grown in attached cultures contained a higher number of total cells and a lower percentage of hematopoietic and endothelial cells, compared to embryoid bodies grown in suspension cultures [56]. Our results in zebrafish embryonic cell cultures are consistent with these findings. In the present study, zebrafish blastocyst cells cultured in basic LDF medium developed a few, large-sized EBs, while cells cultured in LDF medium supplemented with endothelial growth medium, or endothelial growth supplements, developed more numerous, but smaller EBs with a higher percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells. These results suggest a direct relationship between the number of EBs and the percentage of *fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells.

In the adherent cultures, the cells grow in a monolayer around the EBs. Cultures showing few, large-sized EBs contain a higher number of adherent cells in the form of a monolayer. In this monolayer, there is less cell-to-cell contact than there is in the EBs, and it is possible that this relative lack of contact favors the growth or differentiation of cell types other than hematopoietic and endothelial cells, as previously suggested [56]. In contrast, cultures showing more numerous small-size EBs contain a higher number of cells as part of the EBs, where there is more cell-to-cell contact and is apparently favorable for the growth or differentiation of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells. This conclusion is

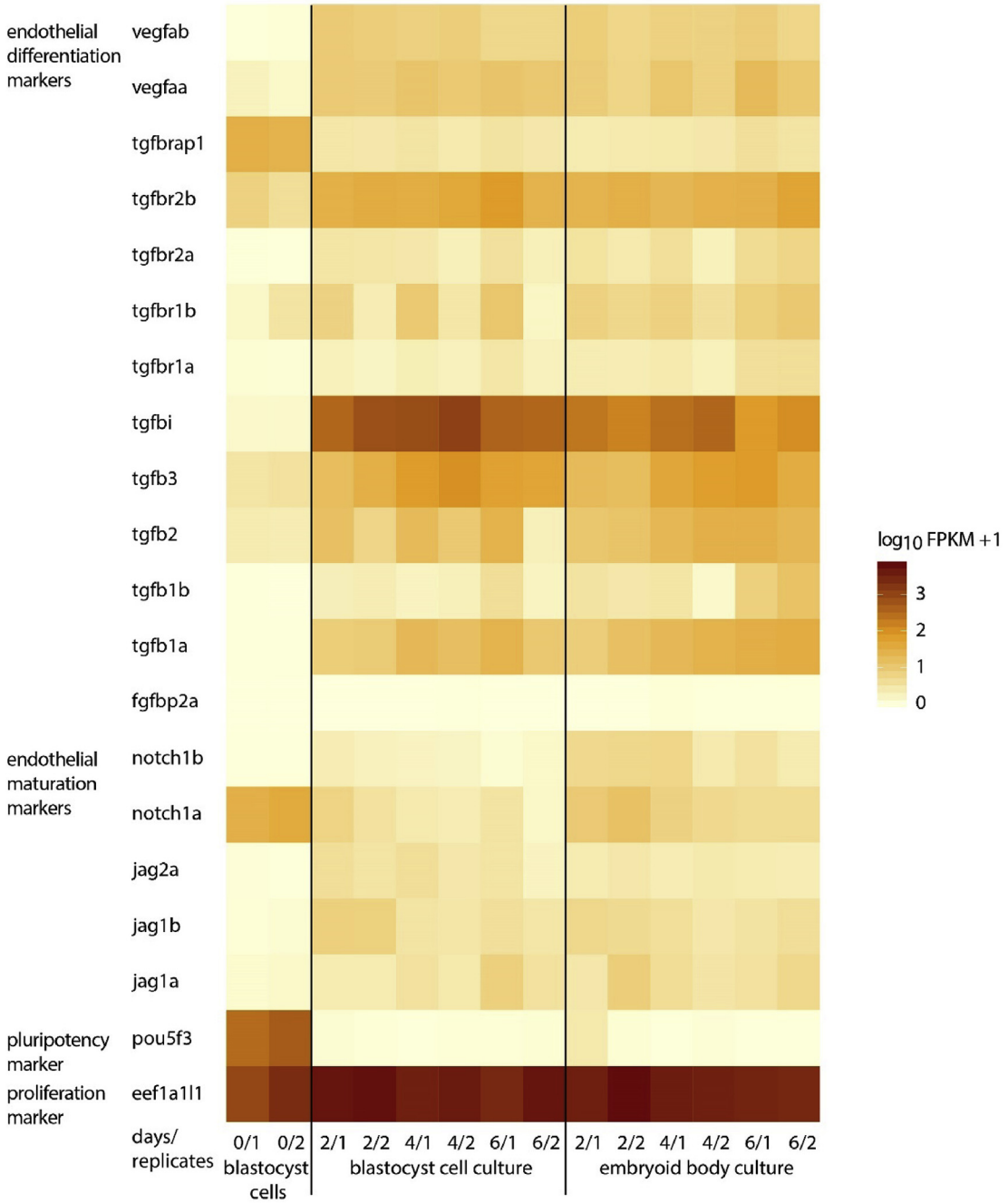

Fig. 11. Heatmap showing the expression values (FPKM) of different genes at subsequent days of blastocyst cells and EB culture. Both culture types were maintained on the same media (LDF:EGS + 40 ng/ml $VEGF_{165}$). The values at day 0 is the expression level of genes in the blastocyst embryos used to initiate both culture types. The number of observations was two for each time-point and culture type.

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. EB intermediate formation has been used as a method of choice to induce specific differentiation in mouse and human ESCs [57].

The transcriptome analysis of endothelial markers of EB and adherent blastocyst cell culture, in our experiments, also showed an increased expression of TGF-β and VEGF in EB cultures. These results suggest that TGF-β and VEGF are candidate genes involved in the differentiation of endothelial-like cells (*fli:GFP*$^+$ or *kdrl:GFP*$^+$ cells), and that their expression is favored by the suspension EB culture. In previous studies, VEGF has been recognized as the main endothelial cell survival and differentiation factor [58]. Similarly, TGF-β has been reported to be involved in the vascular development of early embryos [59]. The transcriptome analysis, in the current study, also showed an increase expression of Notch receptors in the EB cultures. Previous studies have shown that Notch signaling is induced in response to VEGF, promoting the specification of arterial endothelial cells [60]. These results are in accordance with our 3D EB cultures which showed the *kdrl:GFP*$^+$ cells forming vascular network-like structures, unlike the 2D adherent cultures in which the *kdrl:GFP*$^+$ cells showed a less vessels-like morphology, rather growing in a monolayer.

The EB cultures showed a comparatively higher expression of the pluripotency marker (Pou5f) than the blastocyst cell culture on day 2. These results suggest the maintenance of pluripotency for a longer duration in the suspension EB culture compared to the adherent

cultures. This may explain why EB formation has been shown to provide better control over cellular differentiation [57]. Furthermore, reduced proliferation (as was observed in the transcriptome analysis of our EB cultures) has also been reported as a requirement to control the differentiation of ES cells towards specific lineage [57].

Our results show that the zebrafish EB model can be an important tool to study the differentiation of endothelial cells and the formation of vascular networks *in vitro*. It would also be possible to isolate live endothelial (*kdrl:GFP*$^+$) cells from these cultures using fluorescence-activated cell sorting (FACS). The development of vascular networks *in vitro* from these cells under specific conditions can be tracked in real-time using the GFP marker.

4.3. Effects of medium composition

LDF is a commonly-used medium for zebrafish embryonic cell culture [42–45,61]. Zebrafish primary blastocyst cells have more complex nutrient requirements for their growth and attachment [62,63] therefore supplements including FBS, fish embryo extract, fish serum and bFGF are usually added to the medium. A nutrient-rich medium is required, possibly because the initial cell death is high in these cells as a result of embryo sterilization procedure [63]. The blastocyst cells are pluripotent in nature; therefore, specific differentiation pathways can be promoted by selective culture conditions [35]. In some experiments, additional supplementation or a substrate coating may be required to induce specific differentiation in these cells. Examples include sonic hedgehog protein for myocyte differentiation [64], and poly-D-lysine coating for neuron and astrocyte differentiation [44].

Endothelial growth medium (EGM) is usually used to culture human umbilical vein endothelial cells (HUVECs [46,47]), as well as for the differentiation of human pluripotent stem cells into vascular endothelial cells [13]. 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 [12,29] and human [12,31,32] embryonic stem cells. Other components of EGS are heparin and hydrocortisone, which have also been used for endothelial differentiation in human ES cells [32]. Similarly, ascorbic acid found in EGS has also been used in endothelial differentiation medium for mouse ES cell culture [12].

LDF medium supplemented with endothelial growth supplement (EGS) significantly increased the percentage of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in our experiments. LDF is defined as a standard medium in many zebrafish cell culture procedures [42–45,61]. 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 induce maximum differentiation of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells in cultures. The LDF:EGM medium also showed a higher percentage of *fli:GFP*$^+$ cells in our experiments; however, the total number of cells harvested per well from the LDF:EGM medium was lower than the other media that contained LDF as a major component.

To obtain pluripotent embryonic stem cells, zebrafish blastocyst cells have been cultured on a feeder layer of growth-arrested stromal cells in the LDF medium [42,43,65]. Without the support of a feeder layer, the blastocyst cells differentiate into embryoid bodies – that contain various cell types and adherent fibroblast-like cells [62]. In further passages, only the adherent cell type that is best adapted to the medium remains in the culture [61,66]. These studies suggest the suitability of the LDF medium for growth and differentiation of cells other than *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells. However, we found, in this study, 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.

4.4. Effect of substrate composition

Extracellular matrix (ECM) is an important component of tissues *in vivo*, and it directly interacts with cells by receptors and supports their growth and differentiation [67]. Different tissues possess ECM of differing composition and physical properties (stiffness, elasticity, etc.), that influence the behavior and differentiation of cells in that tissue [67]. The same principle applies to the cells *in vitro*. Different ECM substrates have been identified as directing the differentiation of ES cells towards different cell lineages, as is reviewed in Refs. [68]. Some ECM substrates including collagen type I [29,30], collagen-IV [21], and gelatin [12,33], have been used to stimulate endothelial differentiation in mouse embryonic stem cells. Fibronectin has also been used to promote the differentiation of human ES cells along endothelial lineage [32]. In our previous studies, fibronectin substratum was found to increase the attachment of *kdrl:GFP*$^+$ cells recovered from the hearts of 5 dpf zebrafish larvae [39].

In the current study, the blastocyst cells cultured on collagen type-I substratum contained higher percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells, compared to gelatin substratum. However, no significant differences in the percentages of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells were observed between collagen type-I and plastic substrates. This may suggest the suitability of collagen type-I over gelatin for zebrafish cell culture in general. However, it also shows that collagen type-I is not necessary for the differentiation of endothelial cells at the early stages. Research on zebrafish ECM has shown the production of fibronectin and laminin in early developing embryos, and the synthesis of collagen at later stages [69]. Similarly, another study described the role of collagen type I in the development of blood vessels at the latter stages in vascular tube formation [70].

The *in vivo* shift from fibronectin and laminin in early embryos towards collagen type I in later embryos might explain some of our findings *in vitro* (specifically the comparison between blastocyst cultures and EB cultures). In the EB cultures, cells are at an advanced stage of differentiation compared to the blastocyst cell cultures. The *fli:GFP* EB cultures on collagen type-I substratum showed a slow increase in area covered by *fli:GFP*$^+$ cells compared to gelatin and fibrin substrates up to day 6 of culture. And then from day 6 to day 8 a fast increase was recorded on collagen type-I compared to other substrates. The length and connectedness of *kdrl:GFP*$^+$ cell network per EB on the substrates containing both collagen type-I and fibrin was higher compared to pure collagen type-I or Geltrex™ substrates. These results are in accordance with previous studies where the combination of collagen type-I and fibrin has been found to be favorable for vascular network formation from human endothelial progenitor cells [46]. These results may also suggest the requirement of multiple extracellular components for the formation of vascular networks in zebrafish blastocyst cell culture.

4.5. VEGF affects the growth of fli:GFP$^+$ and kdrl:GFP$^+$ cells

VEGF is known to be an important 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 [71]. VEGF has been shown to increase endothelial differentiation in human embryonic stem cell culture [72]. In our experiments, the percentage of *fli:GFP*$^+$ cells was 5.0 fold higher, and *kdrl:GFP*$^+$ cells 2.9 fold higher, in cultures supplemented with 40 ng/ml $VEGF_{165}$ compared to cultures without $VEGF_{165}$. These results are comparable with a previous study on human embryonic stem cells where VEGF at 50 ng/ml has been reported to increase endothelial differentiation by 4.7 fold [72].

5. Conclusions

The growth of endothelial-like (*fli:GFP*$^+$ and *kdrl:GFP*$^+$) cells can be enhanced in zebrafish blastocyst cell cultures by adding endothelial growth supplements and factors to the medium. We describe an

optimized procedure for enhancing differentiation of endothelial-like cells in zebrafish blastocyst cells. The suspension EB culture favored more the differentiation of *fli:GFP*$^+$ and *kdrl:GFP*$^+$ cells and showed a higher expression of endothelial markers, compared to the adherent culture. A combination of different substrate components is required for the formation of vascular-like (*kdrl:GFP*$^+$) networks from zebrafish blastocyst cells *in vitro*. A 3D culture supports the formation of *kdrl:GFP*$^+$ cell networks compared to 2D culture.

CRediT authorship contribution statement

Muhammad Ibrahim: Conceptualization, Methodology, Data curation, Software, Visualization, Writing - original draft. **Bing Xie:** Methodology, Software, Formal analysis. **Michael K. Richardson:** Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexcr.2020.112032.

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