

Sonic Hedgehog promotes angiogenesis and osteogenesis in a co-culture system consisting of primary osteoblasts and outgrowth endothelial cells

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INTRODUCTION: The angiogenic potential of outgrowth endothelial cells (OECs) *in vitro* and *in vivo* has been examined and documented in a number of previous studies(1-3). In co-culture systems using OECs together with primary osteoblasts (pOBs), OECs revealed an organization into microvessel-like structures which increase during the course of co-cultivation. One promising signalling pathway that may contribute to both new vessel formation and bone formation is the Sonic hedgehog (Shh) pathway, which might be used for therapeutical approaches. The purpose of this work is to investigate a possible role for Shh signalling in the co-culture system consisting of pOBs and OECs.

METHODS: OECs were isolated from peripheral blood buffy coats as described previously. Human primary osteoblasts were isolated from human cancellous bone fragments from healthy donors. Co-cultures of different donors were grown on fibronectin-coated Thermanox coverslips, seeding primary osteoblasts first (300.000/well) followed by seeding of OECs (200.000/well) 24 hours later. After 1 week of co-cultivation, cells were treated with 5µg/ml recombinant human Shh-N in EBM-2 with supplements from the kit, 5%FCS/1% P/S for 24 hours. Supernatants were harvested for ELISA and cells were fixed for immunofluorescence staining or lysed for RNA and protein isolation. Quantitative real-time RT-PCR was performed to detect the expression of endothelial marker as well as angiogenic and osteogenic marker expression in the co-culture system.

RESULTS: Recombinant Shh protein was used to treat co-cultures of pOBs and OECs. Cells were co-cultured 1 week before stimulation with 5µg/ml recombinant Shh for 24 hours and stained immunohistochemically for the endothelial marker CD31 (fig.1). cDNA from treated and untreated co-cultures was used for quantitative real-time PCR of different genes that are involved in angiogenesis as well as

osteogenesis (fig.2). Shh treatment leads to a massive increase in the formation of microvessel-like structures in the co-cultures compared to untreated cells, possibly due to an upregulation of angiogenic factors like VEGF, Angiopoietin1 and Angiopoietin2 which could be shown at the mRNA level (fig.2) and measured in an enzyme-linked immunosorbent assay (data not shown).

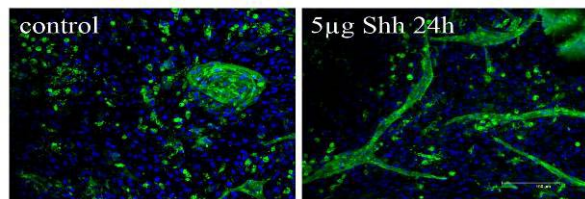


Fig.1: Immunofluorescence staining for CD31 after 24 hours treatment with 5µg/ml Shh compared to control co-cultures. n=4

Shh treatment of co-cultures for 24 hours results in a considerable upregulation of several osteogenic genes as well (fig.2). This could be confirmed by an increase in mineralization evaluated by Alizarin Red and an the increase of the alkaline phosphatase activity in the Shh treated co-culture (data not shown).

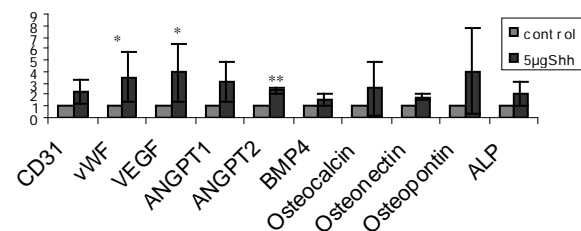


Fig.2: Mean values of relative gene expression of different genes involved in angiogenesis and osteogenesis in Shh treated and untreated co-cultures. RPL13A was taken as endogenous control. n=4

DISCUSSION & CONCLUSIONS: The results shown in figure 1 and 2 clearly support a crucial function of the morphogen Sonic hedgehog in both angiogenesis and osteogenesis in the co-culture system consisting of pOBs and OECs.

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