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3D perfusion culture of human adipose tissue-derived endothelial and osteoblastic progenitors generates osteogenic constructs with intrinsic vascularization capacity

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Abstract

In this study, we aimed at generating osteogenic and vasculogenic constructs starting from the stromal vascular fraction (SVF) of human adipose tissue as single cell source.

SVF cells from human lipoaspirates were seeded and cultured for 5 days in porous hydroxyapatite scaffolds by alternate perfusion through the scaffold pores, bypassing the typical phase of monolayer (2D) culture. The resulting cell-scaffold constructs were either enzymatically treated to extract and characterize the cells, or subcutaneously implanted in nude mice for 8 weeks to assess the capacity to form bone tissue and blood vessels. SVF cells were also expanded in 2D for 5 days and statically loaded in the scaffolds.

The SVF yielded 5.9±3.5x10^5 cells/ml of lipoaspirate, containing both mesenchymal progenitors (5.2±0.9% fibroblastic colony forming units) and endothelial lineage cells (54±6% $CD34^+/CD31^+$ cells). After 5 days, the total cell number was 1.8-fold higher in 2D than in 3D cultures, but the percentage of mesenchymal and endothelial linage cells was similar (i.e., 65-72% of $CD90^+$ cells and 7-9% of $CD34^+/CD31^+$ cells). After implantation, constructs from both conditions contained blood vessels stained for human CD31 and CD34, functionally connected to the host vasculature. Instead, only constructs generated under 3D perfusion, and not by 2D-expanded cells, reproducibly induced bone formation.

In conclusion, direct perfusion of human adipose-derived cells through ceramic scaffolds establishes a 3D culture system for osteoprogenitor and endothelial cells, and generates osteogenic-vasculogenic constructs. It remains to be tested whether the presence of endothelial cells effectively accelerates construct vascularization and thereby enhances implanted cell survival.

Introduction

In the context of tissue engineering and regenerative medicine, there is increasing recognition of the importance of co-culturing different cell types in a three-dimensional (3D) environment in order to generate constructs with increased functionality and engraftment capacity [1, 2]. In particular, the co-culture of tissue-specific cells together with endothelial cells has been proposed as a tool to address one of the main limitations of tissue engineered grafts, namely their rapid vascularization [3, 4]. For example, 3D co-culture of myoblasts and endothelial cells improved blood perfusion and survival of the muscle tissue constructs after transplantation [2]. In addition, capillary-like structures formed in vitro by co-culture of keratinocytes and endothelial cells were able to create a connection with the host vessels upon implantation, thus accelerating blood supply of hypoxic zones within the graft [5]. The approach is currently being extended to generate a prevascular network in engineered bone constructs, in order to possibly favor accelerated vascularization and increased cell survival upon implantation [6, 7]. In all these studies, however, the tissue-specific and endothelial cells are derived from different sources or even species, and thus have limited relevance towards a potential clinical application.

The stromal vascular fraction (SVF) of adipose tissue is known to include progenitor cells with extensive plasticity, capable to differentiate into various musculoskeletal lineages, as well as into neuronal and endothelial cells [8]. In particular, typical osteogenic culture supplements were shown to induce osteoblastic cell differentiation [9, 10], while the endothelial component in SVF was reported to produce vascular-like structures on Matrigel in vitro and to produce neovascularization in an in vivo ischemic mouse model [11]. Thus, adipose tissue could be conceived as a common source of osteoblastic and endothelial cells for the engineering of bone grafts. However, beyond some general traits of osteoblastic differentiation in vitro, the effective capacity of human SVF cells to generate bone tissue in vivo is still controversial and appears to require either pre-culture in osteogenic medium [12] or

transfection with BMP-2 [13]. Moreover, although the endothelial component is present in the SVF, it is rapidly lost in culture [14, 11] and can be induced and/or maintained only by serial changes in substratum and culture medium [15] or by culture in specific media [16, 11].

We recently reported that bone marrow-derived mesenchymal stromal cells can be expanded by direct perfusion of fresh bone marrow cells through the pores of 3D ceramic scaffolds, avoiding monolayer (2D) expansion in plastic dishes [17]. The culture system, as compared to typical 2D expansion, yields a higher fraction of clonogenic mesenchymal cells and generates more reproducibly osteogenic constructs. Moreover, upon medium supplementation with specific additives, it allows the maintenance in culture of non-mesenchymal cell populations, including early hematopoietic progenitors [17]. Thus, a 3D culture system under perfusion has the potential to support co-culture of cell types of different lineages, likely providing appropriate 'niches' for their interaction.

In this study, we aimed at using the SVF of human adipose tissue as a common cell source to (i) establish a 3D co-culture system for osteoblast- and endothelial-lineage cells, and (ii) generate constructs which are both osteogenic and vasculogenic upon ectopic implantation in nude mice. In order to achieve this goal, freshly isolated SVF cells were directly perfused through the pores of 3D ceramic scaffolds, bypassing the typical phase of monolayer culture in plastic dishes.

Materials and methods

Cell isolation

Subcutaneous adipose tissue in the form of lipoaspirates was obtained from 12 healthy donors (20-63 years old, body mass index 19.5-25.1 kg/m^2) during routine lipoaspirations of the inner and/or outer thighs, after informed consent from the patient and following protocol approval by the local ethical committee. The tissue was digested in 0.075 % collagenase type 2 (Worthington) for 45 minutes at 37°C on an orbital shaker. The suspension was thereafter centrifuged at 300g for 10 minutes, and the resulting SVF pellet was washed once with PBS, resuspended in α-MEM medium (Gibco), and finally filtered through a 100 µm strainer (BD Falcon). Bone marrow-derived nucleated cells, from donors with matched age and body mass indexes, were obtained from the iliac crest during routine orthopedic surgical procedures as previously described [18].

Monolayer (2D) cell cultures

SVF cells were seeded onto Petri dishes at a density of 2x10^4 cells /cm^2 in α-MEM containing 10% foetal bovine serum (basic medium), supplemented with 5 ng/ml fibroblast growth factor-2 (FGF-2). The number of clonogenic cells, generally referred to as colony forming units-fibroblastic (CFU-f), was determined by plating 1x10^3 SVF cells, and by staining with crystal violet and counting the colonies after 2 weeks. The proliferation rate was determined by counting the number of cells at the first plating and at the end of this passage, by using a Neubauer chamber. The total number of doublings during the culture period, defined as the basis 2 logarithm of the ratio between final cell number and initial cell number, was divided by the number of days in culture to obtain the number of cell doublings per day. The initial number of CFU-f plated was calculated from the number of nucleated cells plated, by using the independently determined clonogenic frequency.

Osteogenic differentiation

SVF cells, freshly harvested or expanded up to 3 passages, were cultured in osteogenic medium for 3 weeks, with medium changes twice a week. Osteogenic differentiation medium consisted of basic medium supplemented with 100 nM dexamethasone, 10 mM [beta]-glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate. After 3 weeks, celllayers were washed twice with PBS, fixed for 10 minutes with 4% formalin and washed twice with water. Cells were then either incubated for 10 minutes with alizarin red and washed extensively with water, or stained with 5% $AgNO_3$ under UV for 20 minutes (Von Kossa staining) and washed thereafter with water and 5% $Na_2S_2O_3$, 5 H_2O.

Adipogenic differentiation

Adipogenic differentiation was induced in 2D cultures using cycles of treatment with different media as previously described [19]. Briefly, cells were seeded and cultured until confluence. Cells were then treated with 10 g/ml insulin, 1 M dexamethasone, 100 M indomethacin, and 500 M 3-isobutyl-1-methyl xanthine (IBMX) (adipogenic induction medium) for 72 hours and subsequently with 10 g/ml insulin (adipogenic maintenance medium) for 24 hours. The 96-hour treatment cycle was repeated 4 times, and cells were then cultured for an additional week in adipogenic maintenance medium. The onset of adipocytic cells was assessed by Oil red O staining.

Three-dimensional (3D) perfusion cultures

Cell seeding and subsequent culture of freshly isolated cells from adipose tissue in 3D scaffolds was performed using a previously described bioreactor system [20], which is based on the principle of direct perfusion of cell suspensions or culture medium through the pores of 3D scaffolds in alternate directions.

SVF cells were perfused at a velocity of 3 ml/minute through porous hydroxyapatite ceramic scaffolds (Engipore®, Fin-Ceramica Faenza, Italy), with an average porosity of 83% ± 3% and the size of 8 mm-diameter, 4 mm-thick disks ($3x10^6$ cells per disk), in the same medium used for 2D cultures. After 5 days of continuous perfusion, the resulting constructs were either treated enzymatically to extract the cells, or implanted in nude mice as described below.

Cell Extraction from the constructs

After culture in the 3D perfusion system, cells were extracted from the generated constructs by perfusing a solution of 0.3% collagenase type 2, followed by perfusion with 0.05% trypsin/0.53 mM EDTA (Gibco), both at a velocity of 6 ml/minute. Extracted cells were collected in culture medium, counted by using a Neubauer chamber, and characterized by flow cytometry, as described below.

Flow cytometry analysis

SVF, 2D- or 3D-expanded cell suspensions were incubated for 30 minutes at 4°C with fluorochrome-conjugated antibodies against the indicated proteins, or an isotype control. All the antibodies were from Becton Dickinson except the one against CD105 which was from Serotec, and the ones against CD144 (VE-cadherin) and VEGFR-2 which were from R&D laboratories. Cells were washed and resuspended in PBS, and analyzed with a FACSCalibur flow cytometer.

Assessment of bone formation following in vivo implantation

The osteogenicity of scaffold-cell constructs was assessed by ectopic implantation in nude mice (CD-1 nu/nu, 1 month old; Charles River Laboratories), in accordance with institutional guidelines. For each experiment (i.e., using cells from different donors), constructs implanted were generated starting from $3x10^6$ SVF cells, by(i) 3D perfusion culture for 5 days directly

within ceramic scaffolds, (ii) 2D expansion for 5 days and static loading into the scaffolds of the resulting progeny, or (iii) immediate static loading into the scaffolds of the freshly harvested cells, with no further culture. We previously reported that the fraction of cells retained in the scaffolds after seeding by static loading was similar to that obtained using the described perfusion device, although cells seeded statically were less uniformly distributed [20]. Eight weeks after implantation, mice were sacrificed and the constructs were harvested and fixed overnight in 4% formalin, decalcified for 3 hours with Osteodec (Bio-Optica) under agitation at 37°C, paraffin embedded, and sectioned at different levels (7µm-thick sections). Sections were then stained by H&E (haematoxylin/eosin) and observed microscopically for the formation of bone tissue. Some sections were also stained with Oil red O and Safranin-O to assess the formation of adipose or cartilaginous tissue.

Assessment of human blood vessels following in vivo implantation

The presence of blood vessels of human origin was determined in 7 µm-thick paraffin-embedded sections of the same constructs described above, 8 weeks after implantation in nude mice. Human endothelial cells were specifically stained with anti-human CD31 (Dako) and biotin-conjugated anti-human CD34 (Chemicon) antibodies. For the anti-CD31 antibody, the M.O.M™ Kit from Vector Laboratories was used to reduce the unspecific binding of the secondary biotin-conjugated anti-mouse antibody on the sections. After incubation with ABC-alkaline phosphatase complex (Dako), specific staining was revealed by using Fast red (Dako). Sections were counterstained with haematoxylin and mounted.

Results

Characterization of adipose tissue-derived cells in vitro

After the digestion of adipose tissue and subsequent centrifugation to remove the differentiated adipocytes, 5.9 ± 3.5 x 10^5 cells / ml of lipoaspirate were obtained, of which 5.2 ± 0.9 % were clonogenic, as demonstrated by CFU-f assays (n = 7 donors). The proliferation rate of ATSC during monolayer expansion in tissue culture plastic after the first passage averaged 0.92 ± 0.38 cell doublings per day (n = 3 donors), and was in the range of that typically measured for human bone marrow stromal cells (data not shown). Freshly-isolated adipose cells from the SVF were able to deposit abundant mineralized matrix when cultured for 3 weeks in osteogenic medium, as demonstrated by a strong alizarin red and Von Kossa staining (Figure 1A). No changes in the capacity to deposit mineralized matrix were observed in cells expanded for up to 3 passages (data not shown). SVF cells were also efficiently differentiated towards the adipogenic lineage, as indicated by the presence of lipid droplets in the cytoplasm following culture in adipogenic medium (data not shown).

We next seeded 3 x 10^6 SVF cells, corresponding to about 5 ml of lipoaspirate and containing approximately 1.5 x 10^5 CFU-f, either in tissue culture dishes (2D culture) or in porous ceramic scaffolds using a perfusion bioreactor (3D culture) (n = 5 donors). After a 5 day culture period, a significantly higher number of cells was obtained in 2D (4.8 ± 4.1 x 10^5 cells) than in 3D cultures (2.7 ± 2.2 x 10^5 cells) (Figure 1B). Flow cytometry-based analysis of cell phenotype indicated that the freshly isolated SVF included cells of the mesenchymal (positive for CD90, CD44 and/or CD105 and negative for CD133), endothelial (positive for CD34, VEGFR2 and CD31), and haematopoietic (positive for CD45) lineages (Figure 1C, D). After 5 days of culture, most haematopoietic cells were lost and the proportion of cells with mesenchymal and endothelial phenotypes was similar in 2D and 3D cultures. The percentage of mesenchymal lineage cells (here defined as $CD90^+$) was 65 ± 26 % (n = 3) in 2D and 72 ± 29 % (n = 3) in 3D cultures, whereas the percentage of endothelial lineage cells (here defined

as $CD34^+/CD31^+$) was 7 ± 3 % (n = 3) in 2D and 9 ± 6 % (n = 3) in 3D cultures. After 3 weeks of 2D or 3D culture, cells expressing CD45, CD34, VEGFR2 and CD31 represented a negligible fraction of the total cells (data not shown).

Assessment of in vivo formation of bone tissue by adipose tissue-derived cells

Cell-ceramic constructs generated starting from the same number of SVF cells, either directly cultured under 3D perfusion or loaded into the scaffolds following 2D-expansion for the same time (i.e., 5 days), were subcutaneously implanted in nude mice for 8 weeks in order to determine their osteogenic and vasculogenic capacity (n = 5 donors). Constructs generated by 3D cultures displayed abundant formation of bone tissue, organized in structures typically referred to as "ossicles", formed starting from the ceramic surface of the pores (Figure 2A) and including vascular elements (Figure 2B). Instead, constructs generated by 2D-expanded cells generally contained loose interstitial tissue, with no evidence of frank bone tissue formation (Figure 2C, D), with the exception of one case (i.e., cells from one donor), where scattered areas of bone formation were found. Similarly, when SVF cells were loaded onto the scaffold directly after isolation and implanted without culture, only a loose connective tissue was observed (data not shown). Safranin-O (Figure 2E) and Oil red O staining (Figure 2F) of the explanted constructs were negative, indicating that in our experimental conditions cells in the constructs did not acquire a chondrogenic or adipogenic phenotype.

Assessment of in vivo formation of blood vessel by adipose tissue-derived cells

A fraction of the blood vessels formed in implants generated by 3D culture was positively stained by anti-human CD34 or CD31 antibodies (Figure 3), indicating that the cells of the endothelial lineage present in the grafts were capable of generating blood vessels in vivo. Branching of such human blood vessels to host vasculature was observed in the vicinity of larger caliber mouse vessels (Figure 3C). At higher magnification, erythrocytes were

observed in the lumen of the positively stained vessels (Figure 3D), indicating a connection of the human blood vessels to the vasculature of the host. Similar human blood vessels were found in constructs generated by loading of 2D-expanded cells (Figure 4A) or freshly-isolated SVF (Figure 4B), although in the absence of bone formation. Vessels positively stained for human CD34 accounted for 27% to 43% of the total number of vessels counted in tissue cross-sections, and such percentages were similar in constructs generated by 2D cultures, 3D cultures, or freshly implanted SVF cells from the same donors (data not shown).

Discussion

In this study, we demonstrated that 3D perfusion culture of SVF cells from human adipose tissue through the pores of ceramic-based scaffolds results in the generation of constructs containing both osteoblastic- and endothelial-lineage cells. Upon in vivo ectopic implantation, the constructs were both osteogenic and vasculogenic: this property, to the best of our knowledge, has not yet been achieved starting from a single source of cells. One important component of our result was cell culture under direct 3D perfusion, since SVF cells expanded in plastic dishes and subsequently loaded in the same porous scaffolds were not able to reproducibly generate ectopic bone tissue.

A suitable cell source for bone tissue engineering applications requires the availability of a large number of functional cells, under minimally invasive harvest conditions. In this regard, assuming that a clonogenic mesenchymal cell (CFU-f) represents the functional unit for generation of bone tissue [21], our results indicate that the quantity of CFU-f which can be derived from 1 ml of human adipose tissue (i.e., about 30'000) is about 50 to 100 fold higher than the number of CFU-f typically available from 1 ml of human bone marrow (considering a yield of 3 to 6 million nucleated cells and a clonogenicity of 0.01% [17]). Moreover, since harvesting of more than 200 ml of marrow aspirate is challenging without increasing the related morbidity, whereas collecting up to 1 liter of lipoaspirate can be done with a minimal morbidity, the quantity of CFU-f available for clinical applications could be up to 500 fold higher with adipose tissue than with bone marrow. Obviously, an equivalence in osteogenic function between bone marrow- and adipose tissue-derived CFU-f remains to be demonstrated. However, the finding that only 5 days of pre-culture, corresponding to a minimal extent of cell expansion, was sufficient to obtain osteogenic grafts starting from SVF cells, is an indication of the importance of the initially high amount of CFU-f available from adipose tissue.

Our result that adipose tissue-derived cells expanded in 2D were not capable to reproducibly generate bone tissue upon ectopic implantation is consistent with previous studies proposing pre-culture in osteogenic medium [12] or transfection with BMP-2 [13] as possible strategies to use human lipoaspirate cells for bone repair. Interestingly, even constructs immediately implanted following scaffold seeding with SVF cells were not osteogenic, whereas 5 days of 3D perfusion culture were sufficient to achieve reproducible osteogenic properties. Since during the 5 days of 3D perfusion culture a limited extent of expansion was achieved, this result suggests the possibility of a specific osteogenic commitment imparted by the exposure to fluid-induced shear [22], by the integrin-mediated signaling triggered by a mineralized substrate [23], by the establishment of 3D cell-cell interactions [24], or by a combination of these factors.

The SVF of human adipose tissue is known to comprise highly heterogeneous cell populations, including adipose stromal (mesenchymal) cells, blood derived cells, vascular (endothelial) cells, smooth muscle cells and pericytes [25], and most likely several others yet unidentified cell types. Our phenotypic analysis of SVF cells is in general accordance with previous studies [26, 27], although with some differences in the proportions of the different cell types, which indeed are known to greatly vary with parameters such as duration of collagenase digestion, donor site or storage duration [28-30]. A precise identification of all different cell types generated after 2D and 3D culture was beyond the scope of the present study, and the analyses and interpretations were restricted to the two cell populations of interest, namely the stromal mesenchymal/osteoprogenitor cells (characterized by the expression of CD90, CD105 and CD44) and the cells of the endothelial lineage. With regard to the latter cell population, our results indicate that they are neither frank endothelial progenitor cells, which are described to express not only CD34 and CD31, but also CD133 and VEGFR2 [31], nor fully differentiated endothelial cells, which typicallyexpress VE-cadherin (CD144). It is possible that the $CD34^+/CD31^+$ cells that we define as 'endothelial

lineage cells' are a population of endothelial progenitors, since they were functional to generate the endothelial layer of the human blood vessels in vivo, but possibly more committed than those described to be present in peripheral blood [31].

During skeletal development and regeneration, interactions between endothelial cells (EC) and osteoblasts are thought to play a key role [32]. Although transplanted EC have been reported to enhance orthotopic bone formation by bone marrow stromal cells in vivo [33], in vitro studies about the influence of endothelial cells on osteoblastic differentiation have been so far controversial [34-37]. In this context, our perfusion-based culture system represents a relevant model to investigate the 3D interactions between endothelial and osteoblastic cells, as well as the possibility that mutual conditioning of the two cell populations, including a precise control of their self-renewing and differentiation, requires the formation of an appropriate 'niche' by the stromal cell component.

Conclusions

In conclusion, our study provides a prospective for the streamlined manufacturing of osteogenic and vasculogenic grafts in 3D perfusion systems starting from adipose tissue cells, bypassing the constraining 2D culture step which is typically used in tissue engineering approaches. Since cells from the SVF of adipose tissue are being proposed for the regeneration of other tissues with stringent vascularization requirements (e.g., cardiac [38], skeletal muscle [39]), the approach may be extended to other areas of regenerative medicine. It remains to be tested whether in larger, clinically relevant-sized constructs, the co-culture of mesenchymal and endothelial progenitors will accelerate vascularization inside the implanted graft, and thereby enhance cell survival and improve engraftment.

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Figure legends

Figure 1. Characterization of adipose tissue-derived stromal cells.

(**A**) Alizarin red and Von Kossa staining of freshly isolated SVF cells cultured for 3 weeks with osteogenic medium. (**B**) Number of cells after a 5 day culture period, starting from $3x10^6$ nucleated freshly isolated SVF cells expanded either in monolayer (2D) culture or by 3D perfusion within the pores of a ceramic scaffold. (**C**) Flow cytometry analysis for the indicated surface markers of freshly isolated SVF cells, or of cells cultured for 5 days in either 2D or 3D. Results are given as % of positive cells ± standard deviation of 5 independent experiments. (**D**) Flow cytometry histograms of freshly isolated SVF cells from one representative experiment.

Figure 2. In vivo bone formation by adipose tissue-derived cells.

Representative hematoxylin/eosin-stained sections of constructs ectopically implanted for 8 weeks in nude mice and generated by 3D perfusion culture of SVF cells in ceramic scaffolds for 5 days (**A**,**B**), or by static loading in the same scaffolds of SVF cells expanded in 2D for 5 days (**C**,**D**). Constructs including bone tissue were negative for Safranin-O (**E**) and Oil-red O staining (**F**). **b** = bone matrix; **b.v.** = blood vessel; **f.t.** = fibrous tissue.

Figure 3. In vivo formation of human blood vessels by 3D cultured adipose-derived cells.

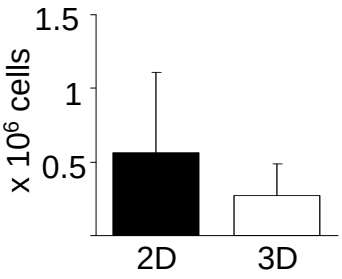
Representative sections of constructs ectopically implanted for 8 weeks in nude mice and generated by 3D perfusion culture of SVF cells in ceramic scaffolds for 5 days. Sections are stained with an anti-human CD34 (**B-D**) or anti-human CD31 (**F**) antibody, or with the corresponding isotype controls (**A, E**).

Figure 4. In vivo formation of human blood vessels by fresh or 2D-expanded adipose-derived cells.

Representative sections of constructs ectopically implanted for 8 weeks in nude mice and generated by static loading into ceramic-based scaffolds of SVF cells expanded in 2D for 5 days (**A**) or freshly harvested (**B**). Sections are stained with an anti-human CD34 antibody.

A

B



C

	SVF	2D	3D
CD44	14 ± 11 % (n = 5)	53 ± 36 % (n = 3)	25 ± 24% (n = 2)
CD90	68 ± 7 % (n = 6)	65 ± 26 % (n = 3)	72 ± 29 % (n = 3)
CD105	49 ± 17 % (n = 6)	70 ± 23 % (n = 3)	47 ± 41 % (n = 3)
CD45	25 ± 9 % (n = 5)	4 ± 3 % (n = 2)	0 % (n = 2)
CD34	74 ± 5 % (n = 6)	69 ± 11 % (n = 3)	52 ± 24 % (n = 3)
CD31	54 ± 6 % (n = 5)	7 ± 3 % (n = 3)	9 ± 6 % (n = 3)
VEGFR2	14 ± 12 % (n = 6)	4 ± 5 % (n = 2)	0 % (n = 3)
CD144	3 ± 4 % (n = 6)	0 % (n = 3)	0 % (n = 3)
CD133	0 % (n = 6)	0 % (n = 2)	0 % (n = 2)

D

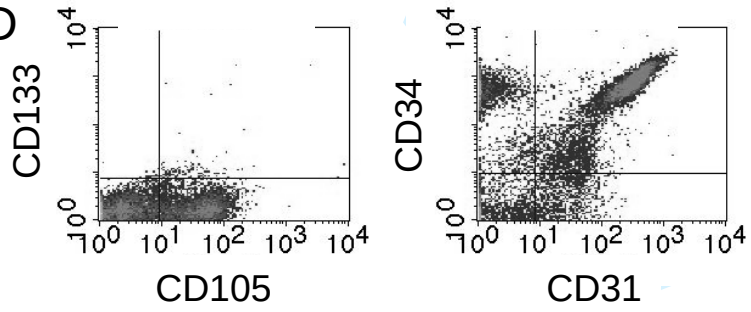


Figure 1.

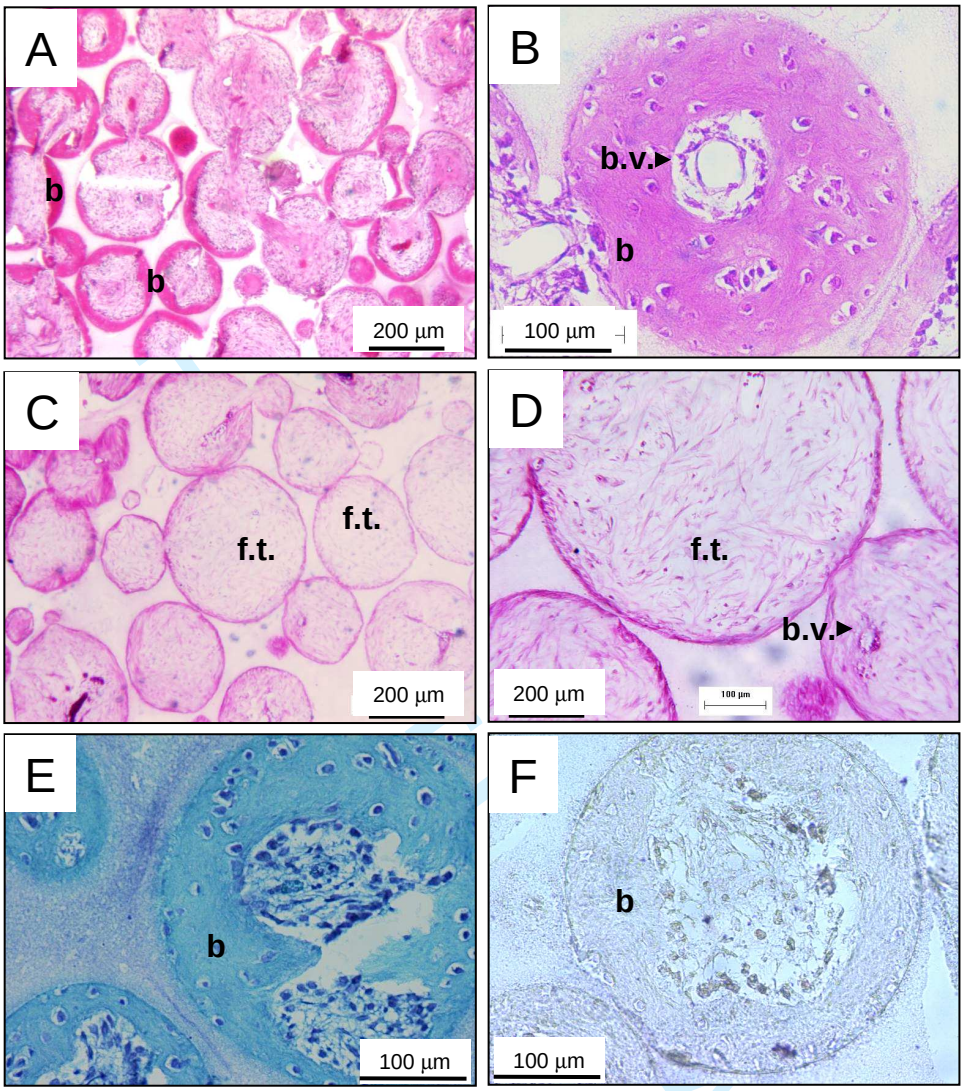

A
b
b
200 µm
B
b.v.►
b
100 µm
C
f.t.
f.t.
200 µm
D
f.t.
b.v.►
200 µm
E
b
100 µm
F
b
100 µm

Figure 2.

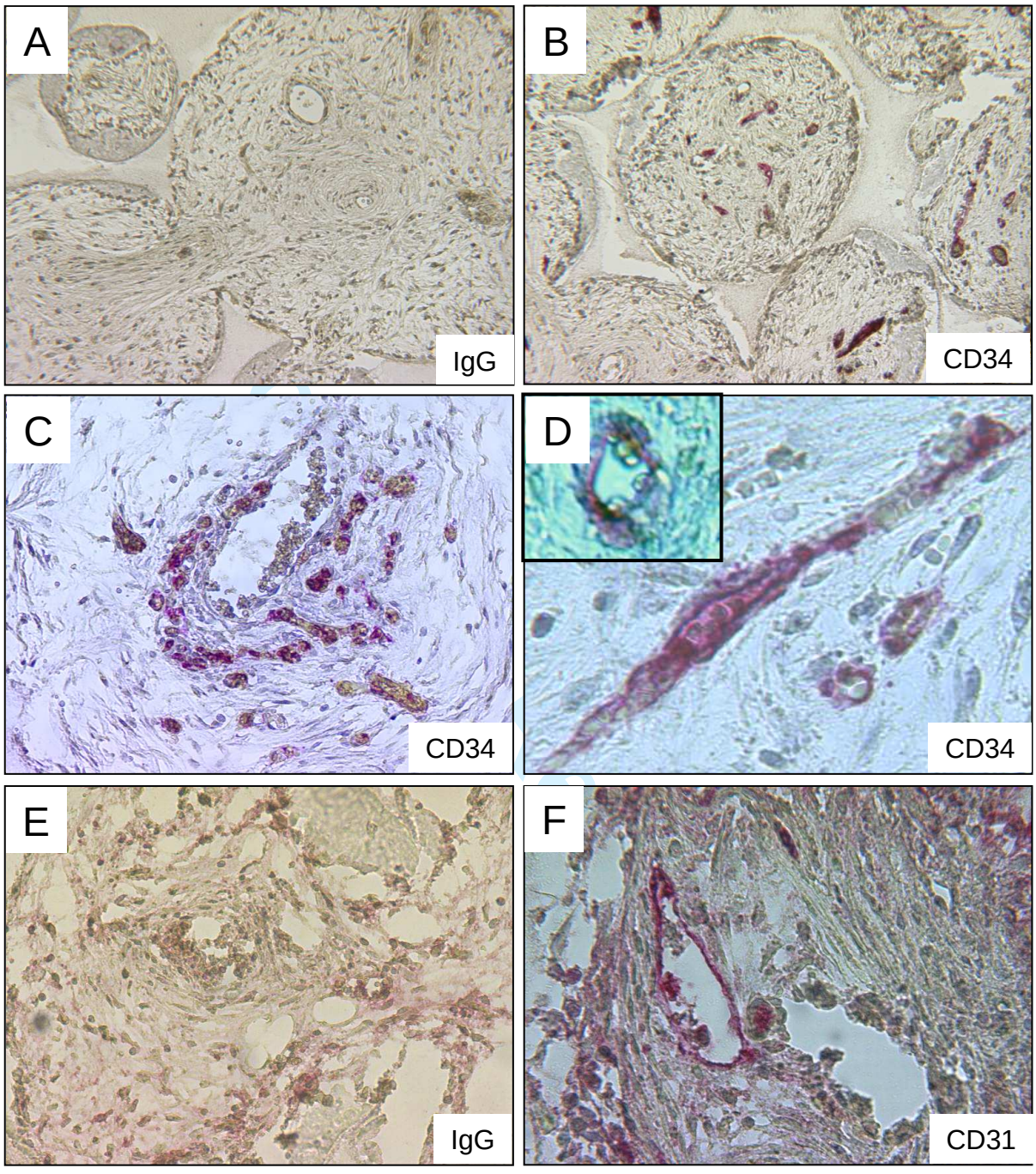


Figure 3.

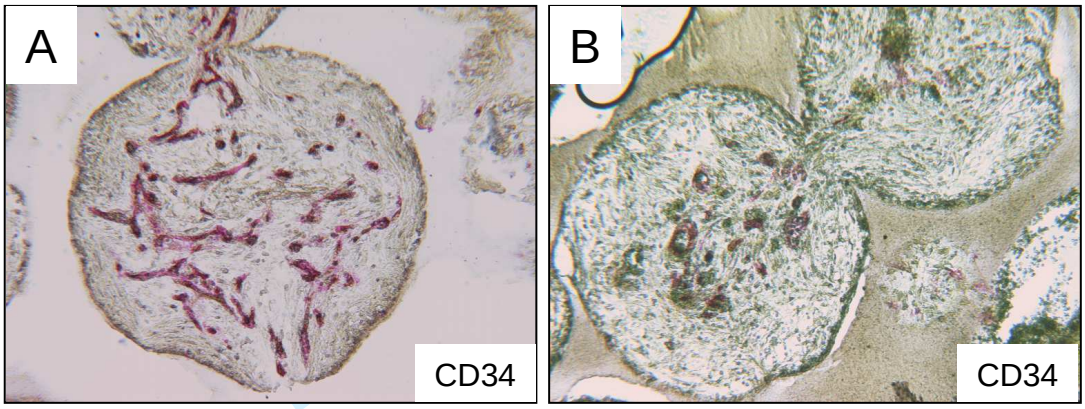


Figure 4.