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Controlling integrin specificity and stem cell differentiation in 2-D and 3-D environments through regulation of fibronectin domain stability

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Abstract

The extracellular matrix (ECM) exerts powerful control over many cellular phenomena, including stem cell differentiation. As such, design and modulation of ECM analogs to ligate specific integrin is a promising approach to control cellular processes *in vitro* and *in vivo* for regenerative medicine strategies. Although fibronectin (FN), a crucial ECM protein in tissue development and repair, and its RGD peptide are widely used for cell adhesion, the promiscuity with which they engage integrins leads to difficulty in control of receptor-specific interactions. Recent simulations of force-mediated unfolding of FN domains and sequences analysis of human versus mouse FN suggest that the structural stability of the FN's central cell-binding domains (FN III9-10) affects its integrin specificity. Through production of FN III9-10 variants with variable stabilities, we obtained ligands that present different specificities for the integrin $\alpha_5\beta_1$ and that can be covalently linked into fibrin matrices. Here, we demonstrate the capacity of $\alpha_5\beta_1$ integrin-specific engagement to influence human mesenchymal stem cell (MSC) behavior in 2D and 3D environments. Our data indicate that $\alpha_5\beta_1$ has an important role in the control of MSC osteogenic differentiation. FN fragments with increased specificity for $\alpha_5\beta_1$ versus $\alpha_v\beta_3$ results in significantly enhanced osteogenic differentiation of MSCs in 2D and in a clinically relevant 3D fibrin matrix system, although attachment/spreading and proliferation were comparable with that on full-length FN. This work shows how integrin-dependent cellular interactions with the ECM can be engineered to control stem cell fate, within a system appropriate for both 3D cell culture and tissue engineering.

Keywords

Fibronectin; Integrins; Recombinant protein; Hydrogel; Mesenchymal stem cell; Osteogenesis

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INTRODUCTION

Biomaterial matrices are being explored to guide stem cell differentiation phenomena, for purposes both *in vitro* and *in vivo* [1]. Many efforts are focused on providing a biofunctional surface for cell adhesion through addition of natural extracellular matrix (ECM) proteins such as fibronectin (FN) or cell-adhesive ligand motifs derived from ECM. FN is a core ECM component of many tissues, where it regulates a variety of cell activities predominantly through direct interactions with cell surface integrin receptors. FN is critically important in vertebrate development [2], since it mediates a wide variety of cellular interactions and plays important roles in cell adhesion, migration, growth and differentiation [3]. The capacity of FN to bind up to 20 distinct integrins provides its influence on multiple tissues and cell types. Moreover, physiological molecular unfolding and refolding of the more than 15 FN Type III repeats has been proposed as a part of FN's control mechanism for integrin-specific binding [4]. Thus, this capacity to bind multiple integrins represents a design challenge when delivering FN to instruct specific cell behaviors. Small protein fragments corresponding to functional FN domains that contain integrin-specific binding sites should be used instead of the full-length protein, which displays low level of specificity, or small FN-derived peptides such as RGD that notoriously display poor integrin specificity.

The integrin $\alpha_5\beta_1$ is an important FN-specific integrin that can be found in different adhesion structures [5], and has been implicated in the control of differentiation of various cell types, such as precursor cell osteogenic differentiation (OD, [6-9]), while its effect on human mesenchymal stem cells (MSC) OD is still unknown [10]. Functionally, $\alpha_5\beta_1$ interaction requires both the traditional integrin-binding sequence (RGD) located in the 10th type III repeat (FN III10) as well as the "synergy sequence" (PHSRN) in the adjacent 9th type III repeat (FN III9), whereas most of other RGD dependant integrins such as $\alpha_v\beta_3$ do not require PHSRN [11]. Interestingly, type III repeats are stabilized only by hydrogen bonding and van der Waals forces [12]. Recent simulations of physiologically-relevant force-mediated unfolding of the FN III9-10 structure predicts the existence of a stable intermediate structural state prior to complete unfolding of the 10th type III repeat [13]. In this stable intermediate, the PHSRN-to-RGD distance has been shown to be too large for both sites to synergistically bind the same receptor [14] suggesting that synergy-dependent binding of $\alpha_5\beta_1$ can be turned off by simply stretching FN III9-10 into this intermediate state. This mechano-sensitive regulation of $\alpha_5\beta_1$ binding is further supported by the fact that the degrees of conformational stability of FN III9 modulate integrin accessibility to the RGD motif [15]. One intriguing example of this effect is the stabilization of the FN III9 domain attributed to a single human to mouse (Leu¹⁴⁰⁸ to Pro) mutation that enhances both conformational stability of FN III9-10 and affinity for $\alpha_5\beta_1$ [16]. Because $\alpha_5\beta_1$ binding requires this critical and sensitive domain conformation, it is clear that $\alpha_5\beta_1$ engagement can not be efficiently accomplished with simple or tandem peptides [17] and can possibly be tuned through molecular modifications which alter conformational stability.

In this study, we investigate how engineered integrin-specific ECM fragments can influence MSC behavior in both 2D and 3D environments, determining the effects of the ligation of integrin $\alpha_5\beta_1$ to various degrees. We determined the integrin specificity of full-length FN compared to recombinant FN III9-10 domains, specifically FN III9-10, the structurally-stabilized FN III9*-10 (Leu¹⁴⁰⁸ to Pro) and FN III10, and their capacity to direct MSC behavior in the context of OD. Cell attachment and spreading, proliferation, and differentiation responses on 2D surfaces and in a clinically relevant 3D fibrin matrix system [18] are reported.

MATERIALS AND METHODS

Cell culture

MSCs from human bone marrow purified according to the protocol of Pierre Charbord laboratory (Université François-Rabelais, Tours, France) were purchased from Biopredic Int. (Rennes, France). Cells were expanded until passage 3 in Minimum Essential Medium Alpha (MEM- α) supplemented with 10% bovine growth serum (BGS, HyClone, Logan, UT, USA), 100 U/mL penicillin, 0.1 mg/mL streptomycin and 2 mM L-glutamine (expansion media). Cells were utilized at passage 4.

MSC integrin expression during OD

MSCs were cultured for 3, 7 and 14 days in expansion medium or osteogenic medium composed of Dulbecco's Modified Eagle's Medium (DMEM) (4 g/L glucose), 100 nM dexamethasone (DEX; Sigma-Aldrich, Buchs, Switzerland), 10 mM β -glycerol phosphate (β -GP; Sigma-Aldrich, Buchs, Switzerland), 0.05 mM L-ascorbic acid phosphate magnesium salt n-hydrate (AsAP, Wako Chemical, Osaka, Japan) and 10% BGS. Surface expression of α_5 , α_2 , α_v , β_1 and β_3 integrin was quantified by flow cytometry (CyAn_{ADP}, DakoCytomation, Glostrup, Denmark). Antibodies: α_5 (clone IIA1), BD Bioscience, San Jose, CA, USA; α_v (clone 313.6F8), Merck KGaA, Darmstadt, Germany; α_2 (clone HAS3), β_1 (clone 4B7R) and $\alpha_v\beta_3$ (clone 23C6), Abcam, Cambridge, UK.

Recombinant FN fragment production

cDNAs encoding FN III9-10, and FN III10 were amplified from the pFH154 plasmid (LGC Promochem, Teddington, UK) by standard PCR. The 5' primers contained the sequence encoding a transglutaminase substrate sequence NQEQVSPL [18]. The amplified sequences were inserted into the pGEX4T-1 expression vector (GE Healthcare, Chalfont St. Giles, UK) and the Leu¹⁴⁰⁸ to Pro mutation (FN III9*-10) was made using the pGEX4T-1-FN III9-10 plasmid following the QuickChange protocol (Stratagen, La Jolla, CA, USA). FN fragments were generated in BL21 *E.coli* and purified by GST affinity chromatography (ÄKTA_{FPLC}, GE Healthcare, Chalfont St. Giles, UK). GST tags were removed using bovine thrombin (Sigma-Aldrich, Buchs, Switzerland) and proteins were verified as > 98% pure by SDS-PAGE and endotoxin level was verified as under 0.1 EU/mL (Limulus Amebocyte Lysate (LAL) QCL-1000 kit, East Rutherford, NJ, USA).

Relative coating efficiencies of full-length FN and FN fragments

Polystyrene cell culture plates (48-well, Costar 3548; Corning Inc., Schiphol Rijk, The Netherlands) were incubated with Hank's Balanced Salt Solutions (HBSS) containing FN or FN III9(*)-10 at increasing concentrations (up to 2 μ M of FN and up to 4 μ M FN III9-10) 1 h at 37°C. Wells were then blocked with 0.1% heat denatured BSA (30 min at RT) and incubated with 0.1 μ g/mL of a mouse monoclonal antibody specific for the domain FN III9-10 (clone 3E3, Millipore, Billerica, MA, USA). After incubation with a rabbit anti-mouse antibody coupled to horseradish peroxidase (0.25 μ g/mL; Invitrogen, Carlsbad, CA, USA), wells were washed with phosphate-buffered saline (PBS) and secondary antibodies (relative to surface protein number) were detected using TMB substrate (GE Healthcare, Chalfont St. Giles, UK) with measurement of the absorbance at 450 nm. Final values were obtained by subtracting the signal due to non-specific binding of secondary antibody. Non-specific binding for each condition was determined by duplicating the experiment without the primary antibody.

MSC adhesion and integrin specificity for FN fragments

Polystyrene plates were coated with human plasma FN (Sigma-Aldrich, Buchs, Switzerland) or FN fragments by adsorption at increasing concentrations (up to 32 μ M) for 1 h at 37 °C in

HBSS and blocked with 0.1% heat denatured BSA (except for the 100% adhesion control). MSCs (500'000/mL) in serum-free medium with HBSS containing calcium and magnesium, (v/v = 1:1) were allowed to attach for 15 min at 37 °C with or without 1 µg of FBA against the integrin(s) $\alpha_5\beta_1$ (clone JBS5, Abcam, Cambridge, UK) and/or $\alpha_v\beta_3$ (clone 23C6; Abcam, Cambridge, UK). After intensive washes with PBS, adherent cells were fixed with 5% glutaraldehyde and cell attachment determined by crystal violet staining. For the 100% adhesion control cells were fixed without washes. Cell adhesion was reported as percent adhesion compared to 100% adhesion control.

Electrical Cell-Impedance Sensing

Wells of gold-plated electrode arrays (Applied Biophysics Inc., Troy, NY, USA) were coated, as in the adhesion assays, with 2 µM of FN fragments and 1 µM of full-length FN. MSCs (500,000/mL, MEM- α) were added to each well and the electrode resistance was recorded. Complete single-layer cell coverage was confirmed microscopically at the end of each experiment. Trace resistance values were adjusted by subtraction of the medium-only baseline. Data is reported as mean resistance (ohm) of three independent experiments over time.

Immunostaining

Glass slides (8-well, Nalge Nunc Int. Rochester, NY, USA) were coated with 5 µM of FN fragments and 0.25 µM of full-length FN and blocked with BSA as in the adhesion assays. After 2 and 6 h incubation at 37°C, 5% CO₂, wells were fixed (4% paraformaldehyde, 0.5% Triton X-100 in PBS) for 10 min. Samples were incubated with mouse anti- α_v subunit (10 µg/mL) and rat anti- α_5 subunit (20 µg/mL, a gift from Dr. K.M. Yamada, National Institutes of Health, NIDCR, USA) for 1 h at RT. Detection was performed using goat anti-mouse AlexaFluor-488 and goat anti-rat AlexaFluor-546 (Invitrogen, Carlsbad, CA, USA). Nuclei were stained by Hoechst 33342 (Invitrogen, Carlsbad, CA, USA). Cells were mounted and examined under a fluorescence confocal microscope (LSM 510, Carl Zeiss, Göttingen, Germany).

Proliferation and differentiation assays in 2D

Polystyrene plates were coated with 2 µM of FN fragments and 0.1 µM of full-length FN and MSCs (4,000 in MEM- α for proliferation and 10,000 in DMEM for differentiation) were plated with or without 0.5 µg of $\alpha_5\beta_1$ and/or $\alpha_v\beta_3$ FBA and allowed to attach and spread for 16 h. Subsequently, BGS was added to 10% for proliferation assays, and osteogenic components (100 nM DEX, 10 mM β -GP, 10% BGS) were added for differentiation assays. On days 4, 7, and 14, Alamar Blue (5%, v/v; AbD serotec, Raleigh, NC, USA) was added to the medium for 5 h. The reduction of Alamar Blue was determined, and cell numbers calculated using standard curves. For differentiation assays, cells were lysed by short sonication on day 14 and alkaline ALP activity was quantified using p-nitrophenyl phosphate (AnaSpec Corp., San Jose, CA, USA) and normalized to cell number. For all proliferation and differentiation assays, the BGS used was depleted of FN using Gelatin Sepharose 4B beads (GE Healthcare, Chalfont St. Giles, UK).

Osteoblast-specific gene expression in 2D

MSCs were cultured on FN proteins identically as 2D differentiation assay for 12 days. Quantitative PCR using IQ SYBR Green Supermix (BioRad, Hercules, CA, USA) was performed using the iCycler IQ Multicolor Real-Time PCR Detection System. Primers for ALP (Genebank #BC126165), CBFA-1 (Genebank #NM_004348), IBSP (Genebank #BC111920), OST (Genebank #BC093033) and β -actin (Genebank #BC002409) were designed with Beacon Designer 4.0 (Premier Biosoft, Palo Alto, CA, USA). Only primers verified by melt-curve analysis with an efficiency above 90% were used (sequences provided on request). Standard

dilution curves (1/5) were produced to determine PCR efficiency ($\epsilon = 10^{(-1/\text{slope})-1}$) and 10 ng of reverse-transcribed mRNA used in each reaction. Ct values for each transcript were normalized to β -actin and analyzed using REST [19] according to the Pfaffl method [20]. Significant differences in relative expression were determined by the pair-wise fixed reallocation randomization test built in REST.

Proliferation and differentiation in 3D fibrin matrices

Fibrin matrices comprised of 8 mg/mL fibrinogen (plasminogen-, von Willebrand factor- and FN-depleted; Enzyme Research Laboratories, South Bend, IN, USA), 2 U/mL factor XIIIa (gifted by Baxter BioSurgery, Vienna, Austria), 17 μ g/mL aprotinin (Roche, Applied Science, Mannheim, Germany), and 20% (v/v) MSCs (7,000/gel for proliferation assays and 15,000/gel for differentiation assays) in Tris-buffered saline) were generated by the addition of 2 U/mL human thrombin (Sigma-Aldrich, Buchs, Switzerland) and 5 mM Ca^{2+} . Matrices were made with a fibrinogen:FN fragment molar ratio of 5:1 or with 250 μ g/mL of FN. Matrices were transferred to a 48-well cell culture plate containing medium (MEM- α for proliferation, DMEM for differentiation) supplemented with 10% BGS. Cell number was assessed at 4, 7 and 14 days as in the 2D experiment. For differentiation, osteogenic components were added after 16 h. For ALP activity, gels were diced and sonicated briefly on day 14. Cell lysates were separated from the fibrin polymer aggregates by centrifugation and ALP activity was quantified as in 2D experiments. For osteoblast-specific gene expression, cells were extracted from gels on day 14 using bovine trypsin (1500 USP units/gel, 15 min at 37°C; EMD Chemicals, Inc. San Diego, CA, USA) and quantitative PCR was performed as in 2D assays.

Statistics

All experimental data represent mean $\pm$ SE of at least 3 independent triplicate experiments and statistical comparisons for all experimental sets were based on ANOVA using Tukey's test for pair-wise comparisons with a $P < 0.05$ considered as significant unless otherwise stated.

RESULTS

Surface expressions of integrins during MSC OD

Flow cytometry was used to detect surface expression of integrins during the initial steps of differentiation of MSCs in an osteoinductive media [21]. In the presence or absence of osteoinductive media, greater than 90% of the cells were positive for the integrin subunits β_1 , α_5 , α_2 and α_v , whereas $\alpha_v\beta_3$ integrin was weakly expressed (20% positive cells). Following 14 days in osteogenic media, no significant phenotypic changes in the population were observed (data not shown). However, the mean fluorescence intensity, representing the surface expression level, significantly increased for α_v , α_5 and β_1 , but not for α_2 over the same time (Fig. 1). Maximum surface expression levels were observed at 7 days for α_5 and β_1 subunits, whereas upregulation of α_v gradually increase during the 14 days. Compared to non-differentiated cells, surface expression levels were 3 ± 0.3 and 2.5 ± 0.3 fold higher for α_5 and β_1 , respectively.

Adhesion/spreading on FN and FN fragments and their specificity for $\alpha_5\beta_1$

FN fragments were generated that would theoretically display differing levels of $\alpha_5\beta_1$ integrin specificity (FN III9*-10 > FN III9-10 > FN III10) based on the presence of the synergy site and the conformational stability of FN III9 (Fig. 2B). Assuming that the FN fragments have similar coating efficiency [22] and using a monoclonal antibody specific for FN III9-10 to determine the coating efficiency of FN versus FN fragments, surfaces were coated in order to present equimolar adhesion molecules (Fig. 2A). FN and FN III9-10 presented equivalent number of absorbed proteins, when initial concentrations of 0.1 μ M and 2 μ M were used,

respectively. No differences between the coating efficiency of FN III9-10 and FN III9*-10 were observed (data not shown). MSCs displayed the highest levels of adhesion to FN and FN III9*-10 ($92.7\% \pm 2.5$ and $87.1\% \pm 1.4$ of total-adhesion, respectively), whereas FN III9-10 supported a medium level of adhesion capacity (66.8 ± 2.4 of total-adhesion) and FN III10 the lowest ($17.5\% \pm 1.8$ of total-adhesion) (Fig. 2C). Electrical cell-impedance sensing was used to measure MSC spreading on the ECM proteins. Both FN and FN III9*-10 supported rapid initial cell spreading rates reaching near maximal spreading state within 60 min. All fragments support cell adhesion and spreading as indicated by equivalent maximal spread states at 6 h, however, MSC spreading rates on FN III9-10 and FN III10 fragments were significant lower (Fig. 2D).

FN fragment specificity for $\alpha_5\beta_1$ was tested by inhibition of adhesion using functional blocking antibodies (FBA) for $\alpha_5\beta_1$ (α_5 subunit targeted) and/or $\alpha_v\beta_3$ (heterodimer targeted) MSC adhesion to FN proteins displayed a dose-response curve (data not shown) that has been used to determine the EC_{50} of each protein (FN protein initial coating concentrations for 50% attached cells after 15 min). Wells were coated with the EC_{50} of FN proteins and cell adhesion in the non-treated controls was used as the 100% adhesion control. FBA for $\alpha_5\beta_1$ strongly inhibited attachment on FN III9*-10 ($43.4 \pm 3.7\%$), and FN III9-10 ($57.4 \pm 4.7\%$), whereas $\alpha_5\beta_1$ FBA only inhibited attachment to FN to $82.3 \pm 6.6\%$ and to FN III10 to $83.4 \pm 4.0\%$ with respect to the control (Fig. 3A). Integrin $\alpha_v\beta_3$ FBA only weakly inhibited cell adhesion on each FN protein. As a second measure of integrin specificity, we further explored the clustering of integrin subunits α_5 and α_v in response to FN proteins. After 6 h, clustering of α_v was observed at focal adhesions more or less for all FN fragments and full-length FN, whereas strong differences were observed with α_5 localization. On FN and FN III9*-10, both α_5 and α_v were clustered and colocalized however, clustering of α_5 was much weaker on FN III9-10 and more so on FN III10 (Fig. 3B). Moreover, within 2 h, integrin α_5 formed clusters at focal adhesion sites predominantly on FN III9*-10. In contrast, on FN III10, integrin α_v clustering was observed but not α_5 , whereas on full-length FN and FN III9-10 both integrins were engaged (data not shown.).

MSC proliferation on FN and FN fragments in 2D

Following MSC attachment and spreading on polystyrene surfaces presenting equimolar FN and FN fragments for 16 h without serum to allow for integrin signaling initiation, proliferation was further stimulated by addition of serum. Significant differences in cell number were observed among proteins after 4 days. FN induced the highest increase in cell number followed by FN III9*-10 and FN III9-10, whereas FN III10 supported significantly lower levels of proliferation (Fig. 4A). The contribution of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins to the proliferative responses was determined using $\alpha_5\beta_1$ and $\alpha_v\beta_3$ FBA. Addition of $\alpha_5\beta_1$ FBA significantly reduced proliferation of cells cultured on FN III9*-10 and FN III9-10 ($P < 0.001$), but not on FN or FN III10. Interestingly, addition of the $\alpha_v\beta_3$ FBA led to a slight increase in the proliferation rate on each FN fragment with a significant increase for FN III10 ($P < 0.05$).

MSC OD on FN and FN fragments in 2D

MSC cultures on equimolar FN and FN fragments in osteogenic conditions were examined after 12 days by quantitative PCR. Alkaline phosphatase (ALP), core binding factor-1 (CBFA-1), integrin-binding sialoprotein (IBSP) and osteopontin (OST) message RNAs were each analyzed. All FN fragments displayed stronger osteoinduction potential compared to FN (Fig. 4B). FN III9*-10 was the most potent FN fragment for specific osteoblastic gene expression, resulting in significantly upregulated ALP (2 fold ± 0.5 , $P < 0.003$), CBFA-1 (1.8 fold ± 0.6 , $P < 0.014$) and IBSP (50.6 fold ± 18.4 , $P < 0.001$). FN III9-10 induced significant upregulation of CBFA-1 (1.8 fold ± 0.6 , $P < 0.048$) and IBSP (17.3 fold ± 6.4 , $P < 0.001$), and FN III10 upregulated CBFA-1 (1.7 fold ± 0.4 , $P < 0.014$) and IBSP (11.5 fold ± 4.9 , $P < 0.001$).

In contrast, only IBSP was significantly upregulated (5 fold $\pm$ 1.2, $P < 0.001$) in cultures on FN. No significant upregulation was observed for OST at 12 days on any FN protein.

Similarly, analysis of cellular ALP activity revealed significant differences at 14 days. ALP activities of MSCs cultured on FN III9*-10 and FN III9-10 were significantly higher compared to FN (Fig. 4C). Cells cultured on FN III9*-10 displayed the highest levels of ALP activity (8.9 ng/10,000 cells $\pm$ 0.8) followed by FN III9-10 (7.5 ng/10,000 cells $\pm$ 0.7), FN III10 (5.4 ng/10,000 cells $\pm$ 0.3) and FN (4.2 ng/10,000 cells $\pm$ 0.7). Addition of $\alpha_5\beta_1$ FBA significantly reduced ALP activity of MSCs in all conditions when compared to the non-FBA treated conditions (FN, $P < 0.001$; FN III9-10, $P < 0.05$; FN III9*-10, $P < 0.05$; Fig. 4C). Moreover, addition of $\alpha_v\beta_3$ FBA resulted in a significant increase in ALP activity on full-length FN ($P < 0.001$), FNIII9-10 ($P < 0.05$) and FN III9*-10 ($P < 0.05$) (Fig. 4C).

MSC proliferation in fibrin matrices functionalized with FN and FN fragments

Fibrin matrices were prepared by addition of thrombin to pure fibrinogen containing either full-length FN or engineered FN fragments, which were crosslinked to the matrix by factor XIIIa through native crosslinking sites for full-length FN and through an engineered substrate sequence (NQEQVSPL) on the N-terminus of FN fragments [18,23,24]. Non-functionalized fibrin was used as a control, since the matrix does not contain specific binding sites for $\alpha_5\beta_1$ [25]. However, MSCs within fibrin gels, displayed only small differences in proliferation within the first week, with total cell numbers nearly equivalent in each condition after 10 days (Fig. 5A).

MSC OD in fibrin matrices functionalized with FN and FN fragments

FN fragments containing FN III9, but not full-length FN and FN III10, displayed significant osteoinductive effects on MSCs, when cells were cultured in a standard osteoinductive media. Despite the osteoinductive properties of both FN III9-10 species, only the conformationally stabilized FN III9*-10 induced significantly higher levels of ALP activity (3.4 ng/10,000 cells $\pm$ 0.2; Fig. 5B) compared to both fibrin matrices containing full-length FN (1.5 ng/10,000 cells $\pm$ 0.3) and fibrin only (2.1 ng/10,000 cells $\pm$ 0.2). The ALP activities of cells cultured in matrices containing FN III9-10 and FN III10 were 2.5 ng/10,000 cells $\pm$ 0.2 and 2.3 ng/10,000 cells $\pm$ 0.3, respectively. As in 2D, even though $\alpha_5\beta_1$ FBA reduced ALP activity in all cultures, significant differences relative to non-FBA treated cells were observed only in matrices containing FN III9-10 ($P < 0.05$) and FN III9*-10 ($P < 0.01$). Addition of $\alpha_v\beta_3$ FBA slightly increased ALP activity in all conditions but not significantly (Fig. 5B).

Similarly, using MSCs cultured in fibrin matrix without additional ECM protein as the baseline, quantitative PCR showed that FN III9*-10 had a significantly stronger osteoinduction potential compared to full-length FN and non-functionalized fibrin matrix (Fig. 5C). Fibrin matrix functionalization with the $\alpha_5\beta_1$ -integrin-specific FN fragment (FN III9*-10), accelerated MSC OD, as demonstrated by a significant upregulation of ALP (3.3 fold $\pm$ 1.6, $P < 0.001$), CBFA-1 (2.8 fold $\pm$ 1, $P < 0.001$) and IBSP (6.2 fold $\pm$ 3.7, $P < 0.001$), while fibrin functionalization with full-length FN did not show to influence MSC OD.

DISCUSSION

Our interest in full-length FN and FN fragments arises from a need to better direct stem cell fate in a physiological context, i.e. within a 3D microenvironment capable of displaying adhesion ligands and matrix-binding growth factors so as to mimic the ECM. Such engineered microenvironments may be useful in basic biological studies as well as in tissue engineering, both with transplanted MSCs as well as in influencing the behavior of endogenous infiltrating MSCs. To date, most efforts in this field have been focused on the development of biofunctional

surfaces, i.e. rigid 2D materials that support cell attachment. To this end, the use of small ECM-derived peptides has proven fruitful, although they often have a poor biological activity in comparison to full-length ECM proteins. Unfortunately, cells delivered in this manner often undergo uncontrolled physiological responses. Our assumption was that the promiscuity of such adhesive peptides, as well as the full-length ECM protein, in the engagement of integrins drives competing intracellular signaling events that are undirected toward a defined goal, such as cell differentiation. Recent evidence of mechano-switches in FN's type III repeats that influence integrin specificity provide an avenue to explore the development of systems that have the capacity to engage specific integrins toward a designed end goal. We have attempted to utilize and merge this new knowledge gained in the field of FN biophysics with cell and molecular biology and biomaterials, establishing a simple system that allows the regulation of binding to integrin $\alpha_5\beta_1$ to various degrees to explore the physiological and bioengineering consequences of directed integrin binding in a 3D context suitable for *in vitro* as well as in tissue engineering applications.

Our initial analysis of MSC surface expression of integrin subunits during OD indicates that MSCs have the necessary machinery to engage the different FN fragments and thus we do not believe that results gleaned from this study are simply an effect of altered cell attachment. Moreover, the parallel upregulation of the subunits α_5 and β_1 observed during the first week of MSC OD (Fig. 1) and, the significant decreases of ALP activities, when $\alpha_5\beta_1$ is blocked (Fig. 4C and 5B), confirm our hypothesis that $\alpha_5\beta_1$ has an important role in the control of MSC OD.

We demonstrated that MSCs have similar initial attachment and spreading rate on FN III9*-10 and full-length FN (Fig. 2C,D). While we observed different attachment and spreading on the different FN proteins, we also observed complete cell attachment and near-equivalent spreading on all proteins after 6 h. Interestingly, significant differences in integrin cluster patterning on the different FN fragments emerged. The clustering of α_5 versus α_v coincides with our adhesion inhibition data that indicate that FN III9-10 and FN III9*-10 (more so) specifically bind $\alpha_5\beta_1$ (Fig. 3B). FN III10, as expected, does not appear to support significant α_5 clustering but rather significant amounts of α_v clustering, again being distributed along the edge of the cells. Wild-type FN III9-10 supports both α_v and α_5 (to a greater extent) promoting a classical focal adhesion phenotype, since the few α_v integrin clusters are localized to the edges of the cell, whereas the predominant α_5 clusters are more interior. In contrast, FN III9*-10 supports an even greater number of α_5 clusters than the wild-type fragment and the distribution of α_v clusters more closely resembles that of cells attached to full-length FN, which display a more typical fibrillar adhesion phenotype. Thus, the FN fragments generated in the present work represent a powerful molecular tool for the engagement of $\alpha_5\beta_1$ to various degrees. Interestingly, while full-length FN displays a large number of α_5 clusters showing that $\alpha_5\beta_1$ is strongly engaged, adhesion to full-length FN was not sensitive to $\alpha_5\beta_1$ FBA. We believe that these data are representative of the poor specificity of the full-length protein, since FN can bind many different integrin and other cell-adhesion receptors such as transmembrane proteoglycans [3].

In 2D, full-length FN and FN fragments that can engage $\alpha_5\beta_1$ sustained better proliferation compared to FN III10 suggesting that $\alpha_5\beta_1$ is important to maintain MSC cellular cycle. However, in contrast to FN III9-10 and FN III9*-10, blocking of $\alpha_5\beta_1$ did not significantly inhibit proliferation on full-length FN (Fig. 4A) suggesting that additional signals provided by the full-length protein are sufficient to maintain MSC proliferation. Hence, $\alpha_5\beta_1$ would be part of these signals without being critical. Unexpectedly, the blocking of $\alpha_v\beta_3$ slightly enhances proliferation in all conditions and more so on FN III10. While the mechanisms underlying this effect remain unclear, it could be that $\alpha_v\beta_3$ engagement inhibits MSC growth. In contrast to 2D, functionalization of fibrin matrices with full-length FN or FN fragments did not

significantly influence MSC proliferation (Fig. 5A), suggesting that native fibrin signals such as its cell binding domains or its degradation products which are known to promote proliferation of several cell types [25] easily support MSC growth in this system.

In opposition to the proliferation response, MSC OD appears to be much more influenced by $\alpha_5\beta_1$ engagement in both 2D and 3D models, since ALP activity in response to FN proteins is reduced when $\alpha_5\beta_1$ is continuously blocked (more so with the more $\alpha_5\beta_1$ -specific proteins i.e. FN III9-10 and FN III9*-10). Moreover, the more $\alpha_5\beta_1$ -specific fragment, FN III9*-10, supports the best OD in both models (Fig. 4B,C and 5B,C). It should be noted that the levels of MSC OD in presence of the FN fragments were correlated to the recombinant protein's degree of specificity for $\alpha_5\beta_1$ (FN III9*-10 > FN III9-10 > FN III10). It could seem contradictory that full-length FN was the least potent ECM protein since the protein strongly engages $\alpha_5\beta_1$ as shown by the integrin clustering assay (Fig. 4B). However, this result supports our main hypothesis, claiming that full-length FN presents less specificity. Indeed, full-length FN activates a number of different cell adhesion receptors and possibly some stemness pathways that could prevent differentiation, since this protein is found in abundance within the MSC niche [26]. In 3D, blocking of $\alpha_v\beta_3$ slightly enhanced MSC OD (Fig. 5B) similarly as in 2D. Taken together, in opposition to the role of $\alpha_5\beta_1$, this suggests that $\alpha_v\beta_3$ could play a role in the prevention of both proliferation and MSC OD. Indeed these unexpected effects of the blocking of $\alpha_v\beta_3$ were intrinsic to MSCs, since the FBA used did not have similar effect on other cell types (data not shown).

It is important to note that initial experiments conducted without osteoinductive conditions demonstrate that full-length FN and FN fragments do not, in and of themselves, have the capacity to specify which differentiation pathway MSCs traverse. Therefore, further experiments are needed to determine whether $\alpha_5\beta_1$ specific engagement via FN III9*-10 is capable of enhancing multiple differentiation pathways or is specific to osteogenic pathways.

CONCLUSION

This study investigates the effect of the degree of $\alpha_5\beta_1$ engagement in the context of MSC OD within 2D and 3D environments. Our data indicate that the integrin $\alpha_5\beta_1$ plays an important role in the OD process of MSCs within both environments but can be mitigated and controlled by the engagement of other integrins. Furthermore, this work extends our basic understanding of the mechanisms controlling integrin specificity to FN and FN fragments (i.e. FN domain stability) and provides new tools for engineering integrin specificity into biomaterials. FN III9*-10, provides more $\alpha_5\beta_1$ -integrin-specific instructions to MSCs capable of supporting proliferation and enhancing differentiation, while maintaining similar attachment and spreading capacities compared to full-length FN. From a design perspective, FN fragments with different integrin specificities represent a more simplified model for incorporating ECM signals into biomaterials, limiting the information to cells such that cell behavior may be more efficiently controlled. Thus, in systems that require specific integrin engagement such as MSC OD, we believe that full-length ECM proteins or standard RGD presenting peptides should be replaced by engineered ligands that are more integrin-specific. This work demonstrates how engineered cellular adhesive interactions with the ECM can lead stem cell differentiation phenomena, implemented in a system appropriate for both 3D cell culture and tissue engineering. Moreover, it gives clues to design microenvironment for the control of MSC fate.

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Fig. 1.

Surface expression of integrins during MSC OD. Surface expression of integrin subunits α_5 , α_v , α_2 , and β_1 were analyzed during MSC OD by flow cytometry. Fold increase in mean fluorescence intensity displayed by cells in osteogenic media compared to standard growth media are reported for 3, 7, and 14 days of culture. Integrin subunits α_5 and β_1 are upregulated during the first week of OD (** $P < 0.01$, *** $P < 0.001$). $n = 4$.

Fig. 2.

Relative coating efficiencies of full-length FN versus FN III9-10 and MSC attachment and spreading on FN proteins. (A) Polystyrene cell culture plates were incubated with FN and FN III9-10 protein solutions (up to 2 μM of FN and up to 4 μM FN III9-10). Absorbed proteins were detected by indirect enzyme-linked immunosorbent assay, using a monoclonal antibody specific for the domain FN III9-10. Results are expressed as secondary antibody signals (absorbance at 450 nm) as a function of initial coating solution concentrations. For example, FN and FN III9-10 presented equivalent number of absorbed proteins, when initial concentrations of 0.1 μM and 2 μM were used, respectively (hashed lines). Above an initial concentration of 0.5 μM of full-length FN, surfaces were saturated of proteins. Data are mean

± SD fitted with the Hill model ($n = 3$). (B) SDS-PAGE of purified FN fragments. FN fragments displayed correct MW (FN III9-10 and FN III9*-10, 21 kDa; FN III10, 11 kDa) and purities above 98%. (C) MSC adhesion on surfaces presenting equimolar FN proteins expressed as percent adhesion relative to a 100% cell adhesion control. FN III9*-10 supported similar attachment capacity compared to full-length FN, whereas attachments on FN III9-10 and on FN III10 were significantly lower ($***P < 0.001$). $n = 4$. (D) MSCs were plated on FN proteins-coated gold electrodes and cell spreading quantified by Electrical Cell-Impedance Sensing. FN and FN III9*-10 supported faster spreading rate compared to FN III9-10 and FN III10. All FN proteins showed near equivalent levels of spreading (maximal spread state indicated by equivalent resistance in (ohm) at 6 h (data not shown).

Fig. 3. Analysis of FN proteins integrin specificity. (A) Surfaces were coated with the EC₅₀ initial coating concentrations of FN proteins and MSC adhesion to coated surfaces was determined in the presence of $\alpha_5\beta_1$ and/or $\alpha_v\beta_3$ FBA Results are expressed as percent adhesion compared to paired untreated controls. In addition to comparisons indicated, a significant difference (Student's t-test, $\#P < 0.05$) was found between $\alpha_v\beta_3$ FBA inhibition of adhesion to FN III9-10 compared to all other FN proteins. (B) Integrin clustering determined by immunofluorescence. MSCs were allowed to initially adhere to and spread on FN protein-coated surfaces for 6 h. Clustering of α_5 and α_v subunits in response to the coated proteins was visualized by double immunostaining. Hashed squares indicate the zoom (4x) positions. Scale bar = 50 μm .

Fig. 4.

Analysis of MSC proliferation and differentiation on FN protein-coated surfaces. (A) Proliferation was induced in MSCs adhered to and spread on FN protein-coated surfaces in the absence or presence of $\alpha_5\beta_1$ or $\alpha_v\beta_3$ FBA. Results are expressed as the number of new cells ($n = 6$, non-treated cultures; $n = 4$, FBA-treated cultures). Significant differences between non-treated samples were found only between FN III10 and all other FN proteins ($***P < 0.001$). A Student's t-test was used to compare FBA-treated and non-treated samples ($\#P < 0.05$; $###P < 0.001$). (B) MSCs osteoblast-specific gene expression (ALP, CBFA-1, IBSP and OST) was examined at 12 days by quantitative PCR. Gene expression levels are expressed as a ratio to a tissue culture plastic coat baseline ($n = 5$, duplicate). Ct values for each transcript were

normalized to β -actin and analyzed using REST. Significant upregulation over baseline (grey box) are shown (* $P < 0.05$, *** $P < 0.001$). (C) OD state was determined by cellular ALP activity at 14 days ($n = 4$). Results are expressed in ng of ALP/10k cells. Significant differences were found between untreated FN and FN III9*-10 (*** $P < 0.001$) or FN III9-10 (** $P < 0.01$), as well as between FN III9*-10 and FN III10 (§§ $P < 0.01$). Statistical comparisons between FBA and non-FBA treated culture were performed using a Student's t-Test (# $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$).

Fig. 5.

Analysis of MSC proliferation and differentiation in functionalized 3D fibrin matrices. (A) MSC proliferation in 3D fibrin matrices functionalized with FN proteins. MSCs were cultured into fibrin matrices functionalized with full-length FN or FN fragments and cell number determined after 4, 7, 10 and 14 days. No constant significant differences were observed during 14 days of culture. Results are expressed as number of new cells ($n = 8, 7, 5$ and 4 for 4, 7, 10 and 14 days, respectively). (B,C) MSC OD in 3D fibrin matrices functionalized with FN proteins. MSC OD is accelerated in presence of the $\alpha_5\beta_1$ integrin specific ligand i.e. FN III9*-10 (B) Cellular ALP was quantified after 14 days of culture to assess OD. Results are expressed as ng of ALP/10k cells ($n = 4$, non-FBA treated cultures; $n = 3$, FBA treated cultures). Significant

differences were found between matrices functionalized with FN and FN III9*-10 (** $P < 0.001$) or FN III9-10 (* $P < 0.01$), as well as between FN III9*-10 and fibrin only matrices (§§ $P < 0.01$). Statistical comparisons between FBA and non-FBA treated culture were performed using a Student's t-Test (# $P < 0.05$; ## $P < 0.01$). (C) MSCs osteoblast-specific gene expression (ALP, CBFA-1, IBSP and OST) was examined at 14 days by quantitative PCR. Gene expression levels are expressed as a ratio to a non-functionalized fibrin matrix baseline ($n = 3$). Ct values for each transcript were normalized to β -actin and analyzed using REST. Significant upregulation over baseline (grey box) are shown (** $P < 0.001$).