Fibrin delivery of molecular variants of Synergy and RGD-containing fibronectin domains that provide integrin-specific instructions to mesenchymal stem cells

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Statement of Purpose: Fibrin is an ideal protein/cell delivery vehicle for repairing damaged tissues since the polymer is derived from the normal haemostatic pathway, is well tolerated, and is FDA-approved. Unfortunately, pure fibrin gels do not sustain adequate non-inflammatory cell attachment. Contaminating fibronectin (FN), a multidomain extracellular matrix (ECM) protein present in fibrinogen preparations, is responsible, through its central cell-binding domain, for cell attachment to fibrin gels. FN has recently been shown to switch between the stimulation of proliferation versus differentiation through modulation of integrin binding affinities[1,2]. One example of such a switch is the binding of $\alpha_5\beta_1$ integrin, which requires binding to the Synergy sequence (PHSRN) on the 9th Type III repeat and RGD on the 10th Type III repeat, versus $\alpha_v\beta_3$ integrin requires only RGD. We hypothesized that the integration of variants of the 9th and 10th Type III repeats with different integrin binding affinities in the place of full-length FN would provide more specific instructions that would direct cells towards proliferation and/or differentiation. In this study, we determine how modified 9th and 10th Type III repeats alter the homeostasis of human mesenchymal stem cells (MSCs) in 2D and incorporated in a fibrin matrix.

Methods: *FN domains:* The 9th and 10th Type III repeats (FN III10 and FNIII9-10) were cloned by standard PCR from human FN cDNA into the pGEX4T-1 expression vector. Addition of the α_2-plasmin inhibitor transglutaminase-sensitive sequence at the N-terminus allows covalent crosslinking into fibrin gels[3]. A point mutation of Leu^{1408} to Pro (FN III9*-10) was made to increase the specificity for $\alpha_5\beta_1$ integrin[4]. *E.coli* BL21 were used for protein production and proteins were purified on an *ÄKTA*FPLC with a GSTPrep column. *Attachment and integrin binding specificity:* 96-well plates were coated with ECM proteins (FN, FN III9-10, FN III9*-10, FN III10 and fibrinogen). MSCs were plated and allowed to attach for 15 min with or without functional blocking antibody (FBA) for the integrin subunit α_5 and/or α_v. Adherent cells were quantified with crystal violet. *Spreading assay:* Electrical Cell Impedance Sensing was used to determine spreading rates of MSCs on ECM-coated (FN, FN III9-10, FN III9*-10, FN III10 and fibrinogen) electrodes. *Proliferation and differentiation in 2D:* 48-well plates were coated as the attachment assay with 2μM of ECM proteins. MSCs, in serum-free media, were plated with or without α_5 FBA and allowed to attach for 16h, after which 10% FBS (FN depleted) alone or in presence of 100nM dexamethasone and 10mM β-glycerol phosphate (to promote osteoblastic differentiation) was added. Cell number was quantified with Alamar blue after 4, 7, 14 and 21 days with pre-established standard curves. Alkaline phosphatase (ALP) activity was determined with p-nitrophenyl phosphate (*p*NPP). *Proliferation and differentiation in fibrin gel:* Fibrin polymers were made with different ratios of ECM molecules to obtain 1 or 5 molecule(s) of fibrinogen per molecule of FN domains (4-12mg/ml fibrinogen, 50-250μg/ml FN/FN domains with 2U/ml thrombin, 1U/ml factor XIII and 17μg/ml aprotinin). MSCs were seeded into the matrices and cell morphology, cell number and ALP activity were quantified respectively by microscopy, Alamar blue and *p*NPP.

Results/Discussion: Differences in MSC binding and spreading appeared between the ECM proteins. FN, FN III9-10 and FN III9*-10 showed the highest binding and spreading rates, FN III10, despite poor initial cellular attachment, displayed near-equivalent spreading rates as full-length FN, whereas fibrinogen demonstrates poor cellular attachment and low spreading kinetics. Attachment assays in the presence of FBA confirmed the specificity of FN III9-10 and FN III9*-10 for the integrin subunit α_5 (FN III9*-10 > FN III9-10), whereas FN III10 showed poor specificity for both α_v and α_5 integrin subunits, probably because of the RGD promiscuity. In 2D, FN was the best substrate for MSC proliferation. Interestingly, the proliferation on FN III9-10 and FN III9*-10 was significantly better than FN III10 and assays with FBA demonstrated that α_5 binding was implied in these differences. The differentiation of MSCs into osteoblasts was significantly accelerated on the recombinant FN domains compared to FN and fibrinogen (FN III9*-10 > FN III9-10 > FN III10 > FN > fibrinogen). Again, α_5 binding appears responsible for these differences since α_5 FBA were capable of significantly reducing these differentiation events. Preliminary results delivering FN domains and FN in fibrin gel displayed similar differences as in 2D.

Conclusions: Replacement of FN by FN domains provides more specific instructions to MSCs directing proliferation and/or differentiation while maintaining similar attachment and spreading capacities of the full length protein. This new generation of fibrin matrix could be used alone or in combination with other factors for cellular process that required specific integrin binding.

References:

1 Lan MA *et al.* Biomaterials. 2005 26(22):4523-31.
2 Stephansson SN *et al*. Biomaterials. 2002 23(12):2527-34.
3 Schense JC and Hubbell JA. Bioconjug Chem. 1999 Jan-Feb;10(1):75-81.
4 Altroff H *et al.* J Biol Chem. 2003 Jan 3;278(1):491-7.