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INTRODUCTION

Angiogenesis, the development of new blood vessels from preexisting vessels, is vital for a variety of processes for normal tissue development and wound healing, but is also associated with a variety of pathological conditions such as cancer, arthritis and diabetes. Due to the importance of angiogenesis in pathologies progression, based on the acquisition or leak of new blood vessels, understanding the pathways that regulate this process is critical to the design of better therapeutics. Angiogenesis involves mostly endothelial cells (EC) sprouting from the parent vessel, followed by migration, proliferation, alignment, tube formation and anastomosis to other vessels; hence, there is substantial interest in the specific features of the EC. Obtaining populations of human EC to recreate, in-vitro and in-vivo, this complex sequence of events under different pathological stimuli is therefore highly desirable. The main EC model for the scientific community are Human Umbilical Vein Endothelial Cells (HUVEC), useful for any research on general properties of Human EC. Umbilical Cord (UC) is the most simple and available source of human physiologic EC. It is a discarded tissue provided by a pain-free collection method. UC allows the isolation of large quantities of cells with highly proliferative features due to their perinatal origin. Our goal is to generate in our Biobank standardized and quality-checked HUVEC batches to be made available to the scientific community. We also aim to identify an "animal-free" expansion protocol without reagents of animal origin as alternative to the "classic" expansion protocol based on the use of Foetal Bovine Serum (FBS).

MATERIALS & METHODS

All HUVEC primary cultures used in this study were derived from discarded human UC of healthy donors after obtaining the donor informed consent. HUVEC were isolated adapting the protocol described by Baudin et al. (1). Briefly: wash the cord from the excess blood (A) and make fresh cuts on both ends of the cord (B). Identify the vein (*) (C), insert a 21 ½ G needle and clamp it (D).

Connect a syringe to the needle, wash the vein with PBS, clamp the end without needle and using an other syringe insert the collagenase solution into the vein (E). Incubate the cord at 37°C for 15 minutes. After incubation cut the end above the bottom clamp (F) and collect the digestion solution coming out from the vein in a tube (G). Spin the tube at 1500 rpm per 5 minutes (I), discard the supernatant, resuspend the pellet in complete media (Medium 199, 2mmol/L L-glutamine, 10% FBS) implemented with angiogenic growth factors (GFs) (100mg/L Heparin, 1mg/L Hydrocortisone, 10µg/L EGF, αFGF and βFGF) and plate in two gelatin pre-coated 6cm ø petri dishes (L). Incubate at 37°C with 5%CO₂ (Fig.1). Cell immunophenotyping was performed using anti-human antibodies against CD106, CD31, CD90 and CD45.

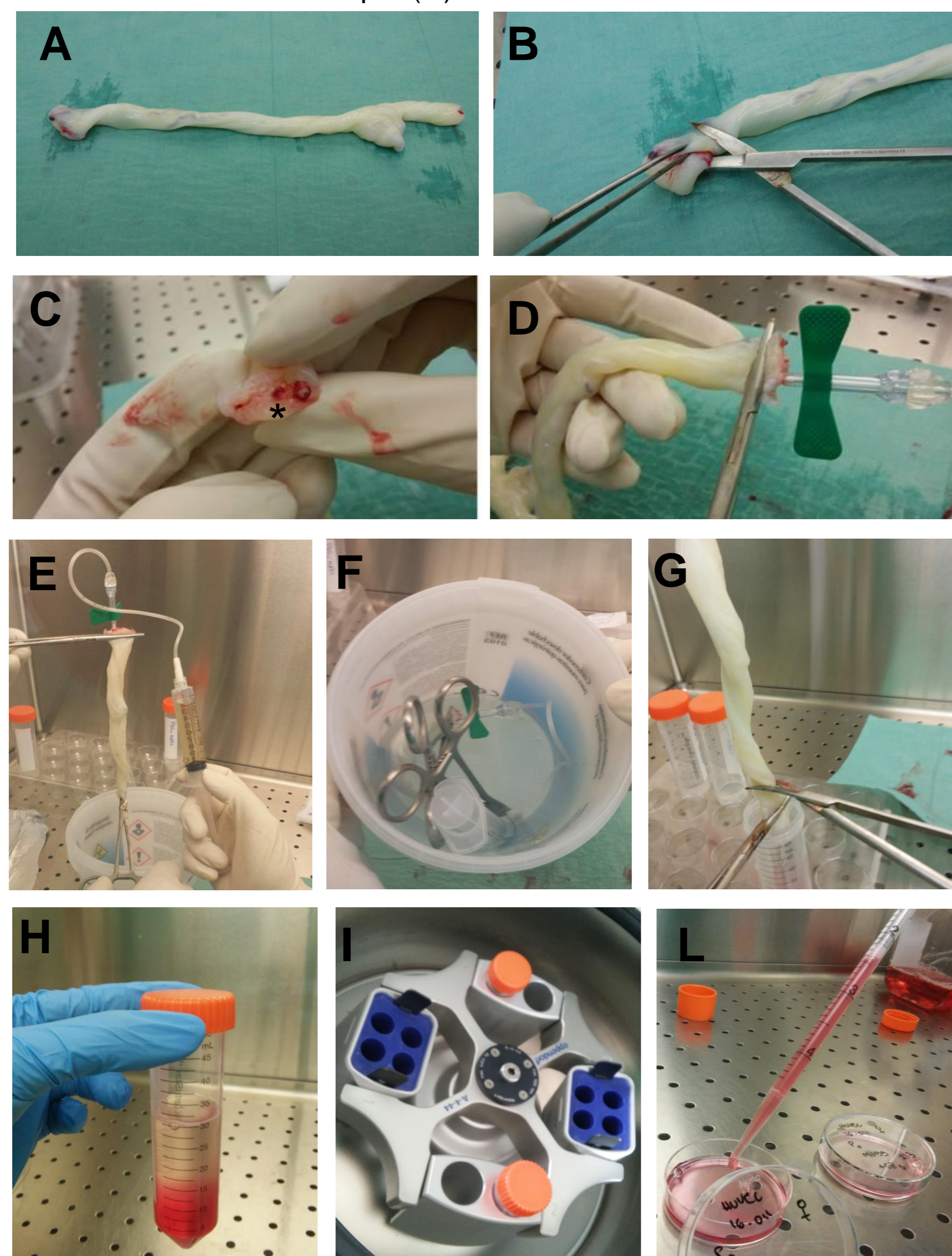


Fig.1 Main phases of HUVEC isolation process.

In order to identify a HUVEC culture protocol devoid of animal derived reagents: 1) we seeded HUVEC not only in porcine gelatin coated dishes but also in human fibronectin coated dishes to test the cell adhesion; 2) we cultured HUVEC adding 5% or 10% of Human Serum (HS) excluding FBS (Fig. 2).

Cell proliferation in different media was evaluated by doubling calculation, seeding the same number of HUVEC in T25 and counting them at 80% confluence for each passage for 14 days. Angiogenic assay was performed using Matrigel with seeded HUVEC to test tube formation capacity in the different culture media.

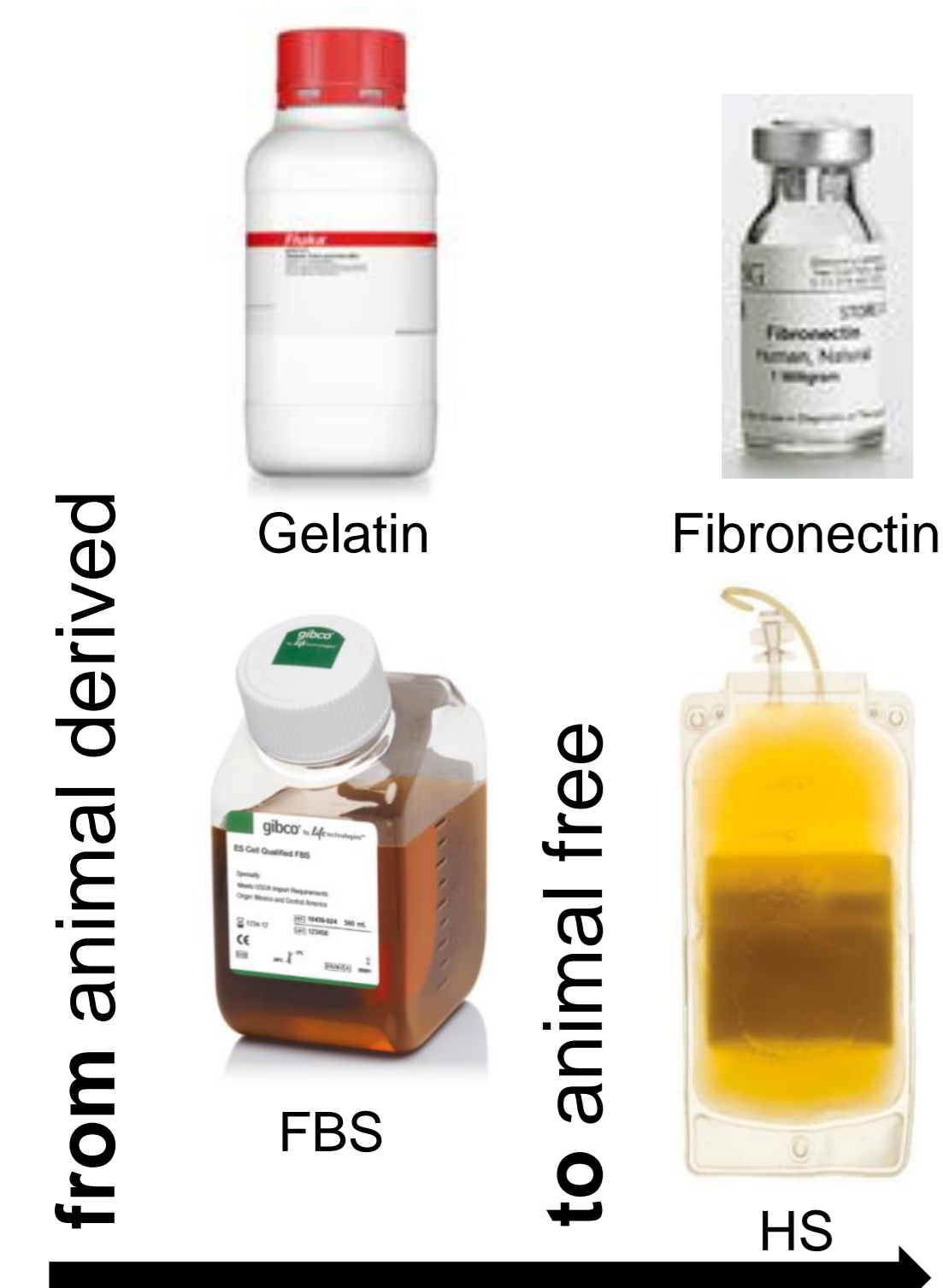


Fig.2 Schematic representation of the animal derived reagents which we aim to substitute with animal free reagents in HUVEC culture protocol: coating by Porcine Gelatin with coating with Human Fibronectin and Foetal Bovine Serum (FBS) with Human Serum (HS).

RESULTS

Phenotype characterization of isolated HUVEC

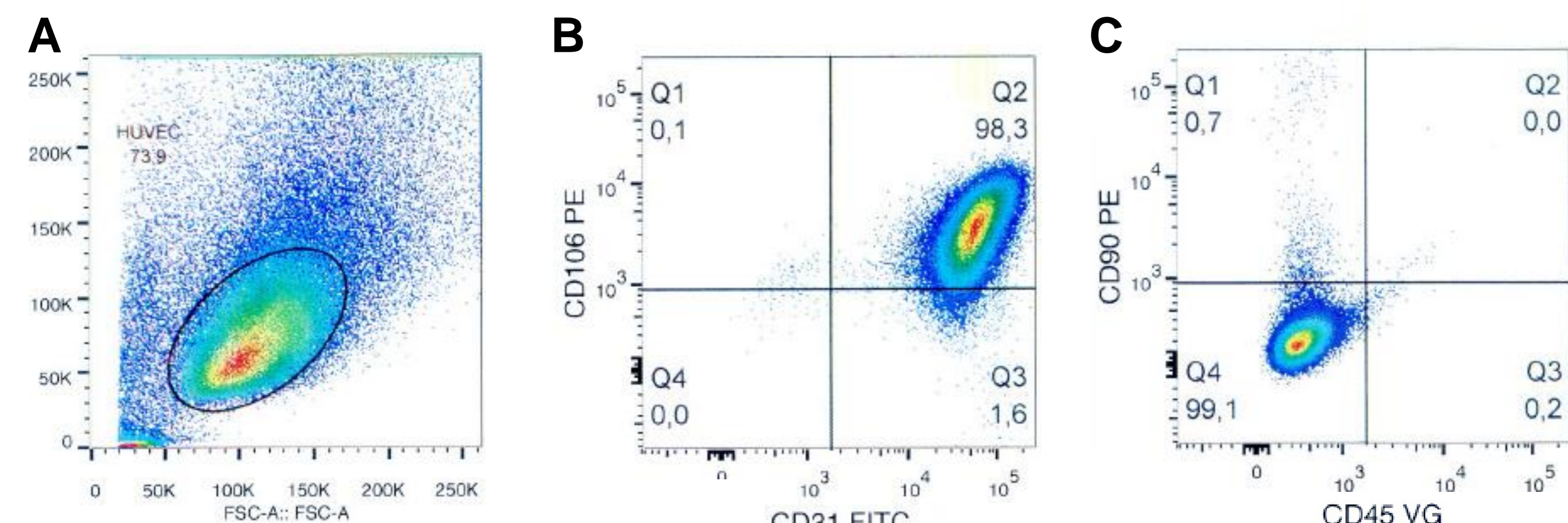


Fig.3 Evaluation of the presence of endothelial (CD106, CD31), fibroblast (CD90) and blood cell (CD45) markers on the surface of hypothetical HUVEC.

To ensure the endothelial origin of isolated cells and avoid contaminant populations we performed a staining for the endothelial markers CD106 and CD31, for the fibroblasts marker CD90 and for blood cells marker CD45. The population isolated (shown in A using physical parameters) is positive for both endothelial markers at 98.3% and negative for CD90 and CD45 markers at 99.1% confirming the success of HUVEC isolation (Fig. 3).

HUVEC morphology and adhesion on different coatings

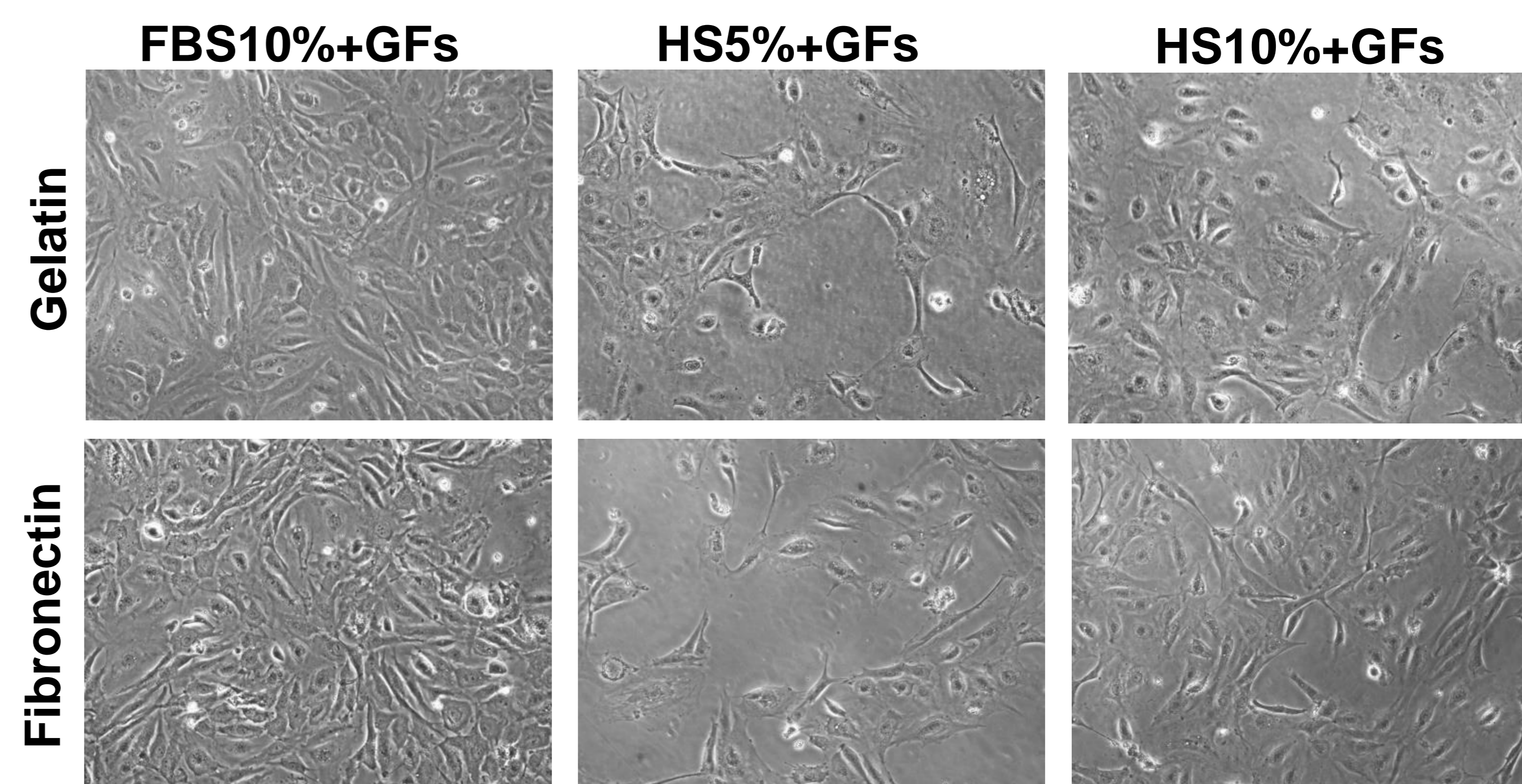


Fig.4 Analysis of HUVEC morphology and adhesion on Porcine Gelatin or Human Fibronectin in presence of media with FBS or HS. 5x magnification.

HUVEC are usually seeded by "classical" protocols on porcine gelatin coated plate, in order to identify an "animal-free" HUVEC culture protocol we tested HUVEC morphology and adhesion on Human Fibronectin in presence of different culture media with (10% FBS) and without animal reagent (5% HS; 10% HS). For all culture media tested no relevant differences were observed in HUVEC morphology and adhesion between gelatin and fibronectin coating (Fig.4). Thus, from these data we can consider the two types of coating equivalent.

HUVEC viability and proliferation in different culture media

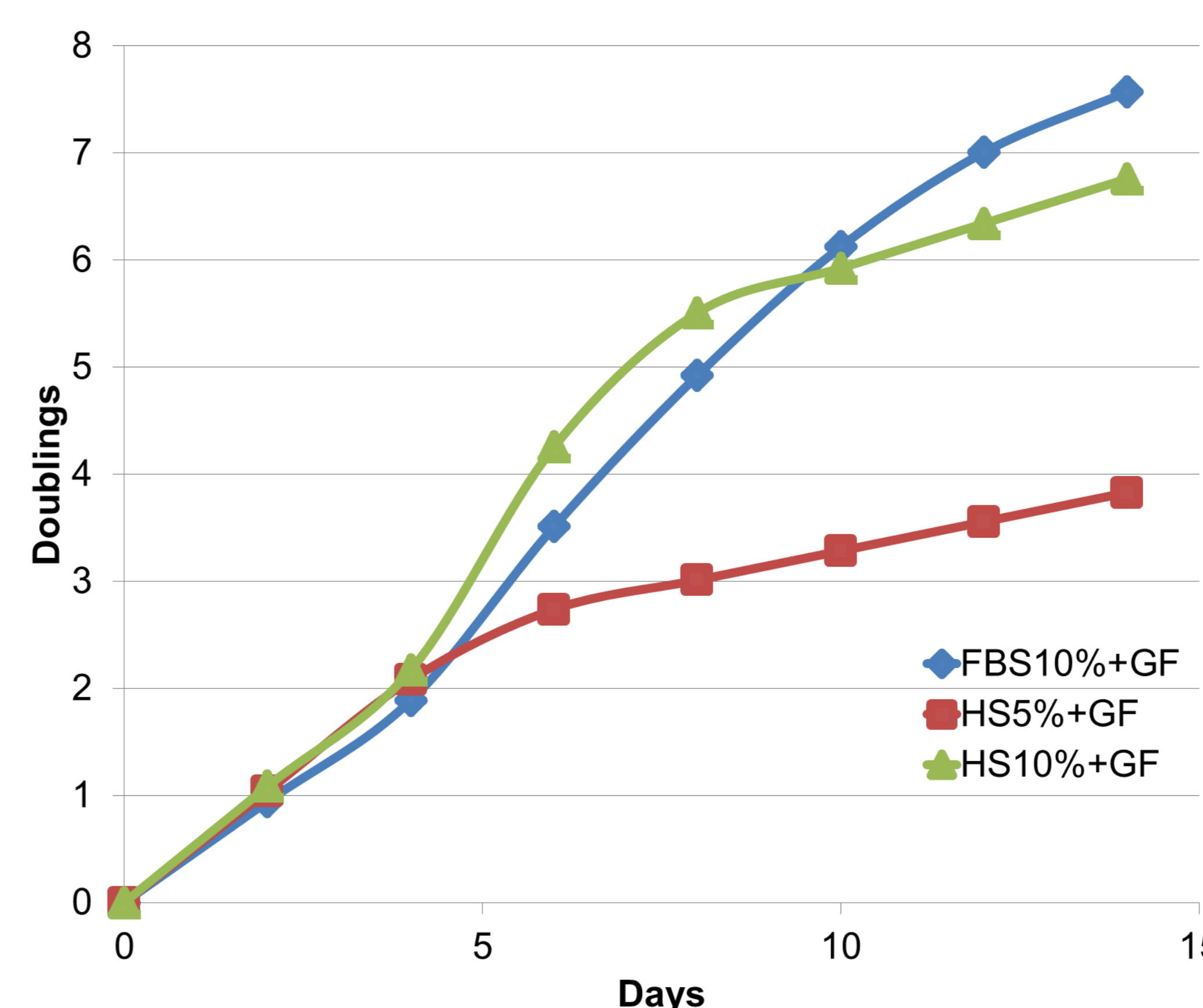


Fig.5 HS effect on HUVEC proliferation. The mitogenic effect of HS added at 5% and 10% in the culture medium was investigated by determining the number of doublings at different HUVEC culture time.

In order to reduce/eliminate the use of FBS as cell culture supplement we checked HUVEC viability and proliferation when cultured with HS. Viable HUVEC number was determined by means of cell counting at different times of cell culture maintained in media supplemented with 5% or 10% of HS or with 10% FCS as control culture. Cell proliferation rates and cell doublings were enhanced in 10% HS cultures as well as 10% FBS control culture, while with 5% HS HUVEC proliferation is reduced in a HS dose-dependent manner (Fig.5).

HUVEC angiogenic differentiation in different culture media

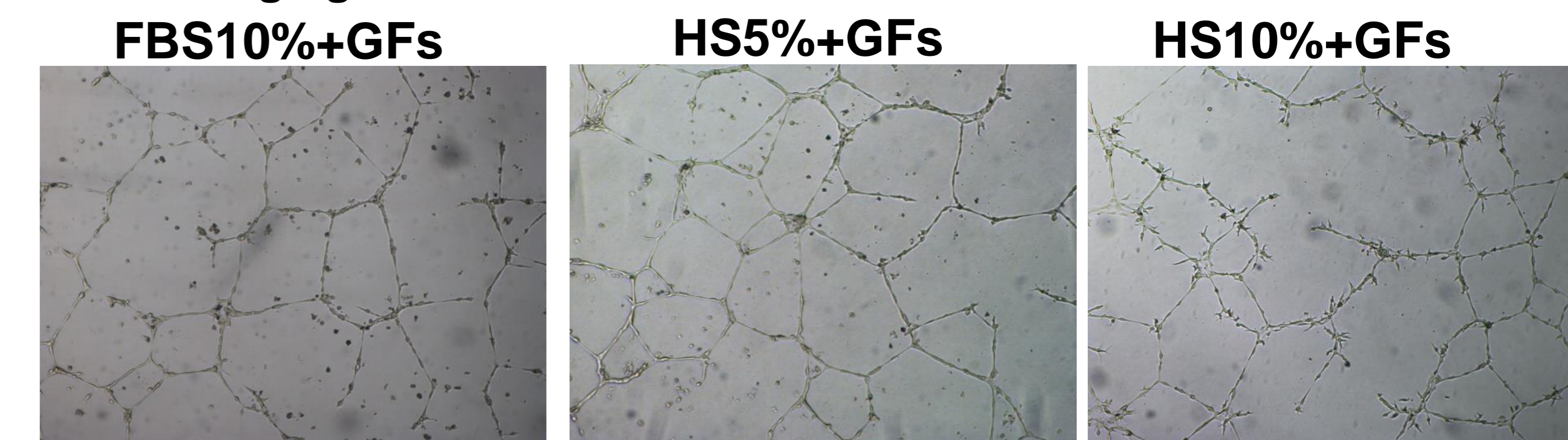


Fig.6 Analysis of HUVEC angiogenic capacity in vitro seeded on a Matrigel matrix and under the angiogenic stimuli given by culture media supplemented 5% and 10% HS or 10% FBS for control culture. 5x magnification.

The maintenance of the angiogenic differentiation capability after the in vitro expansion in the presence of HS was also evaluated. HUVEC expanded with 5% and 10% HS could be induced toward the angiogenic lineage as revealed by the tube-like structures formed in the matrigel matrix as well as the control culture in 10% FBS (Fig.6).

CONCLUSIONS

This work shows the production of high quality HUVEC batches by the Cell Bank of the Genoa Biological Resource Centre. The setting of an optimal isolation and expansion protocol verified by appropriate quality control tests (FACS identification, angiogenesis assay, infections assay) has allowed us to produce several quality checked HUVEC batches. Given the encouraging preliminary results we intend to deepen investigate the development of an animal-free expanding protocol to make available to the scientific community a validated in vitro "humanized" system for human endothelial cell culture.

REFERENCES

(1) Baudin B, Bruneel A, Bosselut N, Vaubourdolle M. "A protocol for isolation and culture of human umbilical vein endothelial cells." *Nat Protoc.* 2007;2(3):481-5.

ACKNOWLEDGMENTS

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Thanks moms!