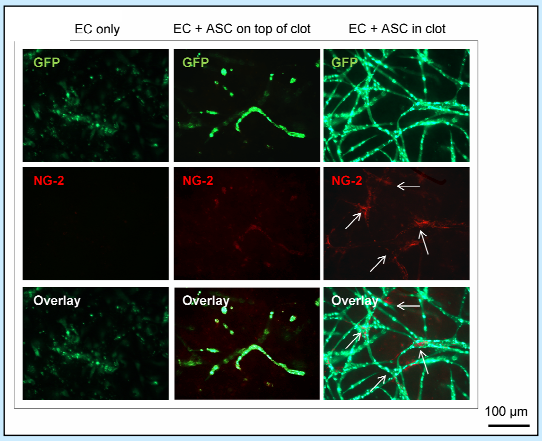
Mechanisms of vasculogenesis in 3D fibrin matrices mediated by the interaction of adipose-derived stem cells and endothelial cells

Sabrina Rohringer[1,2], Severin Mühleder[1,2], Pablo Hofbauer[1,2], Heinz Redl[1,2], Susanne Wolbank[1,2], Wolfgang Holnthoner[1,2]

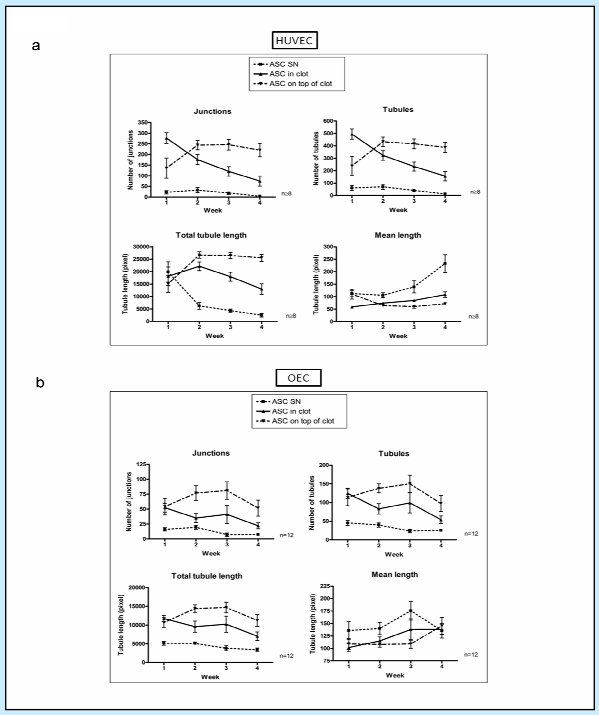
[1]Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, AUVA Research Centre, Vienna, Austria
[2]Austrian Cluster for Tissue Regeneration, Vienna, Austria

Summary: Vascularization of tissue-engineered constructs is essential to provide sufficient nutrient supply and hemostasis after implantation into target sites. Co-cultures of adipose-derived stem cells (ASC) with outgrowth endothelial cells (OEC) in fibrin gels were shown to provide an effective possibility to induce vasculogenesis *in vitro*. However, the mechanisms of the interaction between these two cell types remain unclear so far. The aim of this study was to evaluate differences of direct and indirect stimulation of ASC-induced vasculogenesis, the influence of ASC on network stabilization and molecular mechanisms involved in vascular structure formation. Endothelial cells (EC) were embedded in fibrin gels either containing non-coated or ASC-coated microcarrier beads as well as ASC alone. Moreover, EC-seeded constructs incubated with ASC-conditioned medium were used in addition to constructs with ASC seeded on top. Vascular network formation was visualized by green fluorescent protein (GFP) expressing cells or immunostaining for CD31 and quantified. RT-qPCR of cells derived from co-cultures in fibrin was performed to evaluate changes in the expression of EC marker genes during the first week of culture. Moreover, angiogenesis-related protein levels were measured by performing angiogenesis proteome profiler arrays. The results demonstrate that proximity of endothelial cells and ASC is required for network formation and ASC stabilize EC networks by developing pericyte characteristics. We further showed that ASC induce controlled vessel growth by secreting pro-angiogenic and regulatory proteins. This study reveals angiogenic protein profiles involved in EC/ASC interactions in fibrin matrices and confirms the usability of OEC/ASC co-cultures for autologous vascular tissue engineering.

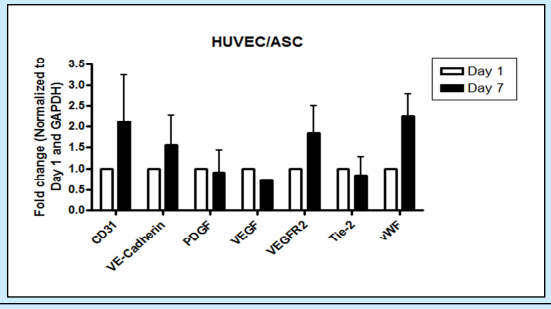
Aim: In this study we compared direct and indirect ASC-induced OEC and HUVEC vascular network formation in fibrin gels over a time period of 4 weeks *in vitro*. Here, we give evidence for mechanisms involved in ASC-induced vasculogenesis *in vitro*.



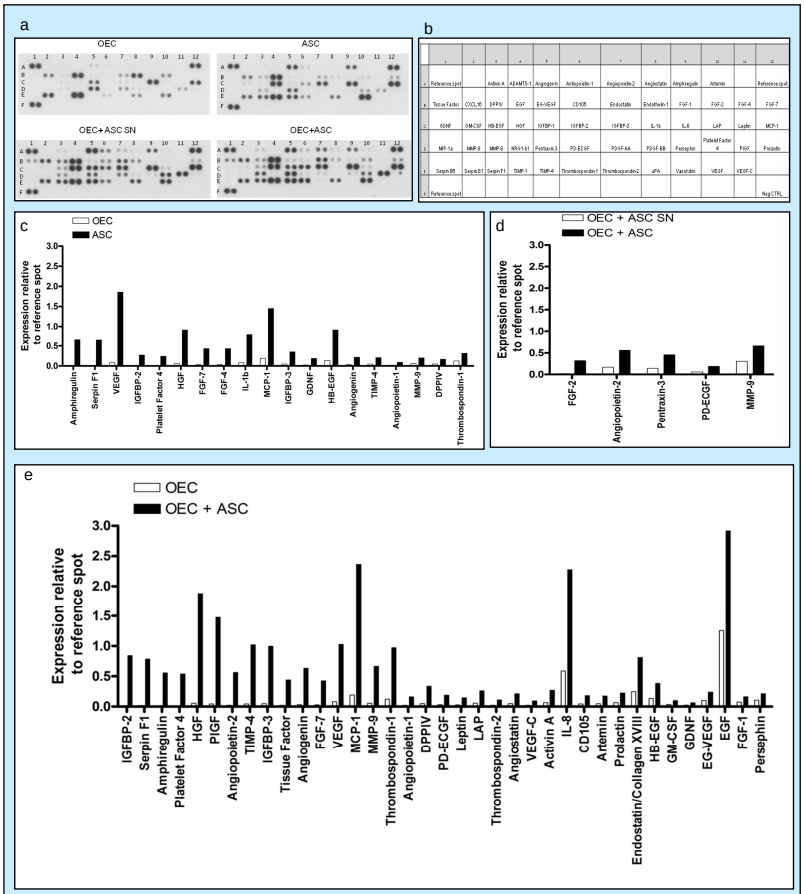
NG-2 staining of ASC in HUVEC/ASC co-cultures. ASC express pericyte marker NG-2 after four weeks of incubation whereas HUVEC are negative for NG-2. ASC are positive for NG-2 just in several regions of the network (arrows)



Quantification of network formation in HUVEC/ASC and OEC/ASC 3D co-cultures. (a) ASC supernatants are not sufficient to induce stable HUVEC network formation. Networks formed by HUVEC start to degrade after one week of culture, whereas vascular structures stimulated by ASC seeded on top of the fibrin clot remain stable for several weeks. The total tubule length decreases whereas the mean tubule length increases. (b) OEC form similar networks either with ASC in the clot or on top of the clot after one week. In contrast to ASC/OEC co-cultures (both cell types seeded into the clot), ASC seeded on top of the clot induce further network growth. While total tubule lengths slightly decrease, the mean tubule lengths show a rise after three weeks. 3 ASC and OEC and 1 GFP HUVEC donor were used. Data shown as mean +/- SEM



Expression of EC marker genes changes of HUVEC/ASC co-cultures in fibrin matrices between day 1 and day 7. Co-cultures showed an increase of CD31, VE-Cadherin, VEGFR2 and vWF levels, whereas PDGF and Tie-2 did not change over time. VEGF expression was slightly reduced after one week of culture. 2 different ASC and HUVEC donors were used. Data shown as mean +/- SEM



(a) Overview of angiogenesis array membranes. Angiogenesis array membranes include 55 different angiogenesis-related cytokines which are spotted in duplicates on nitrocellulose membranes. Membranes were exposed to films for 1 hour and show different condition-dependent spot densities. (b) Allocation of proteins throughout the membranes. (c) Protein expression of angiogenesis-related proteins in OEC, ASC and OEC/ASC co-cultures. (a) Several pro- and anti-angiogenic/regulatory proteins were more than 2-fold upregulated in ASC compared to OEC. (d) Differences of protein expressions comparing OEC/ASC co-cultures with OEC incubated in ASC-conditioned medium showed an upregulation of FGF-2, Angiopoietin-2, Pentraxin-3, PD-ECGF and MMP-9. (e) Comparison of protein expression levels of OEC with direct and indirect ASC stimulation. Co-cultures of ASC with OEC induce much higher expression of pro-angiogenic and regulatory proteins compared to OEC alone.

Conclusion: We show that the direct interaction between adipose-derived stem cells and endothelial cells is a prerequisite for mature vascular network formation in fibrin gels. Established networks started to degrade after the second week of culture unless ASC were seeded on top of the fibrin constructs suggesting that this seeding procedure is the most suitable for prevascularization of tissue-engineered constructs. Furthermore, ASC contribute to vessel stabilization by expressing the pericyte marker NG-2. Stable network formation is induced by increases in CD31, VE-Cadherin, VEGFR2 and vWF gene expression. We identified several pro-angiogenic and regulatory proteins involved in ASC-induced vasculogenesis indicating that ASC stimulate a controlled vessel growth.. Furthermore, *in vivo* studies are necessary to prove the functionality of the generated microcapillaries. Our work provides an insight into interactions between ASC and EC during vasculogenesis *in vitro* and confirms the usability of ASC and OEC for the prevascularization of tissue engineered organs.

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