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Cell-surface markers identify tissue resident multipotential stem/stromal cell subsets in synovial intimal and sub-intimal compartments with distinct chondrogenic properties

K. Sivasubramaniyan †, W.J.L.M. Koevoet ‡, A.A. Hakimiyan §, M. Sande †, E. Farrell ‖, M.J. Hoogduijn ¶, J.A.N. Verhaar †, S. Chubinskaya §, H.-J. Bühring #, G.J.V.M. van Osch † ‡ *

† *Department of Orthopaedics, Erasmus MC University Medical Center, Rotterdam, the Netherlands*
‡ *Department of Otorhinolaryngology, Erasmus MC University Medical Center, Rotterdam, the Netherlands*
§ *Department of Pediatrics, Rush University Medical Center, Chicago, IL, USA*
‖ *Department of Oral and Maxillofacial Surgery, Special Dental Care and Orthodontics, Erasmus MC University Medical Center Rotterdam, Rotterdam, the Netherlands*
¶ *Department of Internal Medicine, Erasmus MC University Medical Center, Rotterdam, the Netherlands*
# *Department of Internal Medicine II, Division of Hematology, University Clinic of Tübingen, Tübingen, Germany*

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SUMMARY

Objective: Synovium contains multipotent progenitor/stromal cells (MPCs) with potential to participate in cartilage repair. Understanding the identity of these MPCs will allow their therapeutic potential to be fully exploited. Hence this study aimed to identify primary synovial MPCs and characterize them in the context of cartilage regeneration.
Methods: Primary MPC/MPC-subset specific markers in synovium were identified by FACS analysis of uncultured cells. MPC-subsets from human synovium obtained from patients undergoing total knee arthroplasty were FACS sorted, cultured, immunophenotyped and chondrogenically differentiated. The anatomical localization of MPCs in synovium was examined using immunohistochemistry. Finally, the presence of these MPC subsets in healthy synovium obtained from human organ donors was examined.
Results: A combination of CD45, CD31, CD73 and CD90 can isolate two distinct MPC-subsets in synovium. These MPC-subsets, freshly isolated from synovium, did not express CD45 or CD31, but expressed CD73. Additionally, a sub-population of $CD73^+$ cells also expressed CD90. $CD45^-CD31^-CD73^+CD90^-$ cells were significantly more chondrogenic than $CD45^-CD31^-CD73^+CD90^+$ cells in the presence of TGFβ1. Interestingly, reduced chondrogenic ability of $CD73^+CD90^+$ cells could be reversed by the addition of BMP2, showing discrete chondrogenic factor requirements by distinct cell-subsets. In addition, these MPCs had distinct anatomical localization; CD73 was expressed both in intimal and sub-intimal region while CD90 was enriched in the sub-intimal region. We further demonstrated that these subsets are also present in healthy synovium.
Conclusions: We provide indications that primary MPCs in synovial intima and sub-intima are phenotypically and functionally distinct with different chondrogenic properties.

Introduction

Multipotential progenitor/stromal cells (MPCs) from native synovium and their culture expanded progeny often referred to as mesenchymal stem/stromal cells (MSCs) represent a promising strategy for treatment of cartilage injuries. These MPCs in synovium are believed to play an important role in maintenance of joint homeostasis[1]. The synovium lining the joint cavity of synovial joints consists of an intimal lining layer and a sub-intimal layer[2]. MPC-like synoviocytes exist together with macrophage-like cells in the lining layer while in the sub-intimal region, MPCs have been described to be present in the perivascular region[3].

* Address correspondence and reprint requests to: G.J.V.M. van Osch, Room Ee1655, Erasmus MC, Wytemaweg 80, 3015 CN Rotterdam, the Netherlands. Tel: 31-107043661.
E-mail address: g.vanosch@erasmusmc.nl (G.J.V.M. van Osch).

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The synovium has a common embryological origin with articular cartilage[4] and synovial MPCs have been described to migrate to the site of cartilage injury to play a role in repair of the defects[5]. Though many studies since have demonstrated synovium as a potential source of MSCs for cartilage repair[6,7], less information exists about the characteristics or identity of MPCs and whether MPCs in lining or in sub-lining layer of synovium contribute to chondrogenic differentiation. In common protocols, unfractionated synovial cells or synovial explants are used as the starting population for the culture of MSCs. This isolation method is based on the adherence of MPCs to the plastic surface. The resulting cells are poorly defined and give rise to heterogeneous MSC populations. In contrast to functional isolation procedures, the prospective isolation of MSCs allows for precise definition and provides biological information on the starting population. Some groups have reported markers for prospective isolation of highly clonogenic or chondrogenic MPCs from primary synovium using combination of one or more markers including CD9, CD90, CD166 and CD271[8,9]. A report demonstrated that CD9/CD90/CD166 triple-positive cells have multipotency for mesenchymal differentiation[8] and another report demonstrated that $CD90^{+}CD271^{+}$ synovial cells have a high chondrogenic potential[9]. However, in both these reports, only one population of MPCs expressing a combination of the studied markers is described. The existence of MPC subsets which are negative for one or more of these markers or the phenotypic and functional properties of the non-marker expressing subsets is not characterized. Also, the anatomical localization of these MPC subsets in synovium is not known. To explore endogenous MPCs or injection of exogenous synovial MSCs as a potential cartilage regeneration strategy, it is essential to understand the biological relevance of these cells and their subpopulations for cartilage repair. To address this gap of knowledge, we screened a large panel of markers and established a combination of markers to identify MPCs and their subsets in synovium. We further show that the isolated MPC subsets differ in their immunophenotype, clonogenicity, chondrogenic differentiation ability and anatomical localization.

Methods

Throughout the manuscript, we refer to native, uncultured cells as MPCs and cultured cells as MSCs.

Synovial sample preparation and characterization

Osteoarthritic (OA) synovial tissue was isolated from a total of 22 patients (50–80 years) with advanced clinical OA, undergoing total knee replacement at Erasmus MC, Rotterdam. Ethical approval was granted by the local ethical committee (left over material after surgical removal using implicit consent and an opt-out procedure; MEC2004-322). Healthy synovial tissue was isolated from knee joints of eight human organ donors (approved by the Rush Institutional Review Board for research with human tissue specimens from deceased donors).

Synovial samples were either fixed in 4% formalin for immunohistochemistry or used to isolate cells. To isolate cells, the tissue was mechanically dissociated with scissors and enzymatically digested in HBSS with Ca^{2+} and Mg^{2+} (Gibco, Waltham, USA) containing 200 μg/ml of Dispase II (Roche, Basel, Switzerland) and 2000 μg/ml Collagenase IV (Sigma–Aldrich, Saint Louis, USA) for 90–120 min at 37°C. The digest was passed thrice through 16G needle, filtered through 100 μm and 40 μm sieves. The cells were centrifuged at 250×*g* for 10 min, washed twice with PBS and kept on ice for further use.

Synovial MSC cell culture and differentiation

MSC cultures were initiated from MPCs or MPC subpopulations in expansion media containing alpha-MEM (Gibco), supplemented with 10% fetal calf serum (FCS), 10^{-4} μg/ml FGF2 (AbD Serotec, Kidlington, UK), 10^{-4} M ascorbic acid-2-phosphate (Sigma–Aldrich), 1.5 μg/ml fungizone, and 50 μg/ml gentamicin. Medium was renewed twice per week. When MSCs neared confluence, they were detached with 0.05% trypsin and passaged at a density of 2300 cells/cm^2. FACS sorted and expanded MSCs in passage 3 were subjected to osteogenesis, adipogenesis and chondrogenesis as previously described ADDIN EN.CITE ADDIN EN.CITE.DATA[10]. The detailed procedure is described in supplementary methods.

Flow cytometric analysis and cell sorting

Cells were sorted (n = 6 donors) on a FACSJazz cell sorter (Becton Dickinson, Erembodegem, Belgium) or analyzed on a FACS Canto II flow cytometer (Becton Dickinson) and FlowJo software (FlowJo, Ashland, USA). The detailed procedure of FACS analysis and sorting and the antibodies used in the process are described in the supplementary methods.

CFU-F assays and population doubling time

CFU-F assays were performed by plating 500 FACS selected MPCs (n = 4 donors) per well of a six well plate in expansion medium. After 10–14 days of culture, adherent cells were washed twice with PBS, fixed with methanol and stained with the Giemsa solution (Merck Millipore, Billerica, USA). CFU-F colonies consisting of at least 50 cells were macroscopically enumerated. MSCs were cultured in expansion media and population doubling times (in days; n = 4 donors) were calculated between P1 and P2[10].

Immunohistochemistry

Pellets were fixed in 4% formalin, paraffin embedded and 6 μm sections were cut and stained with 0.4% thionin solution (Sigma–Aldrich) to detect glycosaminoglycans or immunostained to detect collagen II as previously described[11].

6 μm sections were cut from 4% formalin fixed, paraffin embedded OA-synovium (n = 6 donors) and healthy-synovium (n = 6 donors). Sections were deparaffinised and processed for CD34, CD73, CD90 and SUSD2 staining as described previously[10]. The detailed procedure is described in supplementary methods.

GAGs and collagen II in pellet (percentage of GAGs/collagen II positive area per pellet) and the expression of CD73 and CD90 (percentage of area occupied by CD73 or CD90 expressing cells to the total intimal/vessel area) were quantified. The intimal and vessel regions were marked as shown in supplementary methods.

Data analysis

The study design showing the relation between the aim of the study, results obtained, and donor numbers are shown in Supplementary Table 1. To identify markers for isolation of distinct MPC subsets, we screened four donor samples by FACS analysis. To analyze whether a combination of CD45, CD73 and CD90 can isolate two different MPC subpopulations from synovium using FACS, we used nine OA-patient samples and two healthy donors. To analyze phenotypic and functional difference between sub-populations, we performed CFU-F, chondrogenic differentiation and gene expression analysis in biological triplicate with FACS sorted MPC-subpopulations from each donor, population doubling time was performed in duplicate and data were averaged to obtain one value

for each subpopulation per patient. These experiments were performed using samples from 3 to 4 donors. To detect anatomical localization of MPCs, immunostaining was performed for six different OA-patient and six different healthy donors. IBM SPSS 25.0 was used for statistical analysis. All data are presented as mean ± standard deviation and were deemed statistically significant for $P < 0.05$.

The normal distribution of data (CFU-F and gene expression) or its residuals (population doubling time) was checked with Kolmogorov–Smirnov test and Shapiro–Wilk test. All data were considered normal as it passed either one or both of the tests. CFU-F of the two populations were compared using three samples of each population for each donor using a two-tailed paired Student's *t*-test and we also considered data from all donors in one analyses using a mixed linear model. To take donor variability into account, a mixed linear model was used, with multiple donors (statistically independent values) comparing different subgroups (cell populations from the same donor) that are statistically independent. From the same subgroup, multiple replicates (referred to as biological replicates) were analyzed and these are dependent to each other and the analyzed end point (including GAGs, gene expression, COL2/CD73/CD90 expression, CFU-F, population doubling time) are dependent on the subgroups and treatment condition. Using the model, independent analysis of GAG and collagen II positive area in the pellets was made to compare chondrogenesis in two different cell populations under different treatment conditions. The two different cell populations ($CD45^-CD31^-CD73^+CD90^-$ and $CD45^-CD31^-CD73^+CD90^+$) and different treatment conditions (TGFβ1, TGFβ1 + BMP2, TGFβ1 + BMP6) were defined as fixed factors, while the synovium donor was considered as random factor. Bonferroni correction was used to adjust for multiple comparisons due to use of different treatment conditions (TGFβ1, TGFβ1 + BMP2, TGFβ1 + BMP6). No correction was done for multiple end points (i.e., the different analyses). As the data were normally distributed, gene expression analysis of chondrogenesis associated genes, the expression of CD73 and CD90 using histology and population doubling time were also compared between the two different cell populations using mixed linear model. The two different cell populations ($CD45^-CD31^-CD73^+CD90^-$ and $CD45^-CD31^-CD73^+CD90^+$) was defined as fixed factor, while the synovium donor was considered as random factor.

Results

CD73 and CD90 identify distinct subsets of $CD45^-$ cells in synovium

To identify markers expressed on subsets of MPCs in synovium, we screened the freshly isolated cells obtained from synovium of patients undergoing total knee replacement for the reactivity with CD45 and a panel of stem cell markers and looked for markers specifically enriched on $CD45^-$ non-hematopoietic cells [Fig. 1(A), Supplementary Figs. 1 and 2]. FACS analysis revealed that marker CD73 was enriched on most $CD45^-$ cells and CD90 on a subpopulation of $CD45^-$ cells [Fig. 1(A)]. In the next step, co-staining of CD45, CD73 and CD90 followed by exclusion of dead cells with DAPI staining revealed that CD90 was expressed on a subpopulation of $CD45^-CD73^+$ cells [Fig. 1(B), Table I, $n = 9$ donors]. To check whether $CD45^-CD73^+CD90^{+/-}$ subsets had endothelial cell contamination, four color staining with CD31, CD45, CD73 and CD90 was performed. 10–20% of $CD45^-CD73^+CD90^-$ and 0.5–2% of $CD45^-CD73^+CD90^+$ cells expressed CD31 [Fig. 1(C) and (D)], showing the need of CD31 negative selection to exclude endothelial cells. From this point, CD31 was used along with CD45 for negative selection.

$CD73^+CD90^+$ and $CD73^+CD90^-$ sorted cells give rise to MSCs in culture

$CD45^-CD31^-CD73^+CD90^+$ and $CD45^-CD31^-CD73^+CD90^-$ cells were FACS sorted [Fig. 2(A) and (B)] and the subsets were cultured in MSC expansion medium ($n = 4$ donors). Both the sorted populations gave rise to MSC-like colonies defined as adherent, spindle-shaped, fibroblast-like cells [Fig. 2(C)]. Expansion rates of both MSC subsets were different ($n = 4$ donors; $P = 0.001$): $CD73^+CD90^+$ MSCs have population doubling time of 2.96 ± 0.8 days and $CD73^+CD90^-$ MSCs have a population doubling time of 3.97 ± 1.3 days [Supplementary Fig. 3(A)]. $CD45^-CD31^-CD73^+CD90^+$ cells had an average of 2 (±0.3)-fold ($P < 0.0005$) higher cloning efficiency compared to $CD45^-CD31^-CD73^+CD90^-$ cells [Fig. 2(D), $n = 4$ donors, each donor showed statistical difference between the subpopulations].

The sorted populations were immunophenotyped again after culture expansion for three passages ($n = 3$ donors). Both populations expressed MSC-specific markers including CD73, CD90, CD105, CD140a, CD166 and were negative for hematopoietic markers CD45 and CD11 [Fig. 2(E)]. Notably, the CD90 negative population started gaining CD90 expression after culture as early as passage 1 [$n = 3$ donors; Supplementary Fig. 3(B)] and completely gained CD90 expression in passage 3 [Fig. 2(E)].

$CD73^+CD90^+$ and $CD73^+CD90^-$ subsets are localized at distinct anatomical sites in synovium

To investigate the *in situ* localization of $CD73^+CD90^+$ and $CD73^+CD90^-$ subsets, paraffin sections of synovium ($n = 6$ donors) were stained with antibodies against CD73 or CD90. $CD73^+$ cells were present both in perivascular region and in lining intimal layer (Fig. 3). In contrast, $CD90^+$ cells were very abundant in the perivascular region surrounding capillary endothelium and larger vessels, but not in the intimal layer ($P < 0.0005$; Fig. 3, Supplementary Fig. 4).

Differential adhesion and chemokine/growth factor receptor expression in distinct synovial MPC subsets

To evaluate whether $CD73^+CD90^+$ and $CD73^+CD90^-$ subsets were also different for other markers, we further screened the freshly isolated cells from OA-synovium with an extended panel of markers including adhesion, chemokine/growth factor receptors, which are known to be expressed in stem cells in multiple tissues such as bone marrow, endometrium, adipose tissue and brain[12–15]. After excluding DAPI positive dead cells, as well as CD45 and CD31 positive cells, we evaluated the co-expression of this panel of markers by FACS on $CD73^+CD90^+$ and $CD73^+CD90^-$ MPCs ($n = 2$ donors). CD34 appeared enriched on a subset of $CD73^+CD90^+$ cells while CD349 (Frizzled-9), CD10 and SUSD2 were enriched on most of $CD73^+CD90^+$ cells and not on $CD73^+CD90^-$ cells, showing that both subsets have distinct immunophenotype (Supplementary Fig. 5). The anatomical localization of markers SUSD2 ($n = 4$ donors) and CD34 ($n = 1$ donor) that were specifically enriched on $CD73^+CD90^+$ MPCs, was found in the perivascular region and not in the intimal region, similar to CD90 [(Supplementary Figs. 6 and 7(A)]. CD34 was not only found in the perivascular region but also in the vascular region where it was expressed on endothelial cells. In the vascular region, the endothelial cells were identified by specific staining with the key marker CD31 [(Supplementary

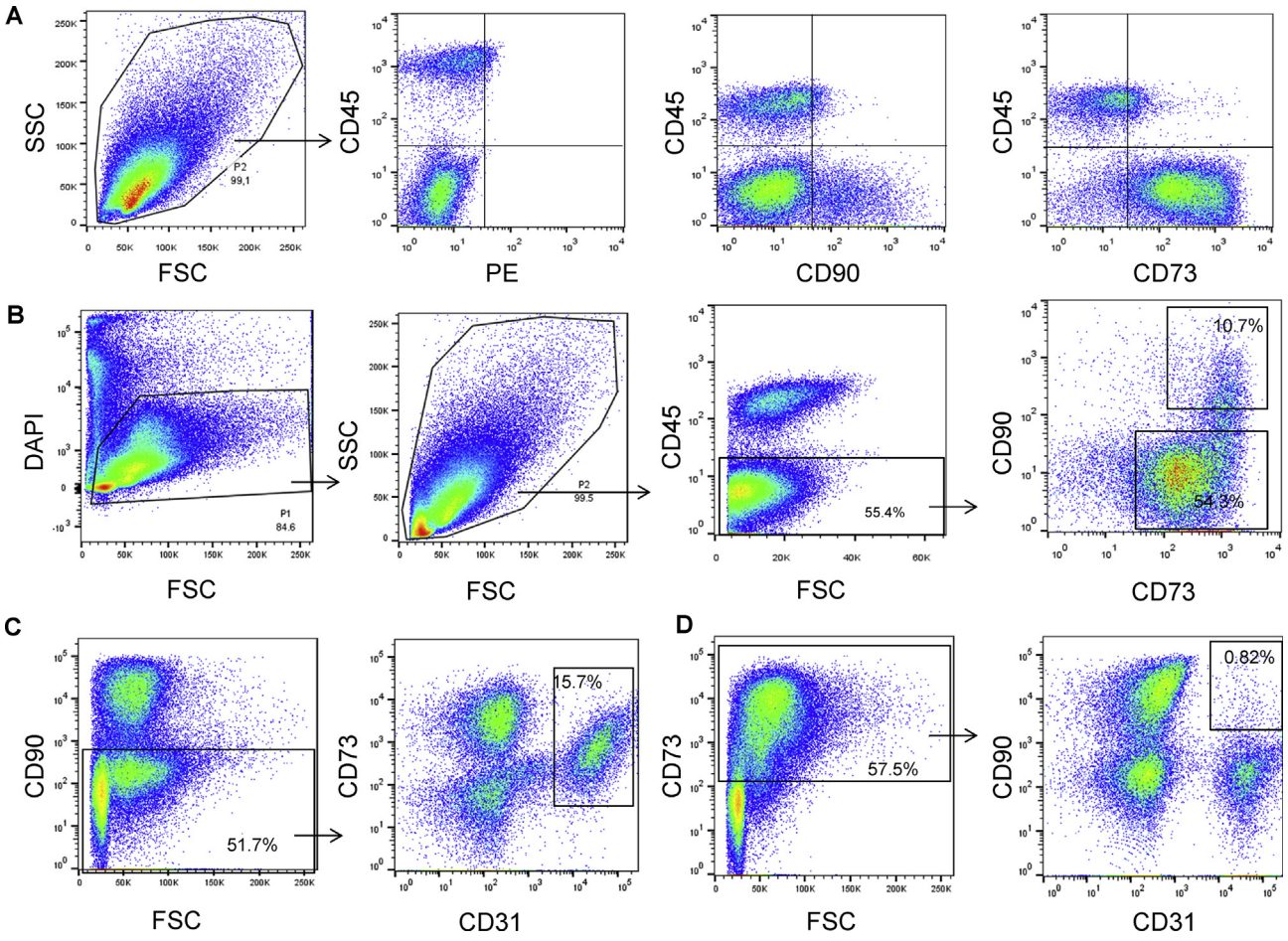

Fig. 1. (A) Identification of markers expressed on MPC subsets in synovium. Freshly isolated cells from synovium from OA patients were stained with CD45 and a panel of markers. Displayed are the two markers CD73 and CD90 which were specifically expressed on most or a sub-population of $CD45^-$ non-hematopoietic cells. **(B) CD90 identifies a distinct subset of $CD73^+$ stromal cells**. Freshly isolated synovial cells were stained with CD45, CD73 and CD90, gated on DAPI negative live cells, followed by gating on $CD45^-$ non-hematopoietic subset and analyzed for expression of CD73 and CD90. **(C & D) CD31 is expressed on a subpopulation of $CD73^+CD90^+$ and $CD73^+CD90^-$ cells**. Synovial cells were stained with CD31, CD45, CD73 and CD90, gated on DAPI and CD45 negative cells, followed by analysis of CD31 expression on (C) $CD73^+CD90^-$ and (D) $CD73^+CD90^+$ cells.

Fig. 7(A)]. This was also confirmed by FACS analysis ($n = 1$ donor) showing that CD34 is expressed on a sub-population of $CD45^-CD31^-CD34^+CD90^+$ MPCs as well as on most of $CD45^-CD31^+CD34^+$ endothelial cells [Supplementary Fig. 7(B)].

Table I
Distribution of synovial MPC subpopulations in different donors

Donors	% of $CD45^-CD73^+$ $CD90^-$ cells	% of $CD45^-CD73^+$ $CD90^+$ cells
OA synovium		
1	50.6	22
2	46.1	28.7
3	37.8	21.9
4	52.6	27.2
5	44.3	23.3
6	54.4	10.7
7	28.1	21.4
8	36.9	18.7
9	47.5	19.9
Mean ± SD	**44.3 ± 8.5**	**21.5 ± 5.18**
Healthy synovium		
1	41.6	39.9
2	46.9	24.5
Mean ± SD	**44.2 ± 3.7**	**32.2 ± 10.8**

Freshly isolated synovial cells were stained with CD31, CD45, CD73 and CD90, gated on propidium iodide/DAPI negative live cells, followed by gating on $CD31^-CD45^-$ subset and analyzed for expression of $CD73^+CD90^-$ and $CD73^+CD90^+$ subset.

Taken together, these results confirmed that CD90 recognizes perivascular MPCs.

$CD73^+CD90^+$ and $CD73^+CD90^-$ cells have distinct differentiation capacities

To examine the osteogenic and adipogenic differentiation of MSC subsets, the FACS sorted cells were expanded in MSC expansion medium and induced to differentiate into osteoblasts and adipocytes. MSCs derived from both the sorted populations after 20 days of culture generated calcifications as visible on von Kossa staining, in the osteogenic differentiation medium [Supplementary Fig. 8(A)]. After 10 days of induction in the adipogenic differentiation medium, clear Oil Red O staining was visible in both the MSC subsets [Supplementary Fig. 8(B)]. Neither population had notable differences in osteogenic and adipogenic differentiation capacity.

Chondrogenic differentiation was initiated by inducing pellet culture in the FACS sorted populations after expansion. After 32–35 days of induction, chondrogenesis was evaluated by glycosaminoglycan deposition with thionin staining ($n = 4$ donors), collagen II staining and gene expression analysis ($n = 2$ donors). $CD45^-CD31^-CD73^+CD90^-$ population was significantly more chondrogenic than $CD45^-CD31^-CD73^+CD90^+$ population ($P < 0.0005$; Fig. 4(A–C)). The $CD45^-CD31^-CD73^-CD90^-$ population poorly underwent chondrogenic differentiation and gave rise

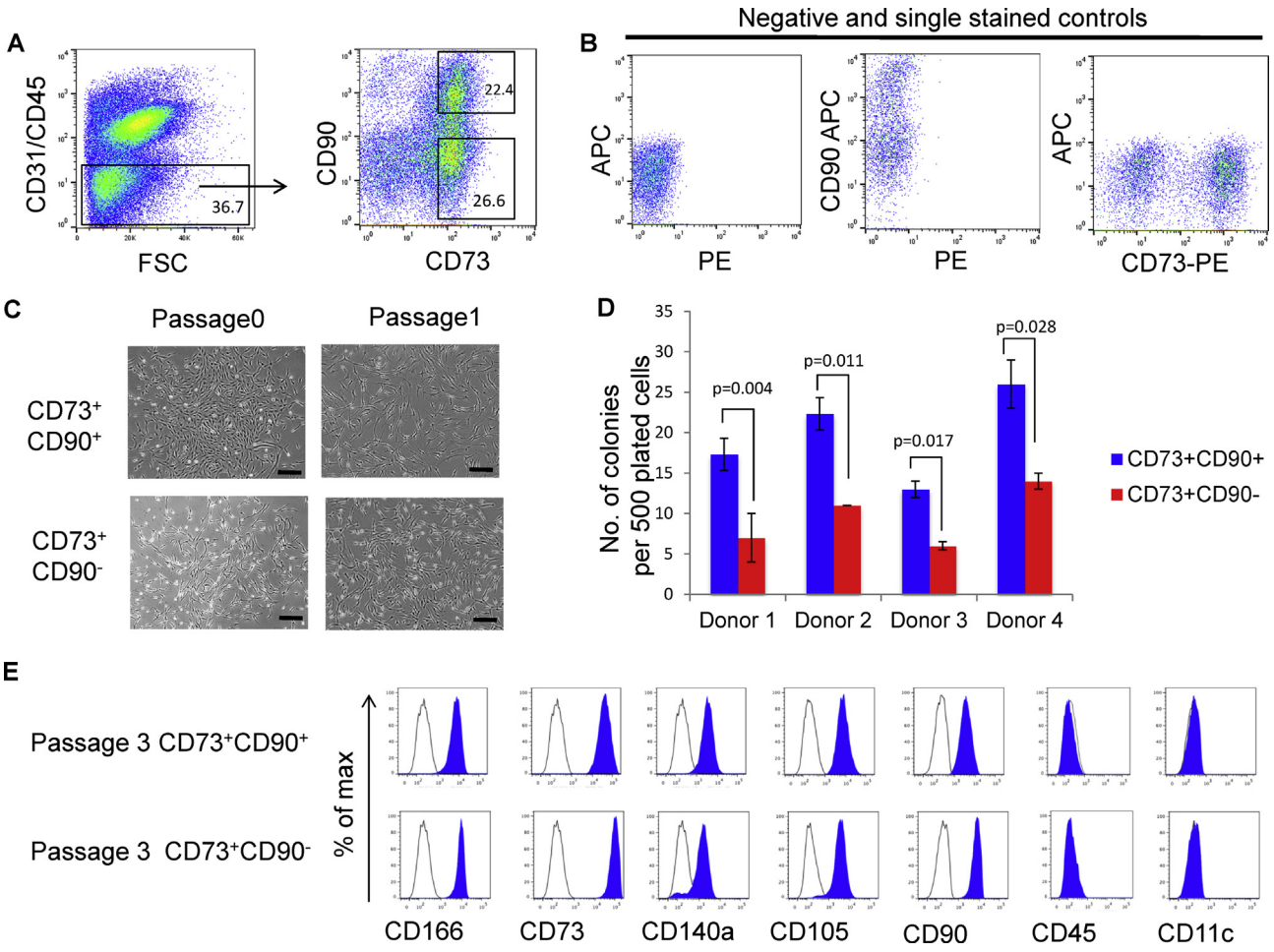

Fig. 2. CD73$^+$CD90$^+$ and CD73$^+$CD90$^-$ cells when cultured display MSC like characteristics. (A & B) Freshly isolated synovial cells were stained with CD31, CD45, CD73 and CD90, gated on propidium iodide negative live cells, followed by gating on CD31$^-$CD45$^-$ subset and analyzed for expression of CD73 and CD90 (A). For controls, cells were stained with CD45/CD31-FITC, CD73-PE or CD90-APC. After exclusion of dead cells with DAPI, the single stained tubes were used to set FACS sorting gates as shown in the CD73 vs CD90 plot (B) and cells were sorted with BD FACSJazz. **(C)** Morphology of MSCs derived from sorted cells. Scale bar = 200 μm. **(D)** CFU-F numbers in two subpopulations per donor. Counted clones were counted in biological triplicates of 500 FACS sorted CD73$^+$CD90$^-$ and CD73$^+$CD90$^+$ cells. The resulting CFU-F were stained and scored 12 days after culture ($n = 4$ donors) ($^{**}P < 0.005$, $^{***}P < 0.0005$). **(E)** Immunophenotype of MSCs cultured for three passages after being derived from sorted MSC subsets.

to many cells with adipose like morphology [$n = 1$ donor; Supplementary Fig. 8(C)]. CD31$^-$CD73$^-$CD90$^+$ fraction contained very few cells that did not form a clear and distinct population and were excluded for differentiation experiments.

To validate whether the poor chondrogenic ability of CD73$^+$CD90$^+$ cells in passage 3 was due to the loss of the chondrogenic potential because of higher population doublings of this population, chondrogenesis of the sorted populations was induced in passage 1. We found that even at lower passage, CD73$^+$CD90$^+$ population was less chondrogenic than the CD73$^+$CD90$^-$ population [Fig. 4(B)]. *COL2a1* was about three times higher in CD45$^-$CD31$^-$CD73$^+$CD90$^-$ compared to CD45$^-$CD31$^-$CD73$^+$CD90+ population ($P < 0.0005$) whereas *ACAN* expression was not different in chondrogenically differentiated pellets after 32–35 days. In addition, *COL10* was higher in pellets made from the CD73$^+$CD90$^-$ population which was in line with better chondrogenic differentiation [Fig. 4(D)]. We further confirmed the difference in chondrogenic ability between the two populations by collagen II immunostaining [Supplementary Fig. 8(D)].

To investigate whether the poor chondrogenic ability of the CD73$^+$CD90$^+$ population is due to the inherent capacity of these cells or if they require different factors for induction of chondrogenesis, we induced chondrogenesis in the presence of other chondrogenic factors of the BMP family (BMP2, BMP6) alone or in combination with TGFβ1 ($n = 2$ donors). We selected BMPs because adipose-derived MSCs are known to require BMP in addition to TGFβ1 for induction of chondrogenesis[16]. Glycosoaminoglycan and collagen II staining revealed that CD73$^+$CD90$^+$ cells, which were poorly chondrogenic in the presence of TGFβ1 alone, turned to be significantly more chondrogenic after the addition of BMP2 (Fig. 5; $P < 0.0005$). Though BMP6 in combination with TGFβ1 also significantly enhanced the chondrogenic ability of CD73$^+$CD90$^+$ cells, it was less effective than BMP2. Pellet sizes were slightly bigger in all conditions with combination of TGFβ1 and BMP2, with maximum size observed in pellets formed with CD73$^+$CD90$^+$ cells. [Supplementary Fig. 9(A–B)]. To confirm the results, we analyzed the gene expression of the sub-populations after induction of chondrogenesis ($n = 1$ donor in triplicates). Similar to the COL2 protein levels, *COL2a1* gene expression in CD73$^+$CD90$^+$ cells was higher when cultured with TGFβ1 + BMP2 than when cultured with TGFβ1 only [Supplementary Fig. 9(C)]. Neither BMP2 nor BMP6 induced chondrogenesis in the absence of TGFβ1 [Supplementary Fig. 9(D)]. These results show that these distinct cell subsets require discrete factors for chondrogenesis. Next we also analyzed whether differential chondrogenic response of CD73$^+$CD90$^-$ and CD73$^+$CD90$^+$ cells is due to difference in TGF and

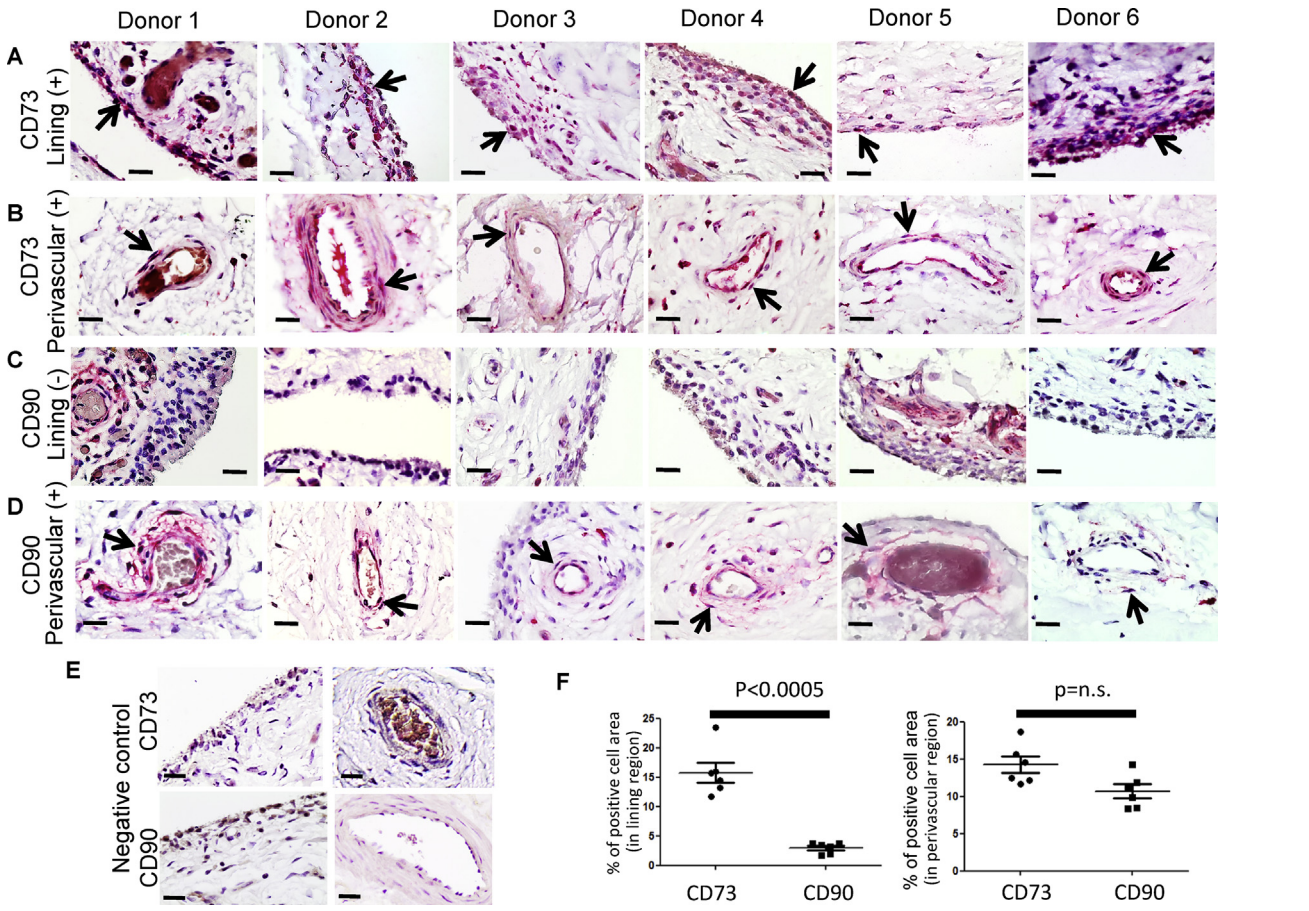

Fig. 3. Cell surface expression of CD73 and CD90 in synovium of osteoarthritic patients. Photomicrographs of OA synovium stained with CD73 and CD90. Images are representatives of sections from six donors, scale bar = 20 μm. Panels **(A)** and **(C)** display intimal region while **(B)** and **(D)** display perivascular region. Note that CD90 is expressed only in the perivascular region and not in the intimal layer while CD73 is expressed in both. **(E)** Representative negative control images stained with isotype control antibodies **(F)** CD73 and CD90 expressing cells were quantified by densitometric analysis of immunohistochemical pictures using ImageJ and expressed as % of positive cell area in the lining and vascular region ($n = 6$ donors).

BMP receptors in these cells and found no difference in the gene expression of these receptors (ALK1, 3, 5, 6, TGFBR2, BMPR2, ACVR2A, ACVR2B) [Supplementary Fig. 10].

CD73 and CD90 identify distinct MSC subsets in synovium from both OA patients and healthy donors

To confirm that CD73 and CD90 identify distinct MPC subsets also in synovium obtained from healthy donors similar to OA-synovium, we analyzed synovium of knee joints without disease. We first assessed the *in situ* localization of CD73 and CD90 in paraffin sections of healthy synovium ($n = 6$ donors). $CD73^+$ cells were present both in perivascular region and in the lining intimal layer while, as in the OA donors, $CD90^+$ cells were enriched in the perivascular region [Fig. 6(A)]. Then freshly isolated cells from healthy-synovium were evaluated for the reactivity with CD45, CD31, CD73 and CD90 with FACS. This confirmed the presence of $CD45^-CD31^-CD73^+CD90^+$ and $CD45^-CD31^-CD73^+CD90^-$ cells in healthy synovium [Fig. 6(B)]. Based on the data of these two healthy donors, the percentage of $CD45^-CD31^-CD73^+CD90^+$ cells in $CD45^-CD31^-$ cell fraction was 32.2 ± 10.8% compared to 21.5 ± 5.18% in OA-synovium ($n = 9$ donors). Similarly, $CD45^-CD31^-CD73^+CD90^-$ cells constituted about 44.2 ± 3.7% of $CD45^-CD31^-$ cell fraction in healthy- and 44.3 ± 10.8% in OA-synovium (Table I). For proper quantitative comparison more donors have to be analyzed.

Discussion

It has been demonstrated in animal models that synovium contains MPCs with chondrogenic differentiation potential that participate in cartilage repair following joint injuries. However, in humans, despite extensive *in vitro* characterization, the equivalent cells within the native tissue *in vivo* have not been characterized in detail. In this study, we have identified a marker combination for isolation of MPC subsets that reside in the human synovium and have elaborately characterized these subsets. We identified $CD31^-CD45^-CD73^+CD90^+$ phenotype as an indicator of perivascular MPC identity in the sub-intimal layer while $CD31^-CD45^-CD73^+CD90^-$ phenotype marks cells within the intimal lining layer in osteoarthritic and healthy synovium. We observed that cultured cells derived from both subsets exhibit a phenotype of MSCs and meet the minimum criteria recommended by the International Society of Cellular Therapy including adhesion to plastic, tripotent differentiation into chondrogenic, osteogenic, and adipogenic phenotypes as well as expression of defined surface markers[17]. Importantly, these anatomically distinct synovial cells had a clearly different response in adopting a chondrogenic fate.

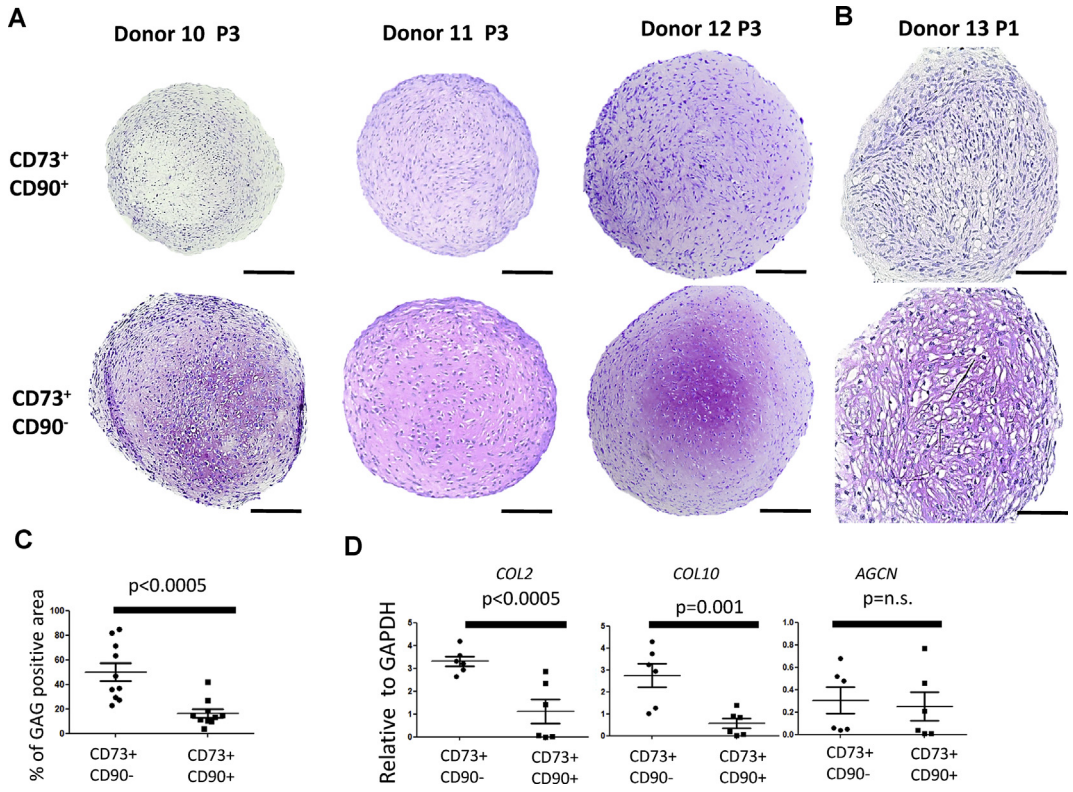

Fig. 4. CD73$^+$CD90$^+$ and CD73$^+$CD90$^-$ cell subsets from synovium display distinct chondrogenic potential. FACS sorted populations that were further expanded in culture for 1–3 passages were subjected to chondrogenic differentiation ($n = 4$ donors). (A & B) GAG deposition was visualized by thionin staining after 32–35 days of induction. (C) GAG levels were quantified by densitometric analysis of histology pictures using ImageJ and expressed as % of positive area of the pellet (2–3 biological replicates per four donors). (D) RNA analysis of the pellets after 32–35 days of induction. Note that CD73$^+$CD90$^-$ cells are more chondrogenic than CD73$^+$CD90$^+$ cells in all donors. Scale bar = 200 μm.

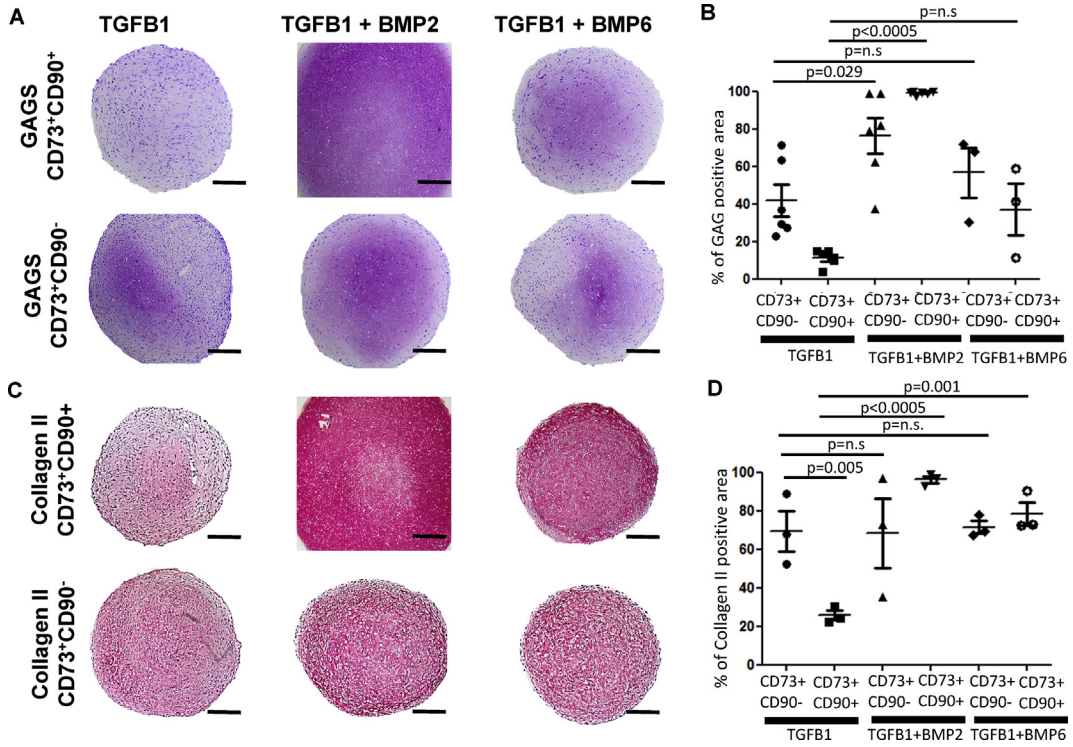

Fig. 5. BMP2 reverses low chondrogenic ability of CD73$^+$CD90$^+$ cells. Chondrogenesis of sorted cells (passage 3) was induced in the presence of TGFB1 alone and in combination with BMP2 or BMP6. **(A & C)** Thionin staining of Glycosaminoglycan deposition and Collagen II staining after 32 days of induction. **(B & D)** Densitometric analysis of GAG and Collagen II stained pictures using ImageJ and expressed as % of positive area of the pellet. Note that the low chondrogenic ability of CD73$^+$CD90$^+$ cells in the presence of TGFB1 alone can be reversed by the addition of BMP2. $n = 2$ donors; three biological replicates per donor. Scale bar = 200 μm.

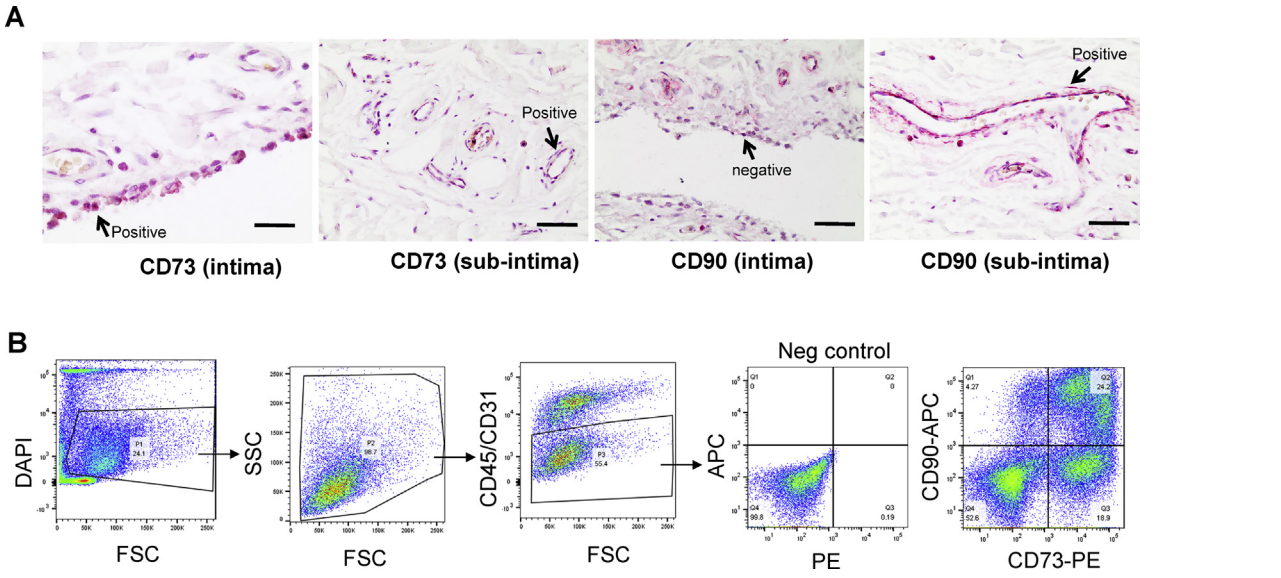

Fig. 6. CD73 and CD90 are expressed in healthy synovium. (A) Freshly isolated synovial cells were stained with CD45, CD73 and CD90, gated on DAPI negative live cells, followed by gating on $CD31^{-}CD45^{-}$ subset and analyzed for expression of CD73 and CD90. **(B)** Photomicrographs of healthy synovium stained with CD73 and CD90. Images are representatives of sections from six donors. Scale bar = 50 μm.

We found that perivascular $CD73^{+}CD90^{+}$ MPCs had a strikingly distinct expression profile and were enriched for markers including CD10, SUSD2 and CD349. Consistent with our finding, CD90, CD349, CD10, SUSD2 are known to be expressed in perivascular cells in multiple tissues such as bone marrow, endometrium, adipose tissue and brain[12–15]. Thus, this confirms the perivascular nature of $CD73^{+}CD90^{+}$ MPCs in synovium. However, there were also a few $CD90^{+}$ cells in the lining layer or beneath the lining layer in most synovial samples. It is known that in humans, CD90 is expressed not only on MPCs but also on other cell types including hematopoietic stem cells, a subset of T cells, keratinocytic stem cells and at varying levels on non-lymphoid tissues such as fibroblasts, neurons and endothelial cells[18,19]. To examine whether CD90 is expressed on hematopoietic cells in synovium, we gated on $CD45^{+}$ cells in healthy and OA-synovium and looked for CD90 expression and found CD90 to be expressed on 0.4–4% of $CD45^{+}$ cells (data not shown). Hence it is very likely that the $CD90^{+}$ cells detected in the intima are of other lineage such as hematopoietic cells.

Other studies have described promising other markers to discriminate MPCs. Recently, Mizuno *et al.* published a study where they identified MPCs in synovial and sub-synovial compartments[20]. They used CD55, an established marker of intimal fibroblast like-synoviocytes to identify MPCs in intimal region and CD271 to identify MPCs in perivascular region. In another study, Corselli *et al.* described two distinct perivascular MSC subsets in multiple tissues, including pericytes and adventitial cells. Adventitial cells were $CD31^{-}CD45^{-}CD34^{+}CD146^{-}$ and pericytes were $CD31^{-}CD45^{-}CD34^{-}CD146^{+}$[21]. $CD34^{+}$ adventitial cells had higher proliferative potential and were suggested to be the precursors of the $CD34^{-}$ subset. In our study we also detected two populations of perivascular MSCs characterized by the $CD31^{-}CD45^{-}CD90^{+}CD34^{-}$ and $CD31^{-}CD45^{-}CD90^{+}CD34^{+}$ phenotype respectively. This observation leads to the speculation that $CD31^{-}CD45^{-}CD90^{+}CD34^{+}$ cells might be adventitial cells and $CD31^{-}CD45^{-}CD90^{+}CD34^{-}$ cells might be pericytes. Surprisingly, we detected very few CD146 expressing cells in freshly isolated $CD73^{+}CD90^{+}$ MPCs from OA-synovium. Since we used only one CD146 antibody clone for the screening, cannot eliminate the possibilities that this observation is linked to the antibody used.

It was previously shown that a variety of surface markers are expressed on cultured MSCs but not on their primary counterparts and vice versa[13], In line with these observations, we noticed that $CD73^{+}CD90^{-}$ MPC subset gained CD90 expression after culturing. Similarly, CD166 and CD105 were almost absent in the freshly isolated $CD45^{-}$ population, but highly expressed in the cultured MSCs derived from both the subsets. This demonstrates that cell phenotypes are altered by exposure to culture conditions and many of these markers are mere artifacts of culturing.

Our study shows that $CD73^{+}CD90^{-}$ cells required only TGFβ1 for chondrogenesis. On the other hand, $CD73^{+}CD90^{+}$ population required BMP2 along with TGFβ1 to undergo chondrogenesis. Hence we speculate that this population resembles AT-MSCs in terms of chondrogenic induction[22] and addition of BMP2 increases the TGFβ based response. Therefore it is important to consider that the chondrogenic ability of autologous transplanted synovial MSCs or their subsets may be partially determined by the growth factors present in the native joint microenvironment. Even though we could not demonstrate differences in expression of TGFβ and BMP-receptors between the subpopulations, differences might occur on receptor protein or activation level. Since healthy synovial sub-populations were not characterized for chondrogenic differentiation, it cannot be excluded that the observed difference in chondrogenic properties in the different sub-populations is OA related.

Hunziker *et al.* showed that, in adult rabbits, synovial MPCs migrate to the site of cartilage injury to play a role in repair of the defects[5]. Similarly, Roelofs *et al.* showed the involvement of intimal and sub-intimal MPCs in cartilage repair in mouse[23]. Hence we postulate that both synovial $CD73^{+}CD90^{-}$ and sub-synovial $CD73^{+}CD90^{+}$ MPC subsets may migrate from the synovium through the synovial fluid and participate in the repair/regeneration of joint tissues. Thus the potential of synovial MPCs can be harnessed for tissue regeneration. Activating stem cells in their native environment by infiltration of growth factors or molecules in a suitable biomaterial scaffold to attract endogenous stem cells is a promising approach for cartilage repair[5,24]. The ultimate goal of this strategy being the manipulation and activation of the regenerative potential of both local stem cells and those recruited from distant

sites. In our study we identified two subsets of endogenous synovial MPCs which show differential expression of chemokine/growth factor receptor Frizzled-9. Frizzled-9 is the receptor for Wnt signaling proteins Wnt2 and Wnt5a[25,26]. Future studies aimed at screening Wnt2/Wnt5a proteins for promoting differential migration of $CD73^{+}CD90^{+}$ MPCs and identifying other factors that can preferentially attract MPC subsets will open new possibilities for use of endogenous synovial MPCs in musculoskeletal repair.

Recent insights into the biology and identity of MPCs from different sources provide new opportunities for therapeutic applications[3,13,21,27]. In this perspective, our results demonstrate that pure synovial MPCs with potent chondrogenic differentiation ability can now be isolated from the heterogeneous cell populations, thus eliminating the interference from cells with no or limited regenerative potential. Robust validation of these cells in animal models and clinical trials is the next required step to verify the potential of these cells for musculoskeletal repair.

Contributions

K. Sivasubramaniyan conceived and designed the presented idea, carried out experiments, data acquisition and manuscript preparation. W. Koevoet performed differentiation assays. M. Sande carried out immunohistochemistry. A. Hakimiyan and S. Chubinskaya acquired and processed healthy synovial samples and S. Chibinskaya revised the manuscript. E. Farrell and M. Hoogduijn provided access to FACS facility for cell sorting and cell analysis and revised the manuscript. H.J. Bühring provided the monoclonal antibodies used in the study and revised the manuscript. J. Verhaar supported the acquisition of osteoarthritic synovial samples, provided feedback on clinical applicability of the data and critically commented on the manuscript. G. van Osch provided critical feedback and helped shape the research, analysis and contributed to the drafting and revision of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary data

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