Proteolytic fragments of fibronectin function as matrikines driving the chemotactic affinity of prostate cancer cells to human bone marrow mesenchymal stromal cells via the $\alpha_5\beta_1$ integrin

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ABSTRACT

The haematopoietic niche is contributed to by bone marrow-resident mesenchymal stromal cells (BM-MSCs) and subverted by prostate cancer cells. To study mechanisms by which BM-MSCs and prostate cancer cells may interact, we assessed the migration, invasion, adhesion and proliferation of bone-derived prostate cancer cells (PC-3) in co-culture with pluripotent human BM-MSCs. We observed a strong adhesive, migratory and invasive phenotype of PC-3 cells with BM-MSC-co-culture and set out to isolate and characterize the bioactive principle. Initial studies indicated that chemotaxis was secondary to a protein residing in the >100kDa fraction. Size-exclusion chromatography (SEC) recovered peak activity in a high-molecular weight fraction containing thrombospondin-1 (TSP1). While TSP1 immunodepletion decreased activity, put-back with purified TSP1 did not reproduce bioactivity. Further purification of the TSP1-containing high-molecular weight fraction of the BM-MSC secretome with heparin-affinity chromatography recovered bioactivity with highly restricted bands on polyacrylamide gel electrophoresis, determined by mass spectroscopy to be proteolytic fragments of fibronectin (FN). Put-back experiments with full-length FN permitted adhesion but failed to induce migration. Monospecific antibodies to FN blocked adhesion. Proteolytic cleavage of FN generated FN fragments which now induced migration. Neutralizing monoclonal antibodies to FN receptors $\alpha_5$ and $\beta_1$ integrins, and $\alpha_5$ knockdown specifically blocked migration and adhesion. Conclusion: Fibronectin fragments (FNFr) function as matrikines driving the chemotactic affinity of prostate cancer cells via the $\alpha_5\beta_1$ integrin. Taken together with the high-frequency of $\alpha_5\beta_1$ expression in disseminated prostate cancer cells in bone marrow aspirates from patients, the FNFr/FN-$\alpha_5\beta_1$ interaction warrants further study as a therapeutic target.

KEYWORDS

adhesion; bone marrow mesenchymal stromal cells; bone metastases; chemotaxis; fibronectin fragments; matrikine; prostate cancer

Introduction

Prostate cancer is unique among solid neoplasms for its proclivity to disseminate with great efficiency within the bone marrow microenvironment, mimicking a leukemic illness. The morbidity and mortality of prostate cancer is highly correlated with this conserved biological behavior described as the lethal phenotype of the disease. The specific advantages endowed by the bone marrow microenvironment for prostate cancer are therefore of great interest as they may offer avenues to effectively interdict the illness at its earliest stages of progression in the metastatic niche, long before evolutionary pressures drive the emergence of variant and resistant biological traits. Currently the only niche-directed therapeutics in prostate cancer is represented by bone-homing radioisotopes that modestly impact survival and skeletal-related events. The clinical observations that bone metastases from solid tumors from infancy to adulthood concur with the centripetal migration of hematopoiesis with aging suggest that bone metastases require subversion of the haematopoietic niche. Although endosteal, perivascular and other locations of the haematopoietic niche continue to be assessed, mesenchymal stromal cells and their osteoblastic derivatives have been previously implicated as architects of the haematopoietic niche. In experimental murine models prostate cancer cells can be demonstrated to compete
with CD45+ haematopoietic stem cells for a niche specified by osteoblasts. Interactions between prostate cancer cells and human bone marrow mesenchymal stromal cells (BM-MSCs) and their osteoblast derivatives likely contribute critically to the well-recognized phenotype of osseus-dominant progression of the disease, a phenotype that cannot be replicated in animal models to date, potentially because of species-specific differences in epithelial-matrix interactions.

In order to generate candidate seed-and-soil mechanisms of disease progression in the bone marrow microenvironment, we studied the interactions of bone-derived human PC-3 prostate cancer cells and pluripotent human bone marrow derived BM-MSCs in co-culture and sought to isolate and characterize the mechanisms driving the strong migratory, invasive and adhesive behavior that was immediately observed.

**Results**

**Human bone-derived prostate cancer cells display strong migratory affinity to human bone-derived BM-MSC in 2-chamber co-culture assays**

When PC-3 prostate cancer cells are co-cultured with BM-MSCs, a strong migratory and invasive behavior is identified. This migratory and invasive behavior is conserved with the BM-MSC secretome along with a strong adhesive phenotype. By contrast, there is apparently little impact on the proliferation of the prostate cancer cells (Fig. 1).

**Initial proteomic characterization of the BM-MSC secretome**

The chemotactic and adhesive bioactivity of the BM-MSC secretome was preserved under serum-free and phenol-red free conditions without the need for protease inhibitors. Trypsinization and heat inactivation suggested that the bioactivity was mediated by a protein. Initial purification efforts with anion-exchange chromatography failed to recover activity. Size separation with spin columns showed that activity was retained by up to 100 kDa filters (Fig. 2a). In order to characterize this further, BM-MSC serum-free conditioned media was concentrated and subjected to size-exclusion chromatography (SEC) (Fig. 2b). The resultant chromatogram allowed recovery of bioactivity in a high-molecular weight peak close to 440 kDa (Fig. 2c). Polyacrylamide gel electrophoresis demonstrated restricted bands (Fig. 2d) which were analyzed by mass spectrometry and a band containing thrombospondin-1 (TSP1) was identified. TSP1 is a 420 kDa glycoprotein comprised of 3 identical polypeptide chains (140 kDa) cross-linked by disulfide bonds and had been previously implicated in the migratory behavior of tumor cells. Immunospecific depletion of TSP1 resulted in consistent loss of bioactivity, however put-back assays with purified platelet-TSP1 following immunodepletion failed to recover bioactivity (Fig. 2e).

**Heparin-affinity chromatography to purify the TSP1-containing BM-MSC secretome recovers bioactivity leading to identification of fibronectin fragments as the putative chemotaxin**

TSP1 is known to bind to a wide range of proteins to mediate cell-cell and cell-matrix interactions. Reasoning that a TSP1 associated protein may be responsible for bioactivity we turned to heparin-affinity chromatography which is routinely employed to purify TSP1 from platelets. We found that BM-MSC bioactivity was depleted by heparin-affinity and elution of the column recovered bioactivity (Fig. 3a) allowing for further purification. Persistent, albeit depleted, bioactivity in the flow-through compared to the input likely resulted from saturation of the heparin-affinity column. The eluate from heparin-affinity was then subjected to SEC with recovery of peak bioactivity in a discrete fraction (Fig. 3b, 3c) with a highly-restricted band on PAGE (Fig. 3d). Mass spectroscopy identified a proteolytic fragment of fibronectin (FN) of approximate mass of 140 kDa (Fig. 3d). With N-terminal Edman sequencing a starting peptide sequence (VATSE) was identified. This matches with the start of the second Type III FN repeat which is a major proteolytic site. Fibronectin and its fragments have been demonstrated to bind or associate with TSP1 in the matrix. Although immunoprecipitation of TSP1 with a FN antibody was demonstrable in the BM-MSC secretome, immunoprecipitation studies of FN and FN fragments with a TSP1 antibody have been inconclusive (Suppl Fig. 1). FN fragments in the BM-MSC secretome may also be purified directly by heparin affinity independent of TSP1 binding. A strong heparin affinity site in fibronectin is found in the C-terminal domain which is conserved in the bioactive FN fragments we isolated.

**The classic fibronectin receptors α5 and β1 integrin mediate the migratory and adhesive behavior of prostate cancer cells to the BM-MSC secretome and fibronectin and its fragments**

To determine the mechanism by which fibronectin fragments (FNFr) signal in prostate cancer cells, the
classic fibronectin receptors a4β1 and a5β1 were first profiled in the prostate cancer cells. We found that PC-3 cells express the αv, α5, α6 and β1 integrins. Monoclonal antibodies to α5 or β1 but not to αv, α4, α6 or β3 blocked the migration and adhesion of PC-3 cells to the BM-MSC secretome (Fig. 4a). Putback assays with FN induced both migration and adhesion and this was also inhibited by monospecific antibodies for α5 or β1 integrin (Fig. 4b). Genetic inactivation of integrin α5 (ITGA5) with shRNA also blocked migration and adhesion of PC-3 cells to BM-MSC secretome (Fig. 4c). Although adhesion was uniformly observed (Fig. 4d), one of several commercial preparations of human plasma FN failed to induce a migratory response in PC-3 cells and when this was compared to 2 other preparations used in earlier put-back assays

Figure 1. BM-MSCs promote PC-3 Migration, Invasion and Adhesion. PC-3 (A) migration toward BM-MSCs in co-culture (****p < 0.0001), (B) migration toward BM-MSC CM (****p < 0.0001), (C) invasion toward BM-MSC CM (****p < 0.0001), (D) adhesion to BM-MSC CM (****p < 0.0001), (E) cell count after 72 h culture in BM-MSC CM (n.s.). Number of migrated and invaded cells reported are mean ± SD of 5 representative fields on the membrane. Proportion adherence reported is mean ± SD (n = 3) of the ratio of adherent to input cells as determined by cell viability (MTS) assay. Cell count reported is mean ± SD (n = 3) of cell viability (MTS). SFM: serum free media, MSC CM: BM-MSC conditioned media.
Fibronectin fragments and not full-length FN induce chemotaxis of prostate cancer cells

Given that the bioactivity we had purified from the BM-MSC contained only fragments of fibronectin, we hypothesized that FNFr and not intact FN was specifically required to induce a migratory response in BM-MSC secretome. Proteolytic cleavage of purified full-length FN with trypsin and chymotrypsin generated FN fragments that now induced migration (Fig. 4d). Together with the primary proteomic studies, this suggested that proteolytic fragments of FN generated by BM-MSCs function as matrikines to specifically drive the chemotactic affinity of prostate cancer cells.

Other prostate cancer cells that express the α5 integrin also demonstrate a chemotactic and adhesive response to the BM-MSC secretome

Prostate cancer cell lines are known to vary in their integrin expression profiles. We found that additional prostate cancer cell lines (DU-145, LnCAP) that express the α5 and β1 integrins by flow cytometry also display an adhesive and migratory response to the BM-MSC secretome that is specifically blocked by monospecific antibodies to the α5 and β1 integrins (Fig. 5). Prostate cancer cell lines (MDA PCa2b, VCAP) that did not express the α5 integrin did not display adhesive or migratory responses to the BM-MSC secretome. Taken together this suggests that the expression of the α5 integrin is likely to be an integral factor in the adhesive, migratory and invasive behavior of prostate cancer cells to the human BM-MSC secretome.
Discussion

We have implicated proteolytic fragments of fibronectin as a key chemotaxin driving the strong migratory and invasive response induced in prostate cancer cells by human bone marrow derived BM-MSCs. As full-length FN induces adhesion alone, FN fragments (FNFr) may function as matrikines in the bone marrow niche. Matrikines can be defined as proteinase-generated fragments of matrix molecules that possess bioactivities not displayed by the native full-length form of the molecule. The virtue of increased solubility of FNFr may allow for diffusion and generation of concentration gradients compared to relatively insoluble full length FN which tends to polymerize into fibrils and aggregate with other proteins in complex assemblies in the extracellular matrix including TSP1. We find however that both FNFr- and FN-driven chemotactic and adhesive responses converge on the classic FN receptor, a5b1.

Several lines of existing evidence lend credence to the a5b1-FNFr/FN interaction as a candidate seed-and-soil principle in prostate cancer and bone metastases. Fibronectin is abundantly expressed in the bone marrow microenvironment by stromal cells. In studies of adhesion-molecule profiles of disseminated tumor cells recovered from bone marrow aspirates of patients with bone metastases, the a5b1 integrin was expressed in nearly 100% of disseminated tumor cells. Interestingly, the alternate FN receptor a4b1 (VLA-4) has been implicated in the homing of haematopoietic stem cells to the marrow-niche; neutralizing antibodies to a4b1 mobilize haematopoietic stem cells into circulation implicating FN as a functional component of the haematopoietic niche. There have been limited efforts targeting the a5b1 integrin in prostate cancer with particular reference to progression in the bone marrow. The a5b1 monoclonal antibody volociximab has been in clinical trials but was abandoned in the absence of discernible clinical efficacy in a range of solid tumors, both as single agent and in combination with cytotoxic chemotherapy.

In other studies, the a5b3 integrin has been proposed as a candidate mediator of prostate cancer progression in bone. Cilengitide, a cyclic peptide that specifically
inhibits αvβ3 and the αvβ5 integrins was studied at 2 dose-levels in metastatic castration-resistant prostate cancer with no discernible impact on clinical outcomes or circulating tumor cells; the short half-life of the peptide following parenteral infusion may have limited target inhibition. A monoclonal antibody against αv integrin slowed the rate of progression of bone metastases but was insufficient to prolong the overall progression-free survival in men with metastatic castration-resistant disease. The αv integrin functions as an alternate but lower-affinity receptor for

Figure 4. The classic fibronectin receptors α5 and β1 integrin mediate the migratory and adhesive behavior of prostate cancer cells to the BM-MSC secretome and fibronectin and its fragments. α5 or β1 integrin neutralization significantly impairs PC3 migration to (A) MSC CM (migration (left): ****p < 0.0001 and ***p < 0.001; adhesion (right): ***p < 0.001 and **p < 0.01 compared to isotype control) and (B) human plasma fibronectin (FN - MP Biomedicals) (migration (left): ****p < 0.0001 and ***p < 0.001; adhesion (right): ***p < 0.0001 and **p < 0.01 compared to isotype control), (C) Shrnα knockdown of α5 significantly impairs migration (CM: ****p < 0.0001, FN: ****p < 0.0001) and adhesion (CM: ***p < 0.001, FN: ***p < 0.001) to BM-MSC CM and FN. (D) Three commercial preparations of FN including FN i, which shows a restricted full length band on western, and FN ii and iii which contain fragments as well as proteolytically derived FN iv and v (30 minutes of trypsin and chymotrypsin digestion of FN i followed by quenching by PMSF) were tested in PC3 adhesion and migration assay. Although adhesion is consistently observed, only FN preparations with fragments induce migration. Integrin neutralizations were performed as described in methods. Number of migrated cells reported are mean ± SD of 5 representative fields on the membrane. Adhesion is reported as proportion adherence, mean ± SD (n = 3) of the ratio of adherent to input cells as determined by cell viability (MTS) assay, or directly as OD or RFU as determined by MTS or AlamarBlue (ThermoFisher). SFM: serum free media, MSC CM: BM-MSC conditioned media, Iso: isotype control (anti-α4), FN: fibronectin.
fibronectin and it is conceivable that these results in part represent a strategy that targets the FN-prostate cancer interaction. In our studies however, although αv integrin was strongly expressed in PC-3 cells, αv integrin blocking antibodies did not inhibit their migration or adhesion to the BM-MSC secretome. In disseminated tumor cells obtained from bone metastases, 100% expressed the αv integrin. Peptide-based or small molecule strategies developed to target α5β1-integrin and fibronectin interactions have not been advanced further in the clinic.

There are significant existing limitations toward assessing integrin-targeting strategies with current preclinical models of prostate cancer and bone metastases. There are no existing animal models of prostate cancer and bone metastases, engineered or xenografted, that are representative of the human phenotype of bone-homing marrow-dominant disease and that allow for reliable predictive preclinical studies. Important species-specific differences in epithelial-stromal interactions may define this modeling challenge which has constrained effective bench-to-bedside translation. A significant gap between murine and human models of prostate cancer and bone metastases exists with starkly contradictory results demonstrable between preclinical data based on mouse model data and clinical translation. Humanized murine models of prostate cancer bone metastases which seek to incorporate a humanized bone marrow niche such as with engineered bone scaffolds seeded with human BM-MSCs, or human fetal or hip bone grafts, or engraftment of human haematopoietic stem cells and BM-MSCs into a murine background may generate predictive preclinical models but these are cumbersome and difficult modeling strategies which are not yet well established.

One strategy to demonstrate proof-of-principle implicating a candidate seed-and-soil mechanism in prostate cancer is to use the “clinic as laboratory” model to circumvent the limitations of existing animal models. For example, an early phase clinical study with a potent neutralizing antibody to α5β1 designed with a primary pharmacodynamic endpoint of preferential mobilization of α5β1-expressing circulating tumor cells into the peripheral blood of men with bone-dominant metastases could permit evidence for the role of the α5β1 pathway in mediating adhesive interactions of prostate cancer cells in the bone marrow. This would be akin to studies demonstrating haematopoietic stem cell mobilization out of the bone marrow niche with CXCR4 and integrin α4 blockade. These type of studies could powerfully address our proposal that the FN-Fr/FN-α5β1 interaction represents a seed-and-soil principle that defines its unique affinity for the bone marrow niche and a potential therapeutic target relevant to the lethal phenotype of the disease.

**Materials**

Standard tissue culture supplies were obtained from Santa Cruz Biotechnology unless otherwise specified. Other tissue culture reagents include phosphate buffered saline (PBS, Corning 21-040-CV), Dulbecco’s modified Eagle’s medium (DMEM, Corning 10-013-CV), Roswell Park Memorial Institute – 1640 with L-glutamine and 25 mM Heps (RPMI, Corning 10-041-CV), fetal bovine serum (FBS, Gibco 10437), penicillin-streptomycin solution (P/S, Corning 30-002-CI). Platelet purified Thrombospordin-1 (TSP1) at 125μg/ml was obtained from Jack Lawler, Beth Israel Deaconess Medical Center, Boston. Purified human plasma fibronectin (FN) preparations i, ii and iii were obtained from Sigma (F2006), R&D (1918-FN-02M) and MP Biomedicals (55913), respectively. Western blots were performed using antibodies to TSP1 (Abcam ab85762) and FN (Pierce PA1-26205).
**Cell culture**

BM-MSCs were obtained from the Kaplan Lab (Tufts University, Medford, MA). These plastic-adherent cells from human bone marrow aspirates (Lonza, MD) were determined to have pluripotent differentiation into osteogenic, adipogenic and chondrogenic lineages and consensus surface phenotypes by flow cytometry. Human prostate cancer lines PC-3, LNCaP, DU145 were obtained from ATCC (Manassas, VA). PC-3, DU145 and BM-MSC were cultured in DMEM supplemented with 10% FBS and P/S. LNCaP were cultured in RPMI supplemented with 10% FBS and P/S. All cells were cultured in a humidified 37°C, 5% CO2 incubator and subcultured by washing 3x with phosphate buffered saline (PBS, Corning 21-040-CV) and trypsinization with 0.25% trypsin, 0.9 mM EDTA (Life Technologies 25200).

**Generation of conditioned media**

BM-MSCs were allowed to reach confluence in a T75 flask, cultured as described above. Cells were washed 3x with PBS before incubation in 10 mL of serum-free culture media. Conditioned media (CM) was harvested after 24 h and gently centrifuged (300 g for 10 minutes) before being stored at 4°C. BM-MSCs were incubated for at least 48h in serum-present culture media between serum starvations.

**Size separation of BM-MSC CM**

BM-MSC CM was subjected to ultracentrifugation (50,000 x g for 3 hrs) and separately to variable molecular-weight exclusion filters: 3 kDa, 10 kDa, 30 kDa, 50 kDa, 100 kDa (Centricon) by centrifugation.

**Cell adhesion assay**

A 96 well plate was plated with 50 uL of adherent substrate in triplicate and incubated at 37°C. Non-specific binding sites were blocked by incubating the wells with 1% BSA for 30 minutes at 37°C. After washing wells 3x with PBS, 10000 cells harvested in log-phase growth were added in 100 uL of serum-free culture media to each well from a homogenous cell suspension. The plate was incubated 1 h at 37°C (6 h for LNCaP) to allow establishment of adherence profiles. To remove non-adherent cells, wells were gently washed 5x with PBS. Adherent cell counts were assayed using MTS reagent (Promega G3582). Adherence was reported either directly as optical density or as percentage adherence (normalized against number of input cells).

**Cell migration and invasion assay**

Cell migration and invasion assays were performed using a Boyden Chamber set up. A 24 well assay plate was prepared by adding 800 uL of chemottractant underneath uncoated 8 micron pore transwell inserts (Corning 353097) [migration] or inserts coated with basement membrane extract (Corning 3458) [invasion] and heated to 37°C. 25,000 cells (harvested in log-phase growth) were added from a homogenous cell suspension to the top of the well to a total volume of 200 uL. The assay was stopped after 24 h at 37°C (48 h for LNCaP) by removal and washing of the membrane inserts with PBS and formalin (Fischer Sci SF100-4) fixation of the cells for 15 minutes. Following another 3x PBS wash, cells were removed from the top of the membranes using cotton-tip swabs and were stained using hematoxylin (Sigma GHS116) and eosin (Sigma HT110116) or calcein AM (ThermoFisherC1430). After drying, membranes were mounted onto microscope slides and cell counts were performed on 5 representative fields of a membrane using a 10x objective.

**Cell count assay**

The effect of BM-MSC-SFCM (serum-free CM) on PC-3 cell count was tested by incubating 2500 PC-3 cells (harvested in log-phase growth) in 100 uL of BM-MSC-SFCM in a 96 well format. After 72 h, viability was assessed using MTS reagent (Promega G3582). Cell count was reported directly as optical density.

**Heparin affinity chromatography**

BM-MSC-SFCM (500 mL) was loaded on a 1.6 × 2.5 cm heparin Sepharose column (GE Healthcare) equilibrated with 5 column volumes of buffer A (15 mM TrisHCL, 2 mM CaCl, and 0.15 M NaCl; pH 7.3), and eluted in a step with buffer B (15 mM TrisHCL, 2 mM CaCl, and 0.55 M NaCl; pH 7.3). The eluate was extensively dialyzed (Slide-A-Lyzer Dialysis Cassettes, 10 K MWCO, Pierce) against PBS and concentrated via reverse osmosis through a semipermeable membrane (10 K MWCO), to a volume of 1 mL filtered (0.22mic, Millipore).

**Size exclusion chromatography**

Concentrated BM-MSC-SFCM elution from heparin affinity column was loaded onto size exclusion chromatography (SEC) column HiLoad16/60 Superdex 200 (GE Healthcare) and eluted isocratically with PBS. Samples from SEC were collected using an automated fraction collector.
Immunodepletion of TSP1 and put-back assays

BM-MSC CM was incubated with 10 μg of α-TSP1 antibody (IgG1k, Invitrogen Life Technologies) or isotype control (Iso) antibody and immune complexes cleared by adding protein G-coupled agarose. A dose-titration of TSP1 (10 ng/ml to 10 μg/ml) was employed to assess induction of bioactivity.

Proteomic identification

The fractionated protein samples from the SEC were subjected to SDS PAGE (4–12% Bis-Tris Protein Gels, ThermoFisher NP0321BOX), and silver stained (ThermoFisher SilverQuest LC6070). Bands from bioactive fractions were excised and submitted for mass spectroscopic identification (MS/MS, Taplin Mass Spectroscopy Facility, Harvard University, Boston, MA). N-terminal Edman sequencing was performed by the Tufts University Core Facility (Boston, MA).

Integrin neutralization

Blocking of surface integrins was performed by incubation 50 μg/mL of anti-integrin monoclonal antibody on ice for 20 minutes before migration and adhesion assays. Anti-β1 clone 6S5 (Millipore MAB2253Z), anti-β3 clone B3A (Millipore MAB2023Z), anti-α4 clone P1H4 (Millipore MAB1 6983Z), anti-α5 clone P1D6 (Millipore MAB1956Z), anti-α6 clone NKI-GoH3 (Millipore MAB1378), anti-αV clone 272–17E6 (Millipore MABT207) were all obtained azide-free from Millipore. As referenced, the neutralizing efficacy of α6 39, β3 40 and αv 41 have been demonstrated.

FN proteolysis

Purified human plasma FN from Sigma (F2006) was proteolytically digested using trypsin (Life Technologies 25200) or chymotrypsin (Sigma C4129) for 30 minutes at 37°C using enzyme unit: substrate ratio of 1:50. The enzyme was quenched and inactivated by addition of 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma P-7626).

shRNA generation and plasmid transfection

Two human ITGA5 shRNAs (shITGA5) were generated using the following primers: shITGA5 #1, 5’-GCTACCTCTCCACAGATAACTCGAAAAGTTATCTG TGAGAGGTAGCCcTTTTTG –3’ (forward) and 5’-AATTCAAAAAggGTACCTCTCCACAGATAACT TGAGATTATCTGTGGAGGGTAGC –3’ (reverse); shITGA5 #2, 5’-GCAGAGAGATGAAGATCTACC

GAAGGTAGATCTTCATCTCTCTGCcTTTTTG –3’ (forward) and 5’-AATTCAAAAAggGCAGAGAGATGAAGATCTACC

Surface integrin expression profiling

Suspended cells were washed (PBS) and incubated with anti-integrin primary antibodies for 1 hour at room temperature and then with fluorescently-labeled secondary antibodies for 1 hour at room temperature. Fluorescence was detected using a CyAn™ ADP Analyzer (Beckman Coulter).

Coimmunoprecipitation (co-IP) assay

Co-IP of FN and TSP1 in MSC conditioned media (CM) was carried out as follows. Equal volume of freshly collected MSC-SFCM (serum-free CM) was incubated with IgG (control), or TSP1 or FN antibody in IP buffer on a rotator at 4°C overnight. Then 20 ul of Pierce™ Protein A/G Plus Agarose (Thermo Fisher Scientific, Rockford, IL, USA) were added, and the incubation continued for another 90 min at 4°C. The immunoprecipitates bound to agarose beads were washed 4 times in IP buffer, heated in 60 μl of 2.5x sample buffer, separated with use of 7.5% SDS-PAGE gels, transferred to polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA), and probed with TSP1 and FN antibodies. The immune complexes were detected by Pierce™ ECL Western Blotting Substrate detection system (ThermoFisher).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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