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Human cell-based 3D *in vitro* model for the testing of drug candidates against venous malformations

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Abstract

Venous malformations (VMs) are one of the most common congenital vascular malformation. Standard treatment is surgery and/or sclerotherapy, however, surgical resection can be impossible due to the size or location of lesions and malformed tissue tends to regrow making the development of medical therapies highly desired. As a breakthrough in the field, recent studies have indicated that the majority of VMs are caused by somatic gain-of-function mutations in TIE2-PI3K signaling pathway in endothelial cells (ECs). The first molecular drug against VMs Sirolimus (also known as a rapamycin) can reduce the full action of Akt *in vitro* via inhibiting mTORC2 and in clinical trials Sirolimus can reduce the VM lesion[1, 2]. Sirolimus has, however, certain limitations; *in vitro* it does not normalize all molecular/cellular VM abnormalities and in humans it has potent immunosuppressant actions. In addition, TIE2/PIK3CA mutation-negative VMs could be caused by mutations in genes that are not directly connected to Akt signaling[3]. As a result, the current clinical treatments are only rarely curative and there are no drugs in clinical use for VMs targeting mutated TIE2 or PIK3CA. Therefore, investigation of new molecular drugs is necessary for the development of effective and personalized VM-targeted medical therapies. Here, we developed VM *in vitro* model using 3D co-culture of human adipose stromal cell (hASC) / umbilical vein endothelial cells (HUVECs) in fibrin gel. We found this model suitable to investigate cellular abnormalities caused VM causative TIE2 mutation (mesenchymal cell differentiation, tube formation, ECM deposition) and for testing of efficacy of drug candidates to normalize cellular abnormalities. Here, we have investigated the effect of rapmycin (as a golden standard), alpelisib (PI3K inhibitor), AZ03 (TIE2 inhibitor-developed by AstraZeneca). The effect of flow on VM ECs was also tested in hASC/HUVEC co-culture set up in microfluidic apparatus designed and manufactured by Finnadvance.

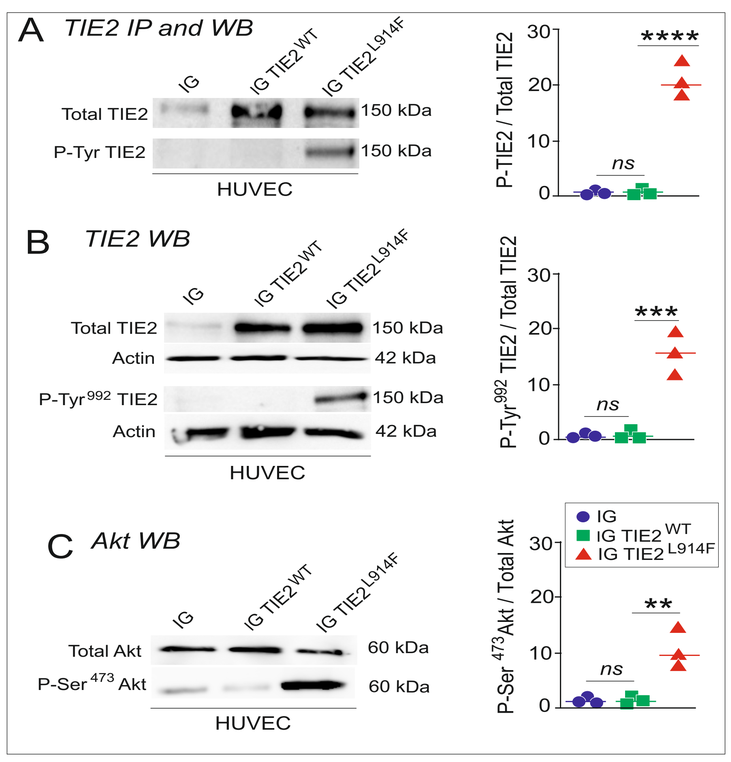

Fig. 1. Western blot analysis of retrovirally transduced HUVECs. Retroviral gene transfer was used express VM causative TIE2-L914F mutation in HUVECs, TIE2-WT and non TIE2 overexpressing HUVECs were used as controls. Cells were transduced with the bicistronic IRES vector so that the transduced cells also expressed GFP marker gene (IG). (A) shows the level of TIE2 expression and phosphorylation in immunoprecipitation, (B, C) show the level of TIE2 and Akt in western blot and anti-actin. On the right side, the quantification of signals from western blot are shown. The whole figure shows that HUVECs IG is like normal HUVECs, the level of TIE2 expression is similar in WT and mutant and the only difference between the WT and mutant is phosphorylation of TIE2 in mutant as we aimed.

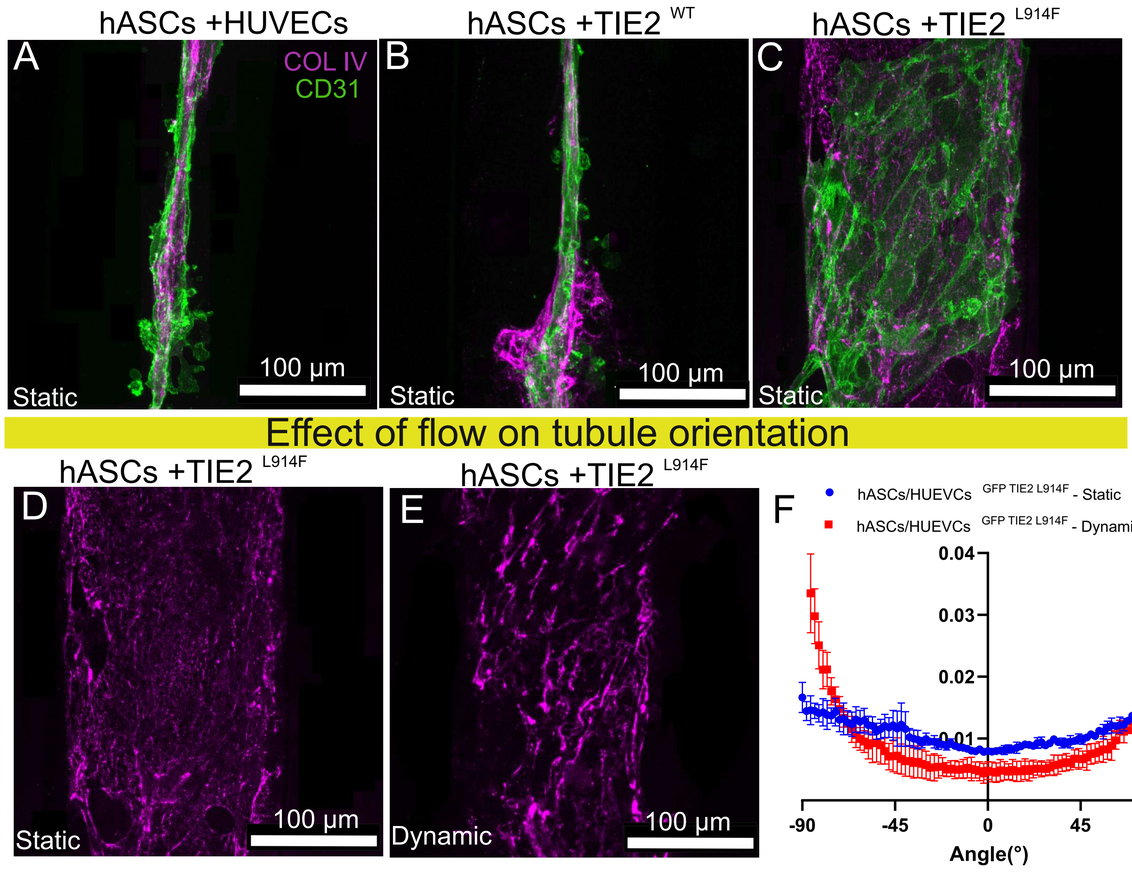

Fig 3. Tubular formation of hASCs and HUVECs mix with collagen type 1 (1mg/mL) loaded in microfluidic channel. A, B & C) show the tubular formation of hASCs in coculture with normal, TIE2 [WT], TIE2 [L914F], respectively. **D, E)** show the COL IV deposited in static & dynamic mode, respectively. **F)** shows the effect of flow on tubular structures orientation on static & dynamic mode.

Methods & Results

In the co-culture model, first, the plate is coated with fibrin gel, then hASCs were loaded (20,000 cell/cm^2) and after a couple of hours, HUVECs (5,000 cell/cm^2) were loaded on top of it. A specific vascular stimulation media developed by FICAM (VA Cure partner organization) was used to enhance EC tube formation. Different drugs were added on day 5 to evaluate their effect on enlarged EC tubules, ECM deposition and SMCs coverage. The co-culture model is then cultured for 7 days in this media and then fixed and imaged with confocal microscope. To check of the flow on the tube formation in co-culture model, microfluidic channels were coated with fibronectin (100 µg/mL) and then hASCs (2×10^6 cell/mL) and HUVECs (2×10^6 cell/mL) were loaded within few hours respectively. The chips were placed both in static mode and in dynamic mode on a rocker. The rocker was adjusted on 25° angle and 2 RPM to exert 0.4-4.0 Pa shear stress on the cells.

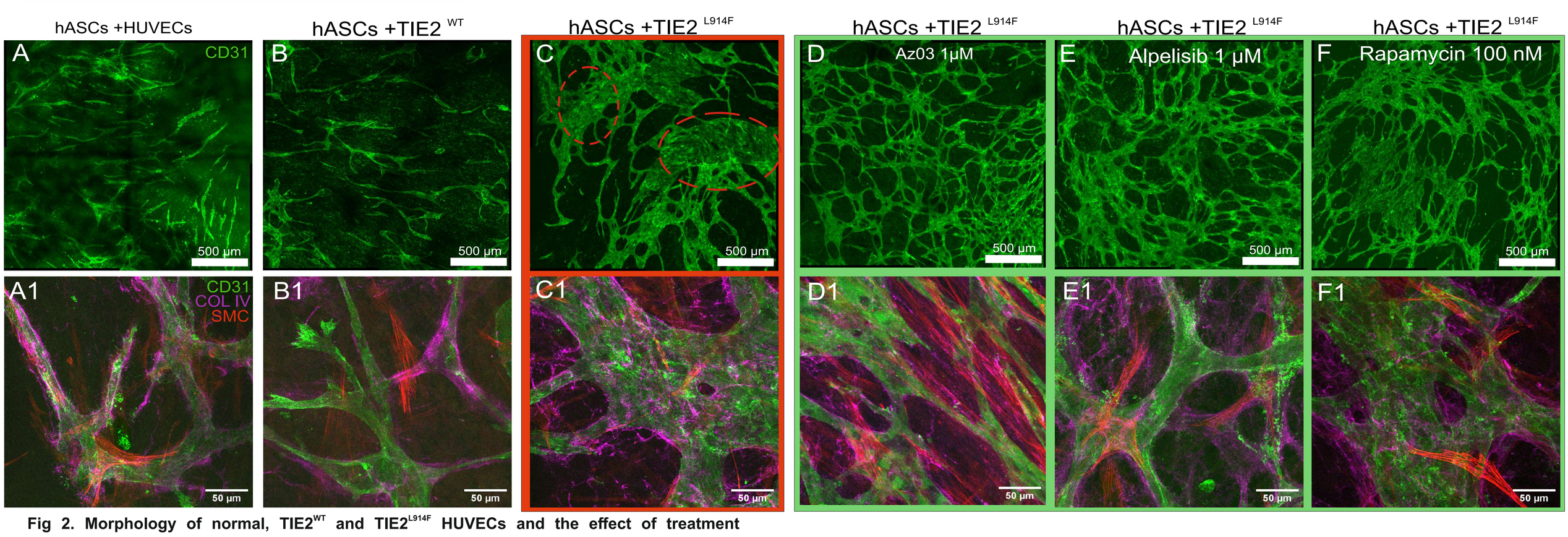

Fig 2. Morphology of normal, TIE2^{WT} and TIE2^{L914F} HUVECs and the effect of treatment (alpelisib, rapamycin and Az03) on vascular structures in human adipocyte stem cell/endothelial cell co-culture model. ECs are stained with CD31, αSMC indicates hASCs differentiation to towards smooth muscle -like cells and ECM stained with Col IV. **A & A1, B & B1**) The normal and TIE2^{WT} HUVECs organized a tubular network as previously shown by others[4]. **C & C1**) TIE2^{L914F} results in abnormally enlarger vascular structures, widely dispersed Col IV and less coverage by SMCs. **D & D1, E & E1, F & F1**) show TIE2^{L914F} vascular structure after treatment against Az03 1µM, alpelisib 1µM, and rapamycin 100 nM for 48 hr.

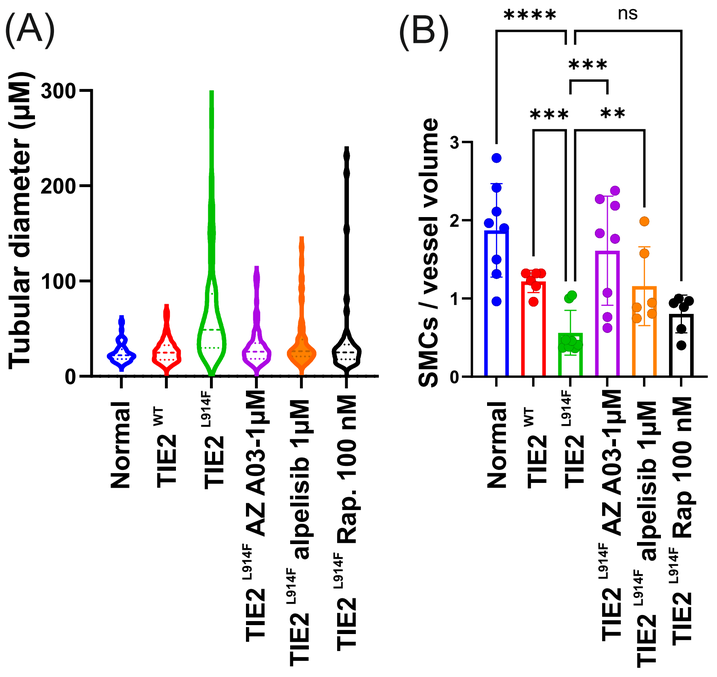

Fig 3. The quantification of vessel's and SMCs's diameters and volumes. A) The tubule (COL IV) diameters measurement show that after treatment with apleisib and AZ03 the number of abnormally enlarged vessel has decreased. **B)** Using 3D rendering in Imaris, all vessels and SMCs are measured and the ratio of SMCs volume to vessel volume is plotted here. This means relative SMCs coverage for vessel which is the lowest in TIE2^{L914F} and has been inhibited in TIE2^{L914F} with alpelisib and AZ A03 TIE2 inhibitors.

Conclusion

In this research, we have developed an *in vitro* 3D co-culture model using hASCs and VM causing retrovirally transduced HUVECs to model venous malformation. This 3D model resembles the venous malformation in three main aspects including enlarged vessel, aberrantly organized perivascular ECM and lower SMC coverage[5]. More interestingly, this model responds to TIE2 inhibitors and shows high potential to be used for further drug screening. Additionally, using microfluidic chip and inducing the flow, we investigated the effect of flow on ECM deposition orientation in this *in vitro* disease model.

References

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