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DOI:
10.3233/BIR-180185

Document Version
Peer reviewed version

Citation for published version (Harvard):
Alanazi, A, Munir, H, Alassiri, M, Ward, LSC, McGettrick, H & Nash, G 2019, 'Comparative adhesive and migratory properties of mesenchymal stem cells from different tissues', *Biorheology*, vol. 56, no. 1, pp. 15-30. https://doi.org/10.3233/BIR-180185

Comparative adhesive and migratory properties of mesenchymal stem cells from different tissues

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Running title:

Adhesion and migration of mesenchymal stem cells

Acknowledgements

Studies were supported by a grant from the British Heart Foundation (PG/14/28/30774). AA and MA were supported by Saudi Government PhD Scholarships; HM was supported by a BBSRC PhD studentship; LSCW was supported by an MRC PhD studentship; HMM was supported by an Arthritis Research UK Career Development Fellowship (19899). Umbilical cords were collected with the assistance of the Birmingham Women's Health Care NHS Trust and Sandwell and West Birmingham Hospitals NHS Trust.

Keywords: mesenchymal stem cells, cell adhesion, cell migration, collagen, fibronectin, umbilical cord, bone marrow

Abstract

BACKGROUND: Mesenchymal stem cells (MSC) are used in therapy, often by injection into the blood.

OBJECTIVE: We aimed to compare the adhesive and migratory properties of MSC from umbilical cords (UCMSC), bone marrow (BMMSC) or trabecular bone (TBMSC), which might influence delivery to injured tissue.

METHODS: MSC were perfused through glass capillaries coated with matrix proteins, collagen or fibronectin, or albumin. Adherent cells were counted microscopically and their spreading analysed over time. MSC migration through 8μm pore filters coated with the same proteins was analysed.

RESULTS: The number of MSC adhering to collagen was greater than fibronectin, decreased as wall shear rate increased from 17 to 70s^{-1}, and was in the order UCMSC>BMMSC>TBMSC. Conversely, spreading was more effective on fibronectin and was in the order BMMSC>TBMSC≥UCMSC. Migration was promoted by coating the lower surface of filters with either matrix protein, with UCMSC migrating more efficiently than BMMSC.

CONCLUSIONS: MSC show origin-dependent variations in their efficiency of capture from flow and subsequent spreading or ability to migrate on matrix proteins. UCMSC showed most efficient capture from flow, which was followed by less spreading, but more rapid migration. These responses might be associated with more effective delivery from the circulation into damaged tissue.

1. Introduction

Mesenchymal stem cells (MSC; also referred to as mesenchymal stromal cells) are multi-potent stem-like cells with the ability to repair damaged tissue. They were extracted first from the bone marrow, but more recently from a range of tissues [1,2]. The therapeutic benefits of MSC are not well understood, but it is believed that MSC promote tissue growth and wound healing, and modulate immune responses [3,4]. We showed, for example, that their cross-talk with endothelial cells could downregulate recruitment of flowing leukocytes [5]. Consequently, MSC have been widely used in clinical trials, in which they are commonly administered by intravenous injection [6]. Therefore, their recruitment from the circulation into the target tissue may be critical for therapeutic efficacy [7]. Nevertheless this recruitment process is not well understood. It has been suggested that like leukocytes, MSC go through a multistep process to cross the endothelium [8,9]. However, we and others have only observed attachment of MSC to endothelial cells from flow at very low shear stress [5,10]. Indeed, we recently found that capture from flow was more efficient for matrix proteins collagen and fibronectin, which might be exposed in damaged tissue, than endothelial cells [11]. Alternatively, MSC may become mechanically trapped in microvessels because of their large diameter (~20μm), particularly in the lungs [12].

Once recruited in vessels, MSC are thought to migrate into tissue, although regulation of this process is again not fully understood [13, for recent review]. They have been found to cross endothelial monolayers cultured on solid surfaces and filters over hours, rather than minutes for leukocytes [14-16]. MSC also migrate across micropore filters, with efficiency that is increased by addition of chemokines and growth factors, and by coating the underside of the filter with fibronectin [17,18]. Collagen and fibronectin have also been shown to promote adhesion and spreading of MSC [18-20], although investigations of

adhesion to matrix proteins under flow are restricted to our earlier study noted above [11]. While attachment from flow is clearly a prerequisite for migration from the circulation, the relationship between adhesion, spreading and migration may not be straight-forward. In general, adhesion-dependent signalling triggers a series of morphological changes of the cell and subsequently produces contractile forces which can support migration [21]. Migration speed depends on strength of attachment, but also the ability to detach in a regulated manner [21,22]. Thus, too strong an attachment may slow migration. In addition, the nature of the adhesive interaction required for capture from flow may be quite different from the attachment developed during spreading and onward migration. These capabilities to spread and migrate may influence therapeutic use of MSC, in terms of effecting stable localisation, autocrine interactions with vascular cells, such as endothelium, or direct contribution to tissue repair.

Much previous work has concentrated on the behaviour of MSC from bone marrow (BMMSC), which may differ from MSC from other sources. Indeed, for therapeutic purposes, MSC from umbilical cords (UCMSC) have some advantages, e.g., related to availability and fetal origin [23]. We recently compared adhesive properties of BMMSC and UCMSC, and also their interactions with blood platelets that could affect MSC delivery in the circulation [11]. UCMSC adhered from flow to matrix proteins more effectively than BMMSC, but also interacted differently with platelets, causing their activation. The two types of MSC consequently behaved quite differently when suspended in blood (human or murine) or injected into mice [11].

The foregoing suggests that to understand the fate of MSC in the circulation and optimize their therapeutic delivery, we need to further define their ability to adhere, spread

and migrate, and consider the effects of tissue origin on these responses. We thus compared BMMSC, UCMSC and also cells grown from fragments of trabecular bone (TBMSC), all of human origin. Cells were suspended in culture medium and perfused through glass capillaries coated with collagen or fibronectin, or allowed to settle and migrate on 8μm pore filters. We observed differences in adhesive properties between different MSC, and also in their spreading and migration, which appeared to be inversely correlated, and might influence behaviour during therapeutic use.

2. Methods

2.1 Ethics and collection of tissues

The study was conducted in compliance with the Declaration of Helsinki. All human samples were obtained with written, informed consent and approval from the Human Biomaterial Resource Centre (Birmingham, UK), the West Midlands and Black Country Research Ethics Committee, or University of Birmingham Local Ethical Review Committee. Umbilical cords were collected from anonymous donors with the assistance of the Birmingham Women's Health Care NHS Trust and Sandwell and West Birmingham Hospitals NHS Trust.

2.2 Isolation, culture and characterisation of MSC

The methods of MSC isolation and culture were as described recently [11,24,25]. Human BMMSC were purchased from Lonza and cultured in the manufacturer's recommended medium, Mesenchymal Stem Cells Growth Medium (MSCGM, BulletKit, Lonza Ltd. Burton on Trent, UK) and used in adhesion and migration experiments reported here between passages 5-7. UCMSC were isolated from umbilical cords as previously described [24,25] and cultured in MSC medium: low glucose DMEM with stable L-

Glutamine (Biosera, ZI du Bousquet, France) supplemented with 10% fetal calf serum (FCS), 100U/ml penicillin and 100µg/ml streptomycin (all from SigmaSigma-Aldrich Company Ltd., Gillingham, UK). UCMSC were used between passages 5-7 in experiments reported here. Trabecular bone explants were obtained from osteoarthritis patients undergoing joint replacement surgery (in collaboration with Dr Andrew Filer, University of Birmingham, UK). Bone explants were transferred to culture flasks and grown in MSC medium for 2 weeks to allow trabecular bone-derived MSC (TBMSC) to migrate away from the tissue, at which point the fragments were removed. Adherent TBMSC were then cultured to confluence and expanded to passage 3 to 6 before use. Finally, cells were counted using a Cellometer (Nexcelom Bioscience Ltd, Manchester, UK) and suspended at $5x10^5$/ml. The Cellometer also measured cell diameter from the microscopic images, using an in-built routine. In this study, experiments were carried out over a two year period and utilised UCMSC and BMSC were from at least 3 donors. TBMSC were from 2 donors. Some sequences of similar experiments were carried out close together and then from a single donor at different passages.

With regard to the different media used for culture, MSCGM (Lonza) is 'a serum containing medium designed to proliferate human bone marrow derived mesenchymal cells in an undifferentiated state'. The serum used is screened to ensure that it does not promotes spontaneous differentiation of cells. Serum used in our MSC medium likewise did not induce differentiation and when we compared proliferation of BMMSC in MSCGM and MSC medium, we obtained similar results: doubling times were 25.7 ± 0.5h vs. 27.1 ± 0.9h in MSCGM vs. MSC medium respectively; mean ± SEM, n=3). In addition, for assays, MSC were dissociated by trypsin-EDTA solution (Sigma) and suspended in MSC medium for all

cell types. Any differences in behaviour of different MSC were thus unlikely to arise from the different media used for expansion.

Cells were identified as MSC based on the criteria of the International Society for Cell Therapies [1]. Their ability to differentiate into osteocytes, adipocytes and chondrocytes, their surface expression of CD44, CD73, CD90, CD105 and CD146, and their lack of expression of CD14, CD20, CD34 and CD45 were verified as recently described [24]. BMMSC and UCMSC were tested repeatedly at passages 3, 5 and 7, where we noted that expression of CD44, CD73, CD90 remained stable, but there was a gradual decrease in expression of CD105 and CD146 [26].

2.3 Adhesion and spreading of MSC on collagen or fibronectin under flow

Adhesion of flowing MSC to chosen substrates was analysed as recently described [11]. Microslides (glass capillaries with rectangular cross-section of width W=3.0mm by depth D=0.3mm) were coated by incubation for two hours at 37°C with required proteins in PBS: 1% bovine serum albumin (BSA); 20µg/ml plasma fibronectin (both from Sigma); 500µg/ml equine tendon collagen (Horm collagen; Axis-Shield, Dundee, UK). Microslides were flushed with 1% BSA and incubated overnight at 4°C to block non-specific protein binding sites. Coating concentration for fibronectin was based on previous studies of MSC attachment and spreading which used ~10µg/ml [18,20] and on studies of the optimal concentration for spreading and migration of CHO cells (10-20µg/ml; [27]), while fibrillar collagen (which is the form found in matrix but rarely used in studies of MSC) was used at the same level as in studies of platelet adhesion from flowing blood [28]. We have recently demonstrated the ability of these coatings to capture flowing MSC [11].

Microslides were attached to a perfusion system and mounted onto the stage of a phase-contrast and fluorescence video-microscope, and events recorded as described [11]. Cell-free culture medium or MSC were drawn through the microslide at chosen flow rate (Q) and hence wall shear rate (γ_w), calculated from the equation $\gamma_w = 6Q/(W.D^2)$. UCMSC, BMMSC or TBMSC at $5x10^5$/ml were perfused over the different surfaces for 4 minutes. Non-adherent cells were washed from the microslide using cell-free MSC medium and video recordings were made of a series of microscope fields along the centreline of the microslide and analysed offline. Adherent cells were counted and converted to a percentage of all MSC perfused to obtain a value for efficiency of attachment corrected for flow rate and hence number perfused, as described previously [11]. Recordings were also made during further washout and images captured and digitised at chosen times offline using Image-Pro Plus software (Media Cybernetics, Marlow, UK). The adherent cells were characterised as spread when phase-contrast images showed transformation from round phase-bright cells to irregular phase-dark cells (see e.g., Figure 2C). The percentage of adherent cells spread was calculated at each time. In addition, the perimeter of cells was drawn using the Image-Pro software, which calculated a value for their diameter and surface area. Values for diameter were only used for cells immediately after washout, while still circular.

In some experiments, we also measured velocities of MSC flowing close to the wall but not adherent, at different wall shear rates. The cells were visible as bright streaks on video playback and the time taken to cross a calibrated distance on the screen was measured and converted to velocity.

2.4 Analysis of MSC migration through Transwell filters

Transwell filters with 8µm-pores (BD falcon) were coated from the top or bottom with 1% BSA, human plasma fibronectin (20g/ml) or equine tendon fibrillar collagen (Horm: 500µg/ml). In order to coat the top, 50µl of the protein solution was placed inside the filter followed by incubation for two hours at 37°C. Following incubation, excess proteins were removed and 1% BSA was used to wash the filter. In order to coat the bottom of filters, the filters were inverted and then the protein solution was pipetted as a 'bead' followed by incubation for two hours at 37°C and rinsing with BSA. Coated filters were placed into 24 well filter-matched plates containing 700µl of MSC medium.

MSC were diluted to $1.5x10^5$/ml in MSC medium, 200µl was added to the upper chamber, and the filter was incubated for 24 hours at 37^oC. After incubation, the medium was collected from above the filters and from the 24 well plates. The top and bottom surfaces of filters were washed once with 200µl and 700µl PBS respectively, with the wash medium added to the collected samples from above and below the filter. Trypsin-EDTA solution was then used to detach cells from upper and lower surfaces of the filter, and these cells were added to the wash samples and made up to a volume of 10ml with MSC medium to make 'TOP' and 'BOTTOM' samples. These isolated cells were counted using a Coulter Counter (Beckman Coulter, Buckinghamshire, UK). Percentage migration was calculated as BOTTOM/(TOP+BOTTOM) x 100%. The Coulter Counter also gave a cell volume distribution which allowed 'gating' of cells according to size in the TOP and BOTTOM samples. We were thus able to analyse the proportion of 'Large' cells that migrated as well as the proportion of the whole population. Large cells were defined as those with volume greater than $1000µm^3$, whereas the whole population contained cells down to volume ~$200µm^3$.

2.5 Statistical analysis

Data are shown as mean ± SEM of (n) replicate experiments. Statistical analysis was performed using Minitab 17 software (Minitab Inc.). Effects of multiple conditions were analysed using analysis of variance (ANOVA) and post hoc comparisons between treatments were made using Bonferroni test. The number of replicates (n) for individual data points are 3-4, not sufficient for testing normality. However, in a given experiment, we compared the data for adhesion or spreading of different MSC as functions of varying surfaces or time. Thus we took the data for a given cell type for each time or surface, expressed it relative to the mean value for that cell type under that condition, and pooled these relative values for the different times or surfaces studied, to give a larger data set. Then we carried out a Kolmogrov-Smirnov test on these relative values for deviation from normality. These test indicate that the data did not deviate from the null hypothesis that the data were normally distributed.

3. Results

3.1 Comparative adhesion of different MSC to matrix proteins as a function of wall shear rate

The effects of shear rate on adhesion of MSC to collagen or fibronectin are shown in Figure 1. Results for TBMSC have been added to data we have previously published for UCMSC and BMMSC [11]. About 5-20% of perfused MSC adhered at the lowest wall shear rate ($18s^{-1}$) and this decreased with increasing shear rate so that only ~1-2% adhered at $70s^{-1}$. Adhesion to collagen (Figure 1A) tended to be greater than to fibronectin (Figure 1B), although both surfaces supported numerous attachments (see e.g., Figure 1D-F). Overall, adhesion varied between MSC type, with UCMSC>BMMSC>TBMSC. Adhesion to these

matrix proteins was specific, in the sense that coating chambers with BSA yielded <1% adhesion even at the lowest shear rate (Figure 1).

3.2 Comparative spreading kinetics of different adherent MSC on matrix proteins

We next analysed the kinetics of spreading by the cells that had adhered to the surfaces after perfusion at a wall shear rate of $35s^{-1}$. Figure 2 shows sequences of images taken over time for the different MSC spreading on collagen (Figure 2A) or fibronectin (Figure 2B). On collagen, there was gradual increase in the proportion of spread cells , with BMMSC spreading more rapidly than UCMSC or TBMSC, which behaved similarly (Figure 3A). On fibronectin, spreading was markedly quicker than on collagen and again, BMMSC spread most rapidly with almost 100% spread in 10-20min (Figure 3B). Initially TBMSC and UCMSC spread at similar rates, but over time the TBMSC spread more efficiently than UCMSC. Overall, spreading was in the order BMMSC>TBMSC≥UCMSC. Examining the area of spread cells, it was evident that on fibronectin cells spread to cover greater areas than on collagen, except for UCMSC which reached similar areas on both surfaces (Figure 3C,D). On collagen, BMMSC attained greater areas than TBMSC and UCMSC, which behaved similarly (Figure 3C). On fibronectin, BMMSC had greater area than TBMSC which had greater area than UCMSC (Figure 3D). Thus in terms of area, the order was again BMMSC>TBMSC≥UCMSC.

3.3 Comparative migration of different MSC on matrix proteins

Having found consistent differences in behaviour of UCMSC and BMMSC (adhesion UCMSC>BMMSC, spreading BMMSC>UCMSC), we compared their ability to migrate across filters coated with collagen or fibronectin. TBMSC were not analysed further because their behaviour was intermediate between the others and they were not as abundantly

available as the other cells. We also compared results when the top or the bottom of the filters was coated with collagen or fibronectin. When the top of the filters was coated, we did not observe consistent differences in the migration of the two cell types, although migration was most effective for fibronectin compared to collagen or albumin (Figure 4A). On fibronectin, UCMSC tended to migrate more than BMMSC (Figure 4A). When the bottom of filters was coated with collagen or fibronectin, UCMSC migrated more effectively than BMMSC; the two migrated similarly on albumin (Figure 4B). Since it appeared that migration was more efficient when the bottom of filters was coated compared to the top, the data are replotted to compare the locations of coating separately for the different cells in Figure 4C,D. This shows clearly that coating the bottom of filters makes migration more efficient, and that especially large numbers of UCMSC migrated when the bottom was coated with either collagen or fibronectin. Also, overall, under most circumstances (except when the top of filters was coated with collagen) UCMSC tended to migrate more efficiently than BMMSC.

3.4 Role of cell size in determining adhesive and migratory behaviour

Adhesion of flowing cells typically ecreases with increasing shear rate because the cells travel with increasing velocity near the wall before attachment, and experience greater shear stress (product of shear rate and fluid viscosity) tending to detach them if adhesive bonds are formed [28,29]. For a given shear rate, the velocity is predicted to be proportional to the cell diameter. To investigate whether cell size affected levels of adhesion, we measured the diameter of adherent cells from the images taken immediately at the end of the perfused bolus when cells were mostly circular in outline (see Figure 1D-F). It was notable that the average diameters were less than those determined for the original samples (Table 1), indicating that smaller cells within a cell population tended to adhere more efficiently. To investigate further, we directly measured velocity of free-flowing MSC perfused over the

different surfaces. As expected there was a linear increase in velocity with increasing shear rate (see e.g., Figure 5A for UCMSC). More interestingly, we also measured mean velocities of samples perfused at wall shear rate of $35s^{-1}$ whose mean cell diameter had been measured before perfusion. Figure 5B indicates that on a cell sample by sample basis, average velocity also correlated with average cell diameter. Comparing the different types of MSC, the data in Table 1 suggested the cell diameter was in the order BMMSC>UCMSC>TBMSC. However, over a larger number of samples measured during the study, diameters (mean ± SEM from n samples) were: BMMSC 21.6 ± 0.4 (n=14); UCMSC 21.2 ± 0.4 (n=26); TBMSC 18.8 ± 0.8 (n=6), with TBMSC being significantly smaller than UCMSC or BMMSC ($p<0.05$ in each case, by unpaired t-test corrected for multiple comparisons). However, levels of adhesion were in the order UCMSC>BMMSC>TBMSC, and so it seems that while diameter influences cell adhesion from flow within a cell population, differences between different cell populations (types) was not attributable to differences in size (see Discussion).

The relationship between cell diameter and kinetics of spreading and migration after attachment is not so clear theoretically. When we compared initial diameter of adherent cells with their spread area after 25min, we found linear correlation (e.g., Figure 5C for BMMSC spreading on fibronectin or collagen) as might be expected. However, the initial diameters of the different cell types did not correlate with their kinetics of spreading or areas attained; e.g. BMMSC spread more rapidly and extensively than UCMSC (see Figure 3), but the two had similar diameters. Thus, again, while size may be linked to spreading ability within a populations of cells, it did not explain differences between types of MSC, or indeed coating surfaces. Finally, for BMMSC and UCMSC we investigated links between cell size and migration through 8μm pore filters. We did this by analysing the proportion of larger cells which transmigrated compared to the whole population. This was possible because the

Coulter Counter used to quantify the number of cells above or below the filter after 24h also measured cell volume, and the counts could be gated (see Methods). For both UCMSC and BMMSC, we found that a lesser proportion of the larger cells in the population migrated compared to the whole population (e.g., Figure5D). However, as noted above, initial diameters of the BMMSC and UCMSC cell populations were similar and thus could not explain the more efficient migration of the UCMSC (see Figure 4).

4. Discussion

The adhesive and migratory properties of MSC may influence their fate and efficacy when injected into the circulation as therapy, and also their behaviour in situ when acting as endogenous repair or immunomodulatory cells. As previously reported [11], UCMSC adhered in greater numbers to collagen or fibronectin from flow than BMMSC. Here we found that TBMSC were less effective at capture by these surfaces than either of the other MSC. Levels of adhesion were similar on collagen or fibronectin, with both proteins being much more adhesive than albumin.

After capture, the majority of cells spread on the surface (transforming from phase-bright to phase-dark in appearance). Spreading was markedly faster and more efficient on fibronectin versus collagen, and for BMMSC compared to other MSC. TBMSC showed similar spreading to UCMSC on collagen, but were more effective on fibronectin. Projected (2-D) surface areas of MSC followed the same pattern as rate of spreading. Cells started at similar diameters/area and then increased 4- to 6-fold for BMMSC on collagen or fibronectin, while UCMSC only increased about two-fold in area even on fibronectin. This spreading behaviour seemed to be inversely related to the adhesion efficiency for BMMSC and UCMSC at least; UCMSC adhered better than BMMSC, but spread less. Regarding the

surfaces, collagen was at least as good, if not better, than fibronectin in supporting capture, but less effective in driving spreading. This illustrates the different natures of the two forms of attachment. Capture from flow requires rapid formation of bonds [28,29], while cell spreading arises from signalling into cells and stabilisation of attachment typically through integrin receptors [30,31]. Here, cells and surfaces which were optimal for capture appeared to be less efficient in subsequent spreading and stabilisation, suggesting the use of different adhesion receptors at each stage.

To pursue the correlations between adhesion, spreading and migration further, we compared migration of BMMSC and UCMSC through differently-coated micropore filters. TBMSC had shown intermediate behaviour between these two types of cell, and practically, were more scarcely available. Migration again showed some clear-cut differences. UCMSC migrated through filters more efficiently than BMMSC under most conditions, and especially when the lower surface was coated with collagen or fibronectin, as opposed to the upper. It was also notable that coating the lower surface itself induced more effective migration. Thus, as well as supporting dynamic capture and spreading, the matrix proteins supported migration better than albumin alone, most evidently when the lower surface was coated. When the lower surfaces were coated, collagen and fibronectin supported similar levels of migration. It may be that coating the lower surface encouraged migration by providing a better anchorage for protrusions made through the pores, thus resembling haptotaxis, which is believed to be important in the guidance of MSC migration [2]. It is also interesting to note that since fetal calf serum was used in culture media (10% FCS, equivalent to about 0.5% BSA), all surfaces of the filters would have albumin deposited on them, even when not specifically pre-treated with any protein. While albumin did not support capture from flow, it did allow migration at significant levels. This illustrates again the difference in the types of adhesion that underlie

capture and migration, with capture requiring relatively fast binding kinetics but migration being supported by slower formation and then detachment or turnover of bonds.

Taken together, our results indicate that migration (or motility) was greater for the cells that spread less. The clearest comparison was between UCMSC and BMMSC, where UCMSC spread more slowly but underwent transmigration more efficiently then BMMSC. UCMSC also adhered in greater numbers than UCMSC, but this phase may be mediated by different adhesion receptors (see below), so that this stage of recruitment does not directly impact on the kinetics of the next stage. In relation to the migration phase, it is well recognised that too high or too low levels of attachment can impair movement over surfaces [21,22,33]. Here it would seem that movement onto matrix proteins was more effective when cells spread less and probably were less avidly bound.

Several previous studies have shown roles for collagen and fibronectin in promoting adhesion or spreading of MSC [e.g., 18-20], but have not compared these surfaces or MSC from different tissues. The ability of collagen and fibronectin to support dynamic attachment and promote migration may be important *in vivo* when it is desirable for infused MSC to become localised in damaged or inflamed blood vessels. Therapeutic efficacy could depend on ability of MSC to penetrate and migrate across the basement membrane which is rich in these proteins. While fibronectin supported more rapid spreading then collagen, the coating concentrations used here were based on previous studies with the proteins [18,20,27], and may not have been optimal to support the behaviours observed. Collagen is likely to be present at higher concentration than fibronectin in subendothelium (as was the coating concentration used here), but their concentrations and relative contributions to MSC behaviour when mixed in vivo is not possible to evaluate. Indeed, comparison of matrix

proteins was not the main purpose of this study which sought to compare behaviours of MSC from different origins, and used different substrates to broaden this comparison. In addition, we were not able for practical reasons to extend the periods over which cells were observed spreading in the flow system to hours. In the longer-term culture on solid substrates, we observe routinely that MSC take on a spindle shape, different from the initial spread morphology. Whether this transformation is faster for one type of MSC than another is not known to us. However, since our purpose was to investigate processes which might occur in vivo, the 30min period observed routinely should be adequate, because after this period, onward migration into tissue is expected [14-16]. To investigate that stage, we transferred to a 24-hour migration assay.

The observed differences in MSC behaviours could arise from various physical or molecular structural variables. Wall shear rate is a fundamental determinant of initial attachment [28,29] and indeed, MSC capture decreased with increasing shear rate as expected. The velocity of the free-flowing cell before binding is a fundamental factor affecting its likelihood of forming an initial bond, and this scales with its diameter as well as the shear rate. Diameter also influences the shear drag on the cell once bonds form, which tends to break bonds. We verified that velocity of cells near the substrate surface increased monotonically with shear rate, and that on a population basis, cells of larger average diameter had higher average velocity (since we could not measure diameter and velocity of each cell at the same time). In consequence, we were not surprised to find that when we measured the diameter of cells that adhered to collagen or fibronectin, they were smaller than the average for the original cell population perfused. This finding shows that variation in adhesiveness within a cell population can be attributed, in part at least, to variation in cell size. However, it cannot explain the differences in adhesion observed for MSC of different origin, as BMMSC

and UCMSC had similar diameters, and capture of the smaller TBMSC was actually the least effective. In relation to cell diameter, it is notable that the range of shear rates over which MSC (diameter ~20µm) adhered is less then the range for leukocytes (diameter ~8µm) which is less than the range for platelets (diameter ~2-3µm) in comparable assays (e.g., [28]). Leukocytes and platelets are adapted for functions where adhesion to the wall of blood vessels is essential, whereas MSC are normally tissue resident rather than circulating cells. Not only will the size of MSC limit their efficiency of adhesion from flow, but they are also unlikely to have evolved specialised capture receptors for this purpose, whereas leukocytes and platelets have such receptors.

We also questioned whether variation in cell size could contribute to differences in spreading behaviour. Here, initial diameter of adherent cells could be correlated with spread area at a fixed later time, on a cell-by-cell basis. Indeed there was a correlation, again contributing to variation within a cell population, but this could not explain the greater spreading e.g., achieved by BMMSC. Finally, in transmigration assays, we noticed that there was a left-shift in size distribution between MSC below the filter versus those above. By gating counts on diameter, we found that larger cells migrated less efficiently than smaller cells. While size is again an interesting determinant of migration within a population, it does not appear to contribute to relative migration of BMMSC and UCMSC.

The foregoing raises the question whether the observed differences in behaviour between the MSC types arise from variations in the molecular interactions used to support adhesion and migration. Integrin receptors are heterotypic glycoprotein dimers with α- and β-subunits, which typically support cell adhesion to collagen and fibronectin [21,34]. We found previously that β1-integrins dominated the capture process for UCMSC and BMMSC on

collagen or fibronectin, with minor contributions from β3-integrins [11]. Others have reported that BMMSC use β1-integrin family members to bind to collagen and fibronectin under static conditions [35]. Whether this applies to spreading and migration is uncertain, although others have shown that on fibronectin, α5β1-integrin was critical in driving adhesion and migration of BMMSC [18]. We did not detect obvious differences in surface expression levels of the major β1- and β3-integrin families between UCMSC and BMMSC [11], but this does not rule out differences in specific α-integrin subunits promoting adhesion, spreading and migration. Further studies might usefully define the exact and likely different integrin dimers supporting the capture and spreading phases of adhesion observed here.

Responses of MSC could also be affected by the number of passages they have undergone during culture, and indeed variations between the original tissue donors. Cells tested here had undergone 5 to 7 passages for BMMSC and UCMSC and 3 to 6 passages for TBMSC. Data for adhesion reported here, and data for BMMSC and UCMSC from our previous report [11] allowed adhesion on collagen or fibronectin at wall shear rate $35s^{-1}$ to be plotted against passage number from 4 to 8. There was no significant correlation between the variables (data not shown). We also had extended data for cell diameter between passages 4 to 9. Again, mean cell diameter did not vary with passage number (data not shown). Standard markers for MSC phenotype were largely maintained between passages 3 to 7 for UCMSC and BMMSC in our hands, and the shifts that did occur were similar for the two cell types [26]. It is thus unlikely that passage number rather than tissue of origin was the source of variation is behaviours of MSC we observed. The number of donors tested was more restricted, and due to their limited expansion, different donors were used typically at different stages of the prolonged study. A much larger population of donors would need to be studied to test any contribution to differences in adhesion and migration from this source. We have

recently been able to attribute some differences in behaviour between BMMSC and UCMSC to the expression of podoplanin by UCMSC only [11]. This marker is absent from UCMSC in a small proportion of donors, but we do not have information on its expression by the isolates used here.

MSC from bone marrow or umbilical cords are widely used for therapy [6]. There is no consensus as to which is better for any specific purpose, although umbilical cord tissue is more easily accessible than bone marrow. We found that both could suppress inflammatory responses of endothelial cells [24]. Effectiveness of delivery will however be critical in any therapy [7,13], including ability to integrate into tissue. Our results illustrate variations in adhesion, spreading and migration behaviours for MSC from three sources. It might be an advantage of UCMSC that they are relatively effective both at adhesion from flow (at least when matrix proteins are revealed in damaged tissue) and onward migration. It is also worth considering that MSC are endogenous cells resident in small numbers in most tissues, with gathering evidence that they play roles in e.g., regulation of immune and inflammatory responses [36-38]. Influence on vascular pathology might be linked to their ability to migrate towards sites, and so migratory responses presented here may also be relevant in that context. However, UCMSC and BMMSC are not from typical sites of vascular inflammation, and comparative studies with perivascular cells from other tissues would be of interest in the future.

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Table 1. Comparison of diameters of cells adherent to different surfaces with cells originally perfused

Cell type	Original sample	Adherent cells - Collagen*	Adherent cells - Fibronectin**
BMMSC	22.3 ± 0.9	18.9 ± 4.2	16.8 ± 4.2
UCMSC	20.3 ± 1.2	17.8 ± 1.2	15.9 ± 1.2
TBMSC	19.3±0.3	15.8 ± 1.3	15.4 ± 3.9

Diameter of the original sample was measured by Cellometer. Diameter of adherent cells was measured using ImagePro software and digitised images. Data are mean ± SEM from 3 experiments where different isolates of MSC were perfused over collagen or fibronectin. Independent analyses carried out with three freshly dispersed sample of UCMSC showed a systematic overestimate of mean cell diameter by the Cellometer compared to ImagePro by 5±1%; values in the table for adherent cells have been multiplied by 1.05 to allow the comparison of the two types of data.

*ANOVA of data for collagen adherent and original sample, showed no effect of cell type but adherent vs. original sample $p<0.05$.

**ANOVA of data for fibronectin adherent and original sample, showed no effect of cell type but adherent vs. original sample $p<0.01$.

Figure Captions

Figure 1. Comparison of adhesion of UCMSC, BMMSC and TBMSC to collagen or fibronectin

MSC were perfused over (A) collagen or (B) fibronectin for 4 min at 37ºC at wall shear rates of 18, 35 or 70s^{-1}, and the number adherent expressed was a percentage of those perfused. Data are the mean ± SEM from three or four experiments. In A and in B, ANOVA showed significant effects of wall shear rate and of cell type on adhesion ($p<0.01$ in both cases). In (A), **=$p<0.01$ for comparison of UCMSC to BMMSC or TBMSC; ++=$p<0.01$ for comparison of UCMSC to TBMSC by Bonferroni test. In (B), **=$p<0.01$ for comparison of UCMSC to BMMSC or TBMSC, and for BMMSC to TBMSC; ++=$p<0.01$ for comparison of UCMSC and BMMSC to TBMSC by Bonferroni test. (C, D and E) representative microscope images of UCMSC, BMMSC and TBMSC, adherent to collagen after perfusion at 35s^{-1} . Results for TBMSC have been added to data we have previously published for UCMSC and BMMSC [11].

Figure 2. Images of adherent UCMSC, BMMSC and TBMSC spreading over time on collagen or fibronectin

UCMSC, BMMSC or TBMSC were perfused over (A) collagen or (B) fibronectin for 4 min at 37ºC at a wall shear rate of 35s^{-1}. Images of fields were captured at 5, 15, 25 and 35 minutes after the start of perfusion.

Figure 3. Comparison of the percentages of adherent UCMSC, BMMSC and TBMSC spread, and of their spread areas, over time on collagen or fibronectin

MSC were perfused over (A,C) collagen or (B,D) fibronectin for 4 min at 37ºC at a wall shear rate of $35s^{-1.}$ Images were captured at 5, 15, 25 and 35 minutes after the start of perfusion and the percentages of adherent cells that were spread analysed for in several fields (A,B). In separate experiments, a single field was recorded over time, and the areas of adherent cells that were spread were analysed at 5, 15 and 25 minutes (C,D). Data are mean ± SEM from 3-4 experiments.

In (A) and in (B), ANOVA showed significant effects of cell type and time on percentage of cells spreading ($p<0.01$ in all cases). In (A), *=$p<0.05$; **=$p<0.01$, for comparison of BMMSC to UCMSC or TBMSC by Bonferroni test. In (B), **=$p<0.01$, for comparison of BMMSC to UCMSC or TBMSC; ++=$p<0.01$ for comparison of BMMSC or TBMSC to UCMSC by Bonferroni test.

In (C), ANOVA showed significant effects of time on spread area ($p<0.01$). In (D), ANOVA showed significant effects of time and of cell type on spread area ($p<0.01$ in both cases); *=$p<0.05$ for comparison of UCMSC to BMMSC or TBMSC; ++=$p<0.01$ for comparison of UCMSC and TBMSC to UCMSC by Bonferroni test.

Figure 4. Comparison of the migration of UCMSC and BMMSC through 8µm pore filters coated with collagen, fibronectin or albumin on top or bottom surfaces.

MSC were settled onto 8µm pore Transwell filters and allowed to migrate at 37°C for 24h. Cells were collected from the above or below the filter and counted by Coulter Counter. Those migrated (below) were expressed as a percentage of the total counted. (A,B) comparisons between BMMSC and UCMSC for filters pre-coated on the top (A) or bottom (B), with collagen, fibronectin or albumin. (C) comparison of migration of BMMSC when

filters were pre-coated on the top vs. the bottom. (D) comparison of migration of UCMSC when filters were pre-coated on the top vs. the bottom. Data are mean ± SEM from 3 experiments.

In (A), ANOVA showed a significant effect of coating protein on migration ($p<0.01$); **=$p<0.01$ for comparison of coating surfaces for UCMSC by Bonferroni test. In (B), ANOVA showed significant effects of coating protein ($p<0.05$) and of cell type ($p<0.01$) on migration; **=$p<0.01$ for comparison of surfaces for UCMSC by Bonferroni test; +=$p<0.05$ for comparison of UCMSC to BMMSC by Bonferroni test. In (C), ANOVA showed significant effects of coating protein ($p<0.05$) and of surface coated (Top or Bottom) ($p<0.01$) on migration; *=$p<0.01$ for comparison of coating protein for Bottom by Bonferroni test; +=$p<0.05$, ++=$p<0.01$ for comparison of Top to Bottom by Bonferroni test. In (D), ANOVA showed significant effects of coating protein ($p<0.05$) and of surface coated (Top or Bottom)($p<0.01$) on migration; **=$p<0.01$ for comparison of coating protein for Bottom by Bonferroni test; ++=$p<0.01$ for comparison of Top to Bottom by Bonferroni test.

Figure 5. Effects of factors linked to cell size on velocity of flowing cells, area of adherent spread cells and efficiency of transmigration

A. Velocity of cells flowing close to the wall as a function of wall shear rate. Data are mean velocities for cells in 3 samples of UCMSC perfused over BSA in separate experiments. The dashed line represents the predicted velocities of spherical particles with diameter equal to the mean of the samples' diameters, flowing in a linear velocity field with shear rate equal to the experimental wall shear rate [39].

B. Velocity of cells flowing close to the wall as a function of cell diameter for constant wall shear rate. Data are mean velocities for cells in samples of MSC perfused at $35s^{-1}$. Each point is a pooled average for a cell sample of known mean diameter perfused over one or

more surfaces in a single experiment. The dashed line (.....) represents the predicted velocities of spherical particles with the experimental diameters, flowing in a linear velocity field with shear rate equal to $35s^{-1}$ [39]. The solid line is fit to the data by linear regression.

C. Final spread area of adherent cells as a function of their initial diameter. Data are for individual cells in samples of BMMSC adherent to collagen or fibronectin, imaged 5min (initial) or 25 min (final) after start of perfusion at $35s^{-1}$. Lines are fitted by linear regression: for collagen $R=0.43$, $p=0.056$; for fibronectin $R=0.63$, $p<0.01$. BMMSC spread to a great degree on fibronectin than collagen (see Figure 3).

D. Transmigration through 8μm pore filters for the whole population of MSC compared to a 'Large' subpopulation detected by Coulter Counter. Data are mean ± SEM from 3 experiments. ANOVA showed significant effect of cell size on migration ($p<0.01$); *=$p<0.05$, **=$p<0.01$ for comparison of sizes by Bonferroni test. A similar trend was observed for BMMSC (not shown) which migrated less efficiently than UCMSC.

Figure 1

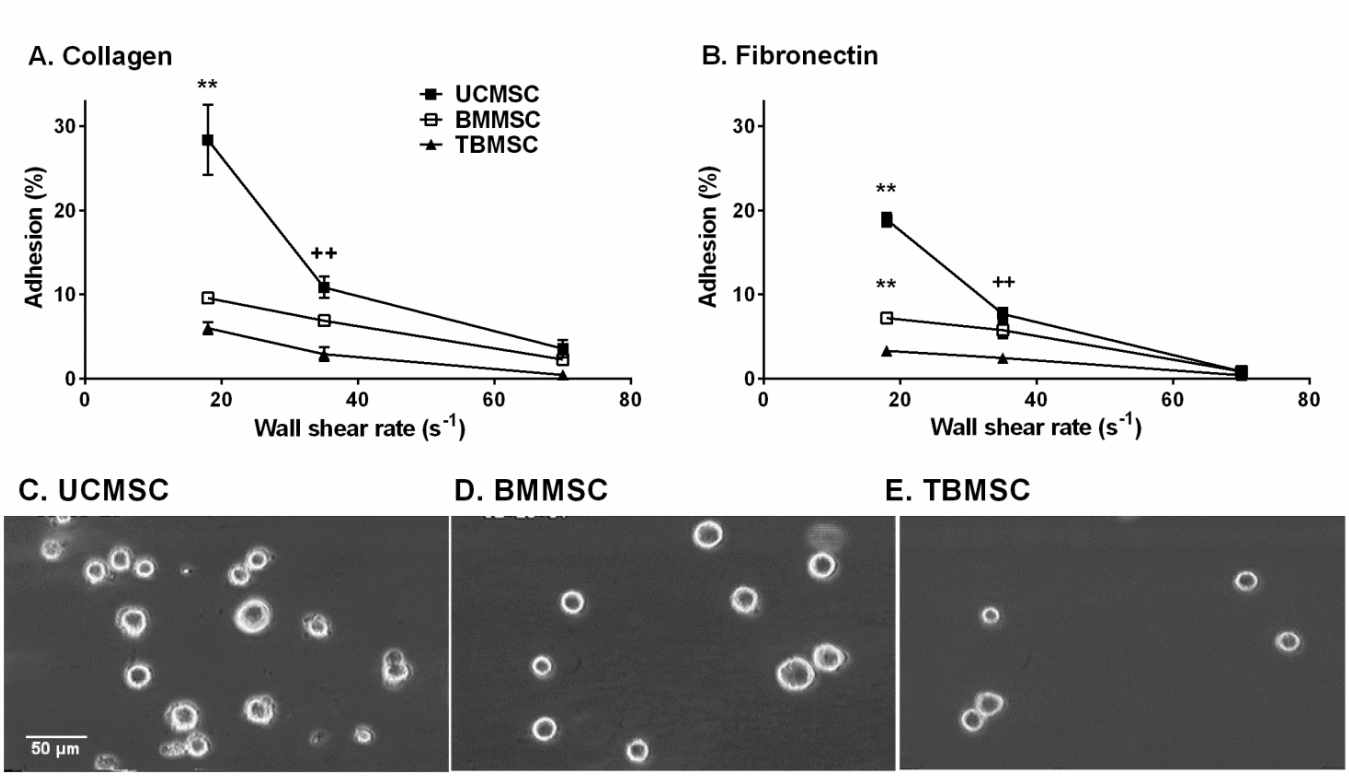

A. Collagen
B. Fibronectin
UCMSC
BMMSC
TBMSC
Adhesion (%)
Wall shear rate (s-1)
**
++
C. UCMSC
D. BMMSC
E. TBMSC
50 µm

Figure 2

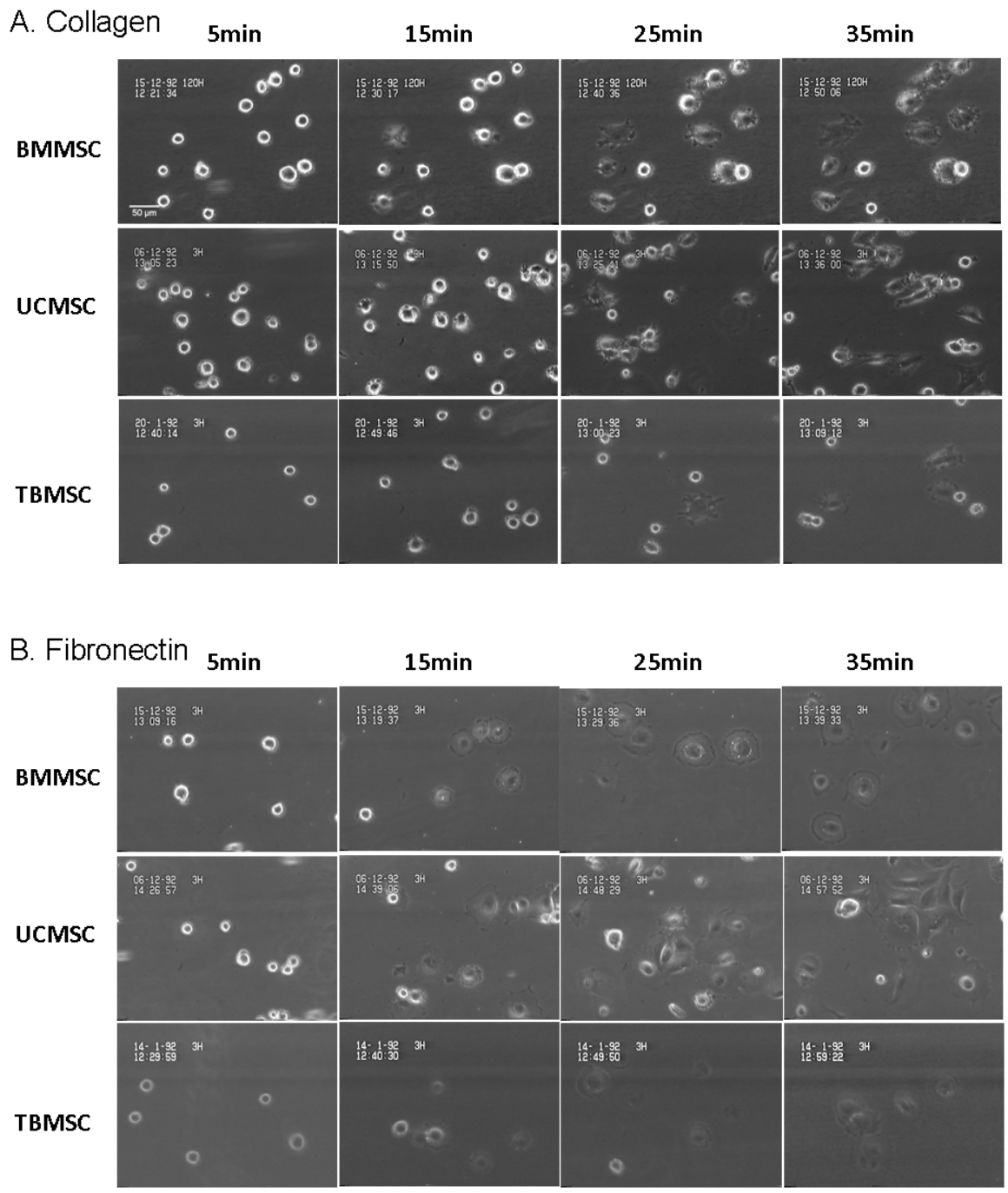

A. Collagen
5min
15min
25min
35min
BMMSC
UCMSC
TBMSC
B. Fibronectin
5min
15min
25min
35min
BMMSC
UCMSC
TBMSC

Figure 3

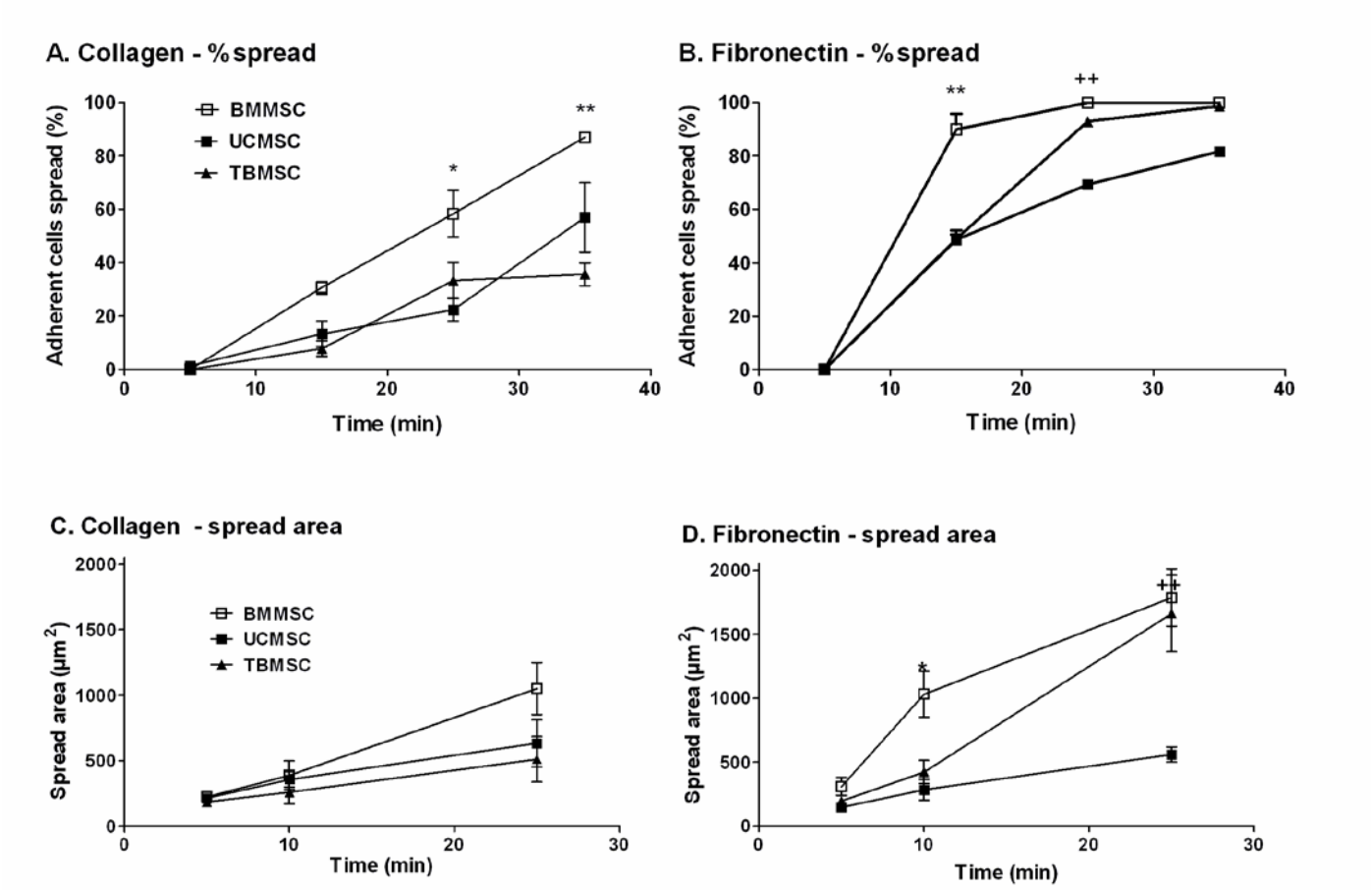

A. Collagen - % spread
Adherent cells spread (%)
BMMSC
UCMSC
TBMSC
*
**
Time (min)
B. Fibronectin - % spread
Adherent cells spread (%)
**
++
Time (min)
C. Collagen - spread area
Spread area (µm2)
BMMSC
UCMSC
TBMSC
Time (min)
D. Fibronectin - spread area
Spread area (µm2)
*
++
Time (min)

Figure 4

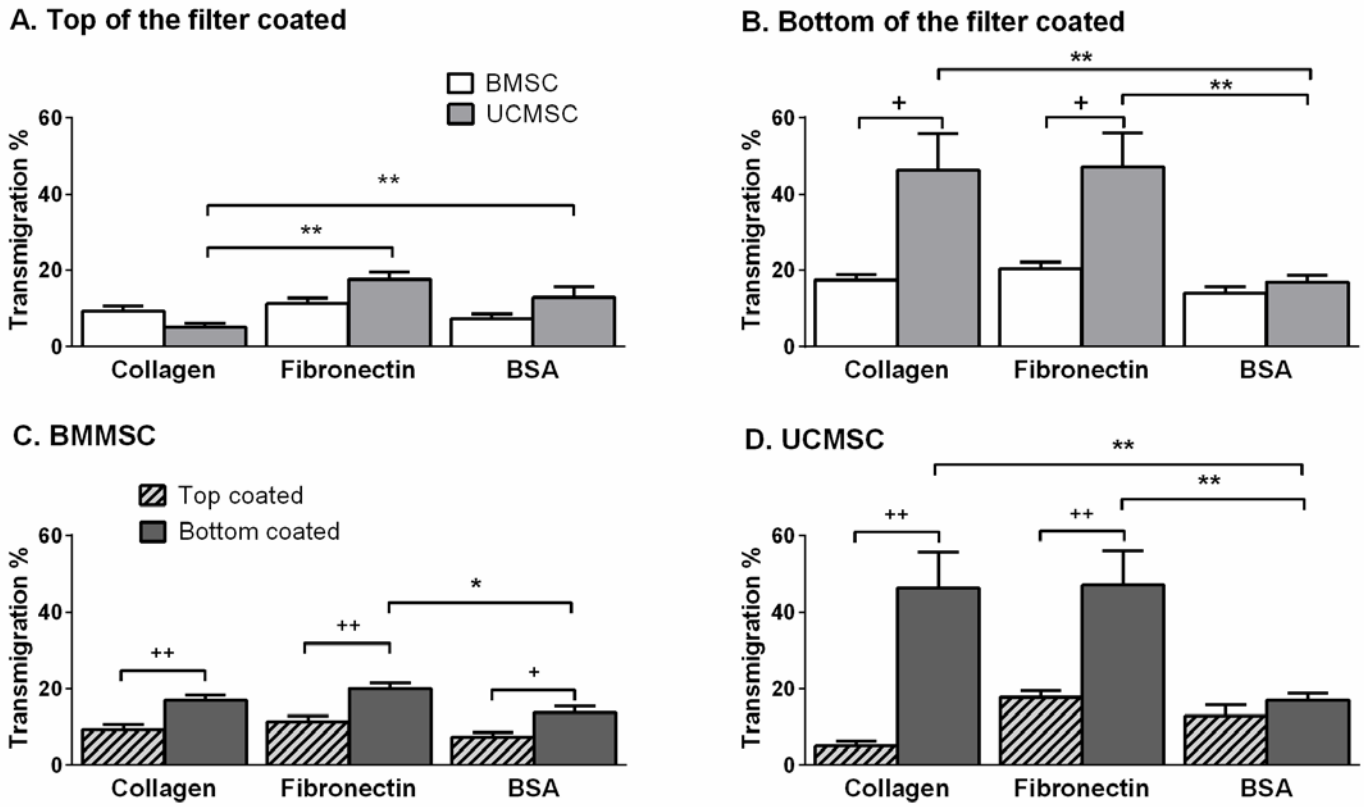
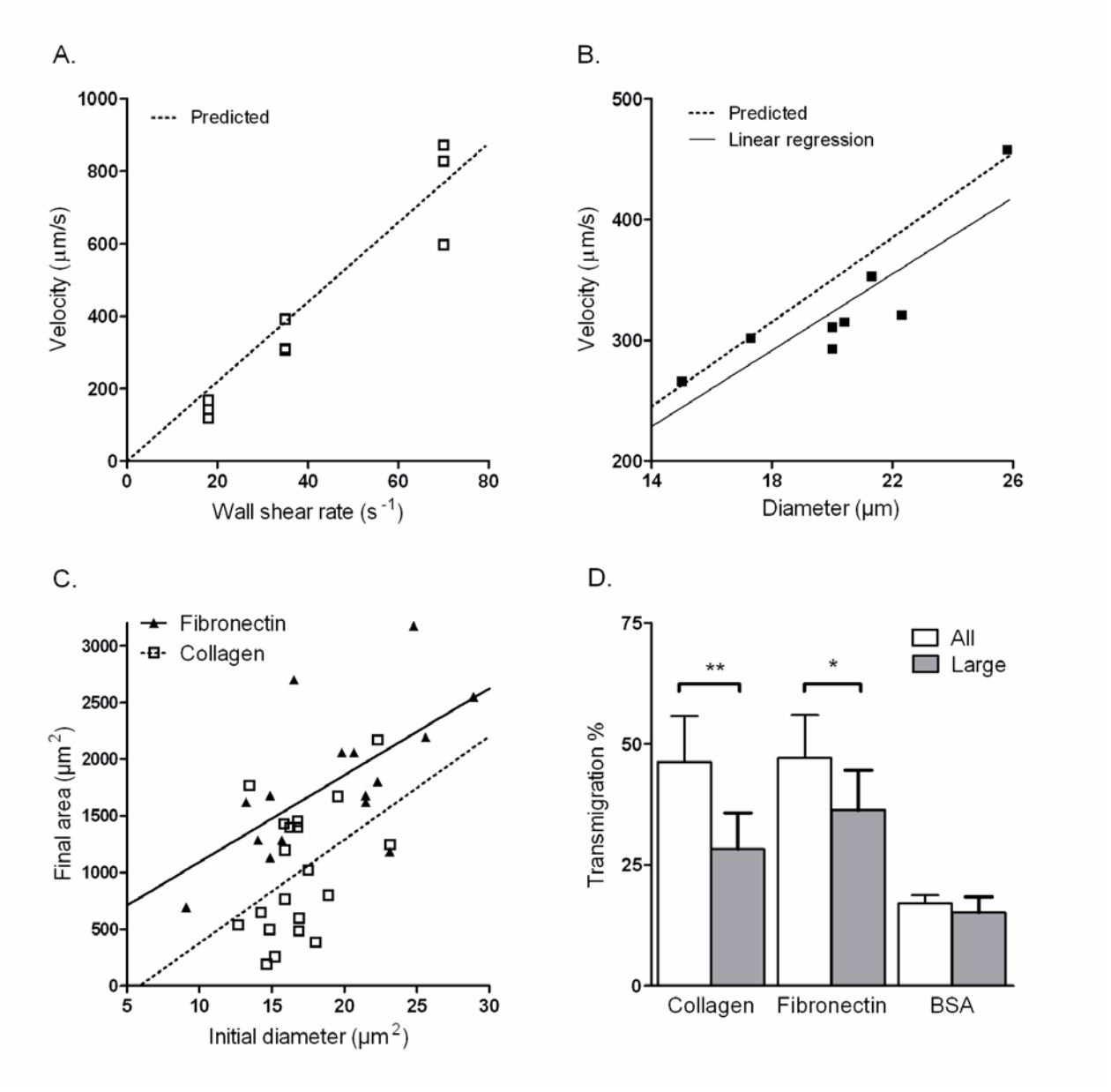

A. Top of the filter coated
BMSC
UCMSC
Transmigration %
60
40
20
0
**
**
Collagen
Fibronectin
BSA
B. Bottom of the filter coated
**
**
+
+
Transmigration %
60
40
20
0
Collagen
Fibronectin
BSA
C. BMMSC
Top coated
Bottom coated
Transmigration %
60
40
20
0
++
++
*
+
Collagen
Fibronectin
BSA
D. UCMSC
**
**
++
++
Transmigration %
60
40
20
0
Collagen
Fibronectin
BSA

Figure 5


A.
Predicted
Velocity (µm/s)
Wall shear rate (s -1)
B.
Predicted
Linear regression
Velocity (µm/s)
Diameter (µm)
C.
Fibronectin
Collagen
Final area (µm 2)
Initial diameter (µm 2)
D.
All
Large
Transmigration %
**
*
Collagen
Fibronectin
BSA