Responders vs. non-responders to mesenchymal stromal cells in knee osteoarthritis patients: mechanistic correlates of donor cell attributes and putative patient features

Running title: Mechanistic correlates to MSCs in knee OA

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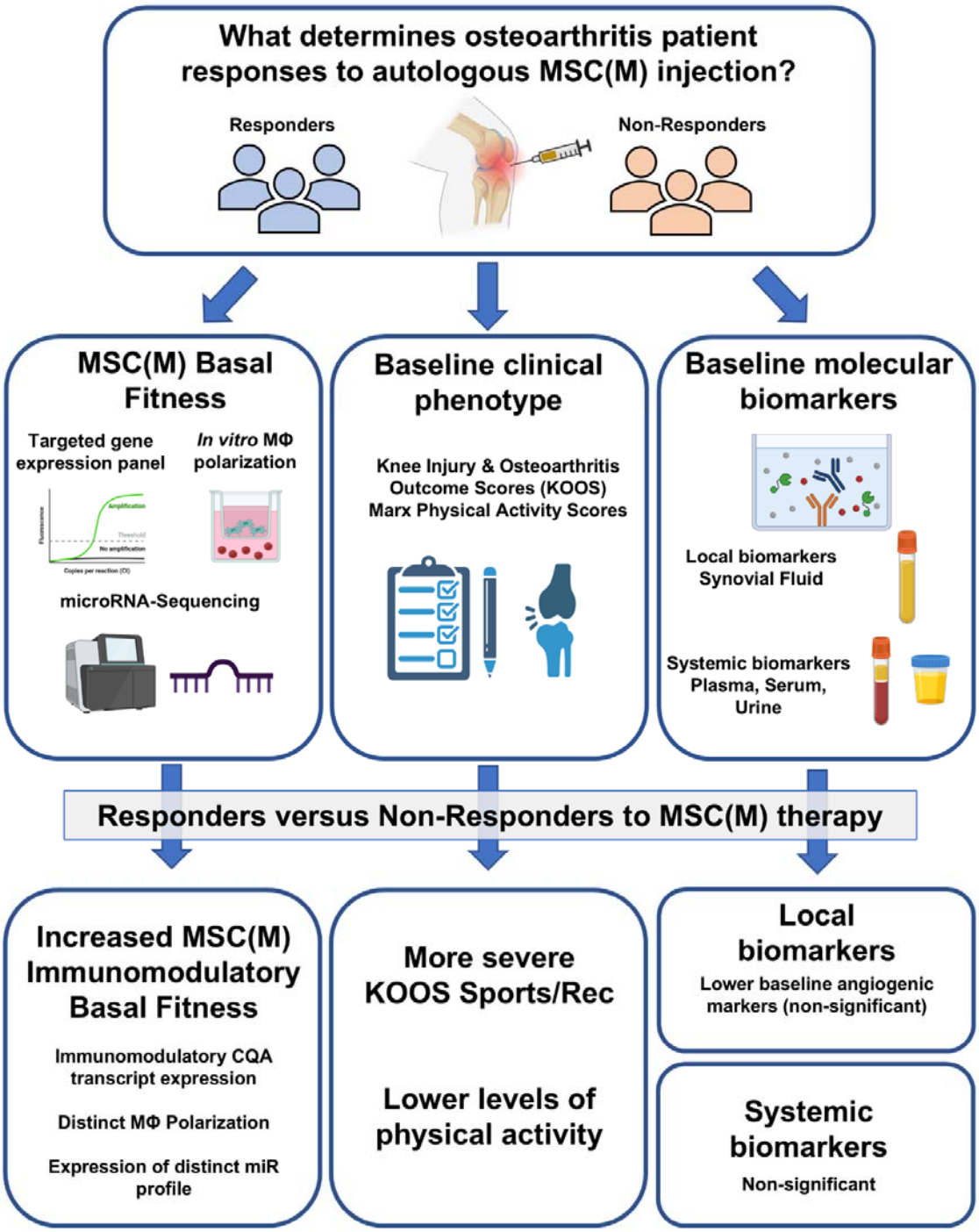
SUMMARY

Mesenchymal stromal cells (MSCs) have resulted in heterogenous clinical effectiveness in knee osteoarthritis (KOA). Herein, a framework that dually correlates KOA patient responsiveness to baseline bone marrow-derived MSC(M) donor batch attributes and baseline clinical and biomarker features is provided. Using previous clinical trial data, we demonstrated that MSCs with increased immunomodulatory potency are more efficacious. Multivariable MSC(M) genes correlated strongly with responder status and to 12- and 24-month improvements in Knee Osteoarthritis and Outcome Scores. Responder MSC(M) donor batches had differential microRNA expression and ability to polarize CD14+ monocytes *in vitro*. KOA Responders had more severe OA and lower physical activity. Baseline biomarkers showed trending correlations to patient responsiveness. 62.5% of KOA patients remained Responders at 8-10 years, speaking to longer-term effects of single MSC(M) injections. Thus, our analytical methodology defines critical quality attributes of potent MSC donor batches and identifies putative KOA patient theratypes to MSC treatments.

Graphical Abstract



INTRODUCTION

Knee osteoarthritis (KOA) affects 16% of adults over 40 years of age worldwide [1], with no available disease-modifying drugs. Pathophysiological mechanisms of KOA are heterogeneous and fluctuate temporally [2]. Culture-expanded mesenchymal stromal cells (MSCs) are being investigated for their multimodal mechanisms of action, including immunomodulatory functions [3,4]; however, there has been conflicting clinical efficacy reported. Equivocal results show no difference in 12-month improvements between umbilical cord-derived MSCs vs. first-line corticosteroids treatments [5]; conversely, other reports [6–9], meta-analyses [10,11], and randomized controlled trials [12,13] showed symptom and structural improvements with MSC treatments. This suggests heterogeneous responsiveness, stressing the need to identify signatures that distinguish responder from non-responder donor batches to deliver effective MSC treatments to KOA patients.

Heterogeneous responsiveness to MSC treatments is due to an interplay of both basal potency attributes of donor MSC batches and basal patient disease status [4]. Heterogeneity exists among MSC investigational products due to intrinsic variations in donors [4,14] and manufacturing parameters [15,16]; well-defined critical quality attributes (CQAs) that are sensitive, quantitative and relevant to disease-specific mechanism of action are thus needed [17].

Using reported [8], and new follow-up data from a phase I/IIa dose-escalation clinical trial, we conduct in-depth analyses of donor MSC batches and baseline/follow-up KOA patient characterization to provide an integrated algorithm to analyze heterogenous KOA patient responsiveness to MSC treatments. Using a previously reported gene panel that correlated to *in vitro* functional activity [18], there is significant discrimination of responder vs. non-responders MSC(M) donor batches from marrow tissue. MicroRNAs, important mediators of MSC [19] further discriminate MSC(M) from responders vs. non-responders.

KOA is recognized not as a single disease but a constellation of clinical phenotypes [20,21] and divergent molecular endotypes [22]. Here we report an exploratory analytical framework that combines clinical phenotypes and molecular endotypes that can be used as the basis for identifying KOA patient responsiveness to MSC(M) treatments.

MATERIALS AND METHODS

Clinical trial and patient data

KOA patients (Kellgren Lawrence (KL) grade 3-4) were recruited to a nonrandomized, open-label, dose-escalation, Health Canada-authorized phase I/IIa clinical trial as reported [8] (NCT02351011). Ethics approvals including for long-term follow up were granted (REB 14–7909;20-5066;24-5463) [8]. Complete clinical follow-up was performed 24-months after MSC(M) injection for all patients. Additional follow-up,at 8-10 years was obtained for 10/12 patients; the remaining 2 patients were lost to follow-up. Calculation of patient-reported outcome measures (PROMs), including the inverted Knee Injury and Osteoarthritis Outcome Score (KOOS) and the Marx Activity Rating Scale (MARS), as described in **Suppl. File 1**. Contrast-enhanced MRI analysis at baseline, 6 and 12-months were conducted on all patients as previously described [8]; whole organ MRI scores (WORMS) and MRI synovitis scores were measured and calculated as previously described [23].

Responder vs. Non-Responder Criteria

Patient clinical responder status was based on the Outcome Measures in Rheumatology (OMERACT) and Osteoarthritis Research Society International (OARSI) Standing Committee for Clinical Trials Response Criteria Initiative (OMERACT-OARSI criteria), based on data from large, powered placebo-controlled drug trials for hip and KOA [2]. These criteria categorize patients as responders according to the following: i) improvement in pain or in function ≥50% and absolute change ≥ 20; OR, ii) improvement (≥20% and absolute change ≥ 10) in at least two of the following: a) pain, b) function, c) patient global assessment; the latter was not measured in our trial. Given the limited sample size and lack of patient global assessment metrics in our previous clinical trial, we categorized patient responder status using the twelve-month follow-up data based on changes in KOOS function (activities of daily living (ADL) subscale) and/or KOOS Pain only, applying OMERACT-OARSI criteria thresholds for function and pain scores of delta value ≥10 AND percentage change ≥20% [2] . The validated KOOS instrument measures responses to interventions in KOA with high test-retest reliability and improved sensitivity in pain and function scoring relative to the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) scoring system [2,4]. Baseline demographics, radiographic KL-grade, magnetic resonance imaging (MRI) total cartilage volume (WORMS, [23] and synovitis [24] scores are provided in both Function Responder vs. Non-Responder donor categories (Table 1).

Biomarker measurements

Levels of curated biomarkers in baseline serum (**Table S2**) were measured previously [8]; updated analyses and experimental questions were applied to the dataset in the present work. Additional inflammatory biomarkers were measured in baseline biobanked patient serum and plasma (analytes specified in **Table S2**). Synovial fluid collection was feasible for only nine of twelve patients. Immune cell profiling was done only on available (five), paired baseline and follow-up synovial fluid samples and is thus considered exploratory [8].

Statistical analyses

GraphPad Prism 6.0 and JMP Pro 17 software were used for statistical analyses and to create plots. Statistical tests are specified in figure and table captions. Spearman's correlation was selected as a non-parametric statistical test to investigate associations between inverted KOOS changes and other variables, given that the data violated assumptions of normality and homoscedasticity. P values derived from Spearman's correlation were corrected for multiple comparisons using the Benjamini-Hochberg False Discovery Rate (FDR) method. Sex, age, body mass index (BMI), KL-grade, MSC dose and baseline MRI WORMs and synovitis scores were not considered as covariates given lack of significant correlation between these factors with Function Responder status (Table 1). Given the limited sample size and exploratory nature of analysis, Spearman's correlations were considered statistically significant when $p<0.05$ (uncorrected) and $p<0.1$, post-FDR.

RESULTS

Patient responder status

We systematically investigated multivariable factors affecting KOA patient responder status using 12- and 24-month patient-reported inverted KOOS data [8]. We applied OMERACT-OARSI criteria [25] to determine responder status, with modifications. Patients were classified as Responders vs. Non-Responders. Within Responders, we identified three sub-categories (**Table S3**) including: i) Function-Pain Responders (exceeding thresholds in <u>both</u> KOOS ADL and Pain); ii) Function Responders (exceeding thresholds in ADL), iii) Pain Responders (exceeding thresholds in Pain); Non-Responders did not exceed thresholds in either ADL or Pain (**Table S3**). Of twelve patients, eight total Responders (all categories) and four Non-Responders met these criteria. While the sample size is limited, the binary criteria

was useful in determining factors affecting patient clinical responses. However, correlations with all KOOS subscale scores were included to mitigate the limited power of binary analysis. There was no observed dose-dependency; responders existed across all three MSC(M) doses including the 1 x 10^6 (2/4), 10 x 10^6 (1/4), and 50 x 10^6 (3/4) (**Table 1**). We report on Function Responders; other Responder category data are included in the supplementary files. We checked for associations between covariates including sex, age, BMI, KL-grade, MSC dose and MRI baseline WORMs and synovitis scores and Function Responder status and observed no significant correlations (**Table 1**).

12-month Responder vs. Non-Responder classification holds at 24-months and 8–10-year follow-up

Function Responders at 12 months displayed significantly improved inverted KOOS scores in all subscales at 12- and 24-months follow-up (**Fig. 1A**), supportive of persistent effects of a single injection of autologous MSC(M). Patients categorized as Non-Responders similarly showed no significant changes at 12- and 24-months, indicating stable clinical phenotype. No significant differences were observed when comparing outcomes at 12 vs. 24-months for any KOOS subscale in Responders or Non-Responders. Similar results were obtained with Function-Pain (**Fig. S1A**) and Pain (**Fig. S1B**) Responder categories.

We further analyzed patient follow-up at 8-10 years after single MSC(M) injection. 12-month Responders maintained significant improvement in multiple KOOS subscales at 8-10 years follow-up (**Fig. S2**). Specifically, 7/8 Responders maintained their Responder status at 24 months (**Fig. 1B, 1C**). Of these, 5/7 maintained Responder status at 8-10 years, and one patient (underwent knee arthroplasty) did not provide long-term KOOS data. 2/8 Responders received no additional interventions; 2/8 received viscosupplementation or platelet-rich plasma; 3/8 patients underwent knee arthroplasty surgery.

4/4 12-month Non-Responder patients maintained status at 24-months, and one patient was lost to follow-up at the 8–10-years (**Fig. 1B, 1C**). 3/4 patients maintained Non-Responder status at 8-10 years, with 1/4 patients reporting no additional interventions and 2/4 patients reporting viscosupplementation.

Immunomodulatory gene panel discriminates between clinically effective and ineffective MSC(M) donor batches

Using biobanked MSC(M) samples cultured under pro-inflammatory licensing conditions, we measured expression of a gene panel that correlated with *in vitro* immunomodulatory functionality [18]. Pro-inflammatory cytokine licensing conditions were used as a common method to measure immunomodulatory genes in MSC(M) [26].

Principal Component (PC) analysis revealed distinct unsupervised clustering between Function Responder vs. Non-Responder MSC(M) donor batches along the PC1 axis (29.3% of total variance), with higher expression of *CCN2* and lower expression of angiogenic markers *THBS1*, *CXCL8*, and *ANGPT1* contributing strongly to higher PC1 scores (**Fig. 2A**, **Fig. S3A**). PC1 scores were significantly lower for all categories of Responders vs. Non-Responders (**Fig. 2B**, **Fig. S3B**), confirming the discriminatory power of this curated gene panel. To ensure that individual donor batches were not skewing results, given limited sample size, outlier analysis was performed by calculating T^2 statistic, identifying three outlier data points (two Responder, one Non-Responder). However, the PCA with these data points removed, returned similar results; clear separation of Responder/Non-Responders was observed across the expected eigenvectors (**Fig. S3C**).

PC1 scores, indexed as a composite score for the basal immunomodulatory gene expression profile, additionally showed statistically significant robust negative correlations with improvements in 12-and 24-month mean KOOS and KOOS pain and/or ADL sub-scores. function at 12- and 24-mo (**Fig. 2C**, **Table S4**).

Individual MSC(M) genes, *ANGPT1* and *CCN2* showed significant correlations with 12- and/or 24-month KOOS pain and ADL subscores and mean KOOS (**Table S5**) that were lost after adjustment for multiple comparisons.

In vitro immunomodulation by donor MSC(M) batches correlates to patient responder status

Functional *in vitro* monocyte polarization using mismatched human peripheral blood monocytes further assessed baseline MSC(M) immunomodulatory potency (**Fig. 3A**). Discriminant analysis demonstrated distinct clustering and greater separation of monocyte phenotypic profiles between Responder vs. Non-Responder donor MSC(M) batches (**Fig. 3B**). Co-cultures of monocytes with Responder MSC(M) donor batches significantly

upregulated expression of the inflammation-resolving markers *IL10* and *STAB1,* and reduced expression of *CD86* compared to Non-Responders batches (**Fig. 3B**). The immunosuppressive marker, *CD274*, was upregulated by Non-Responder vs. Responder MSC(M) donor batches. Overall, Responder MSC(M) donor batches modulated *in vitro* monocyte polarization towards a distinct pro-resolving phenotype. Discriminant analysis showed reduced separation with Function-Pain Responder (**Fig. S4A**) or Pain Responder (**Fig. S4B**) donor MSC(M) batches as indicated by greater misclassified data and reduced Entropy R^2 values (**Table S6**).

Specific microRNAs distinguish responder donor MSC(M) batches

Unbiased baseline differences in MSC(M) donor batches after synovial fluid stimulation were probed with microRNA-sequencing. PCA demonstrated clustering by donor batch and responder group rather than by synovial fluid stimulation (**Fig. 4A**). Thirty-three significant, differentially expressed microRNAs were identified between Responder vs. Non-Responder MSC(M) donor batches after correction for multiple comparisons; 11 microRNAs were upregulated, and 22 microRNAs were downregulated (**Fig. 4B**, **Table S7**). Gene targets related to cell cycle (GTPases), phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), transforming growth factor beta (TGF-β), VEGF, SMAD proteins, apoptosis, and other pathways were identified (**Fig. 4C,D**; **Suppl. File 2)**. Gene ontology analysis identified biological processes linked to neuronal processes, histone modification, cell growth, ossification, Wnt signaling (**Fig. 4E,F**; **Suppl. File 2**).

Predicted gene targets did not include genes within the curated mRNA panel. Limited correlations between the top 10 differentially expressed microRNAs with the curated gene panel were identified, but significance was lost after correction for multiple comparisons ($p<0.05$, FDR>0.1; hsa-miR-377-3p vs *ACTA2*; hsa-miR-1307-3p vs *THBS1*; hsa-miR-4775 vs *THBS1)* correlations (**Table S8**), suggesting putative indirect modulation of select MSC(M) genes by differentially expressed microRNAs that needs to be confirmed in larger datasets. Of the top 10 miRNA, miRNA-483-5p and miRNA-642a-5p positively and negatively correlated, respectively, with improvements in 12-and 24-month KOOS pain and function sub-scores or mean KOOS (Fig 4G; **Table S9**). The microRNA-sequencing analysis provides further characterization and potential mechanistic insights into MSC(M) therapeutic effects in KOA patients.

Baseline clinical phenotype correlates with 12- and 24-month and 8–10-year improvements

We conducted exploratory analysis between baseline inverted KOOS subscales with subsequent responder status (positive delta or percent change values indicate improvement; **Fig. 5**). Function Responders displayed significantly elevated mean KOOSKOOS Sports/Rec at baseline compared to Non-Responders, supportive of more severe baseline clinical presentations in directing MSC responsiveness (**Fig. 5A**). Other responder categories however, displayed no significant differences (**Fig. S5A,S5B**).

Next, we analyzed correlations between the baseline scores for all KOOS subscales with changes in these scores at 12- and 24-months (**Table S10**). Only baseline Sports/Rec scores positively correlated with improvements in KOOS ADL at 12- and 24-months; however, significance was lost after correction for multiple comparisons.

Given the findings on KOOS Sports/Rec, we probed the relationship with physical activity levels using the MARS scores [27]. Higher baseline MARS scores (i.e., more frequent physical activity) were significantly associated with lower (i.e., less severe) baseline KOOS Sports/Rec scores (**Fig. 5C**). Interestingly, higher MARS scores significantly inversely correlated with improvements in the 12-month KOOS Pain sub-score (**Fig. 5C**, **Table S11**).

Baseline MRI scores did not correlate with responder status

Baseline MRI WORMS and synovitis scores, previously calculated [8] did not correlate with Function Responder or Non-Responder classifications (**Table 1**) or to improvements in 12- and 24-month KOOS (**Tables S12, S13**). We had previously reported no significant improvements in MRI WORMS or synovitis scores, relative to baseline based on blinded analysis by two radiologists [8].

Baseline synovial fluid biomarkers do not correlate with 6, 12- and 24-month patient improvements

The levels of baseline inflammatory, angiogenic, metabolic and cartilage extracellular matrix-associated biomarkers in synovial fluid from a subset of nine patients were previously reported [8]; here we analyzed correlations with 6, 12- and 24-month KOOS improvements (**Tables S14, S15**). There were **negative** correlations for baseline HGF and VEGFA with greater improvements in KOOS Pain subscores at 12-months, but significance was lost after adjustment for multiple comparisons (**Table S14**) and no significant correlations between

other local biomarkers including TIMP1, resistin, adipsin, leptin, adiponectin, soluble (s)CD14, sCD163, MMP1, MMP9, IL6, IL8, CXCL1, CCL2, CX3CL1 with mean KOOS and KOOS pain or function sub-scores for all timepoints. [8].

Exploratory analysis of baseline synovial fluid immune cell phenotypes

As additional exploration of KOA patient molecular endotype, we analyzed synovial fluid immune cells using previous data [8]. While this analysis was feasible for a subset of five patients due to limited synovial fluid volumes, our data provides proof-of-concept for probing baseline levels of $CD14^{+}CD16^{+}$ and $CD14^{+}CD163^{+}$ monocyte/macrophages (MΦs) (**Fig. S6A**) as well as $CD3^{+}CD4^{+}CD69^{+}$ and $CD3^{+}CD4^{+}CD25^{+}$ T helper cells (**Fig. S6B**) as putative immunophenotypes of interest. Higher baseline frequencies of Mϕs and activated T cells are present in Non-Responders, although not significant given the limited sampling.

Baseline systemic biomarker levels do not correlate with patient improvements

Systemic baseline inflammatory biomarkers were measured in the present study (sCD163; CRP; S100A8/A9, TNFα; IL-6) alongside previous data on systemic biomarkers of cartilage breakdown [8] (**Table S16, S17**). Higher levels of baseline plasma TNFα significantly positively correlated to percentage changes in KOOS Pain subscores at 6 months (**Fig. S7**, **Table S17**), but this significance was lost with multiple comparison corrections. Thus, the selected panel of local and systemic biomarkers of different categories showed limited capacity to correlate with patient-reported outcomes.

DISCUSSION

Statistically powered clinical trials using MSCs have reported conflicting results in KOA patients [5,12]. To address underlying factors that drive heterogeneous responsiveness to MSCs, we analyzed our test clinical dataset [8] and identified putative MSC(M) donor batch characteristics and responsive KOA patient features that correlated with improvements in PROMs at 12- and 24-months. We anticipate that this combinatorial analysis will serve as an integrated algorithm that can be tested in prospective KOA clinical trials (and retrospectively on existing datasets) to i) screen potent donor/batches of MSCs; and ii) to identify characteristics of responsive KOA recipients. Our data suggests that MSC(M) donor batches with high basal immunomodulatory fitness as measured by reduced levels of angiogenic

genes, higher monocyte modulatory activity and a distinct signature of microRNAs will be more clinically effective. Our analysis of responsive KOA patients was more exploratory given the limited dataset. KOA patients with reduced knee functionality associated with sports and recreation activities and reduced physical activity levels were more responsive to MSC(M) injections. Biomarker analysis did not show significant correlations but there were trends pointing toward reduced levels of soluble angiogenic factors in the local joint environment at baseline that need to be further investigated in larger clinical datasets with matched placebo controls.

We used a previous clinical trial [8] with twelve manufactured, patient-specific lots of MSC(M), which was data-rich with multiple baseline biomarkers, and clinical readouts and offered an excellent test dataset to study MSC(M) batch variations. We provided longitudinal 12-, 24-month and 8–10-year follow-up and are one of the first groups to report such long-term clinical data. Responders showed significant improvements in PROMs at 12- and 24-months, and significantly sustained Responder (62.5%) and Non-Responder (75%) status to 8-10 years, including those lost to follow-ups. We note that 2/8 (25%) Responders (all categories) and 2/4 (50%) Non-Responders received additional injections over the intervening years; 3/8 Responders received or were awaiting a total knee replacement (TKRs). Additionally, 3/8 Responders (37.5%) and 1/4 Non-Responders (25%) did not receive additional interventions. The higher number of TKRs in the Responder group may be related to differences in sample size (N=8 Responders vs. N=4 Non-Responders). Given the extended lag in the follow-up period, it is not possible to confirm if the single MSC(M) injections delayed knee replacements, nor can we rule out the effects of additional interventions that may have contributed to the 8-10-year outcome measures.

Notably, our analysis suggesting that patients with severe KOA were more responsive to MSC(M) treatment aligns with findings in acute graft-versus-host disease [28], myocardial infarction [29] and ulcerative colitis [30], where more severe disease patients responded better to MSC treatments. Function Responders showed correlations to MSC(M) donor immunomodulatory potency and a distinct baseline clinical phenotype (especially KOOS Sports/Rec sub-scores). Although immunomodulatory therapies may be expected to induce greater analgesic effects [31], MSCs act through multimodal mechanisms with pain and functional improvements commonly documented [34]. KOA patients with higher disease severity, especially reduced levels of high-level knee functionality had greater improvements

in daily functioning of the knee (KOOS ADL). Patients with higher baseline MARS scores had lesser improvements, suggesting that patients who may have persisted through pain in physical activity may have exacerbated disease progression [31].

We focused on basal immunomodulatory properties of MSC(M) and their ability to modulate monocytes, given their importance as a clinically relevant mechanistic target in KOA [4,7,8,32]. A previously identified immunomodulatory gene panel for MSC(AT) [18] robustly distinguished MSC(M) donors classified under all categories of Responders. Dimension-reduced PC1 scores capturing multivariable genes were strongly and significantly correlated inversely to patient improvements at 12-, 24-monthsand are thus more reliable than the use of individual genes. Notably, this panel included predominantly angiogenic markers that were previously inversely correlated to monocyte polarization toward inflammation-resolving phenotypes [18], supportive of inverse relationships between angiogenic and immunomodulatory fitness of MSC(M) [33,34]. While the transcriptome of MSC(AT) and MSC(M) are distinct, the genes identified for MSC(AT) are valid for MSC(M) as well, speaking to their broad utility to serve as MSC CQAs [7]. These data support our hypothesis that MSCs with high basal immunomodulatory fitness produce greater improvements in knee function. Nonetheless, the use of autologous MSCs [7,8] adds a layer of complexity, given that systemic factors such as age, BMI, and other health conditions were dually accounted for in both basal fitness of the cells [35,36], as well as baseline patient disease status [37].

Responder MSC(M) donor batches modulated $CD14^+$ monocytes to more pro-resolving subtypes *in vitro*. The importance of monocyte/macrophage modulation in OA has previously been shown [8,38–40], given the role of synovitis in OA progression and pathology. Although MSC(M) co-culture resulted in preferential upregulation of pro-resolving $CD14^+$ monocyte markers, there was also upregulation of pro-inflammatory markers. Reliance on dichotomized gene expression markers is not indicative of the plurality of monocyte functionality [41]; rather induction of mixed monocyte phenotypes by MSCs is important for achieving therapeutic effects [42]. Importantly, we have shown that $CD14^+$ monocytes in OA synovial fluid demonstrate a mixed phenotype, which is not reflective of functionality [43].

Function Responder MSC(M) donor batches had a significantly different microRNA signature compared to Non-Responders. Pathways related to inflammation, fibrosis, angiogenesis, and ossification, relevant to KOA pathophysiology were identified. We also identified enrichment of pathways involved in cell cycle. Surprisingly, our analysis identified

enrichment of several neuronal processes; however, given the role of innervation and neuronal signaling in joint pain in KOA [44], these microRNAs may be implicated in mediating analgesic effects of MSC(M). The functionality of differentially expressed microRNAs requires further study, but they may also serve as additional CQAs for identifying basally fit MSC(M).

Using a curated panel of inflammatory, angiogenic, and metabolic proteins and proteases, we could not discern a signature of local biomarkers that definitively identified KOA patients that were responsive to MSC(M). Responders displayed putatively lower local baseline levels of angiogenic biomarkers, HGF and VEGFA, but this lost significance after multiple comparisons correction. Systemically, there was limited short-term positive correlations between TNFα (plasma) levels and PROM improvements, albeit significance was also lost after multiple comparisons correction. Baseline serum CRP levels, an inflammatory biomarker in multiple inflammatory conditions [45] and cartilage breakdown markers (shown to correlate with worsening KOA pain [46]) failed to correlate with improvements in KOOS at all time points. Overall, our analyses failed to show significant correlations to MSC(M) treatment responsiveness with any of the biomarkers tested. However, a trend towards a mixed profile with increased pro-inflammatory systemic TNFα but reduced local angiogenic proteins (HGF and VEGF) emerged, reminiscent of mixed biomarker profile in rheumatoid arthritis patients, responsive to allogenic MSCs [47]. This mixed local vs. systemic molecular endotype profile needs to be further tested in larger datasets.

Identifying molecular endotypes of OA has proven to be extremely challenging, and multi-consortium efforts have not been able to definitively identify molecular endotypes when profiling local synovial fluid, even with large, covariate-adjusted datasets [48]. This heterogeneity in OA patients is a key reason for failure of multiple drugs in late-phase, powered clinical trials [49,50]. The absence of stratification biomarkers to identify OA theratypes for which specific treatments can be tailored to specific clinical phenotypes and endotypes has been identified as a critical gap impeding the development of disease modifying drugs in the field [51]. Although systemic biomarkers of inflammation and/or cartilage degradation are beginning to emerge to cluster patients [52], their utility in defining patient responsiveness to different therapies remains untested. In our hands, many of the previously reported inflammatory and cartilage degradation biomarkers [8,52] did not correlate with improvements in patient-reported outcomes to MSC(M) treatments.

Limitations of this study include small sample size, which allowed us to perform an exploratory but not definitive analysis. Indeed, baseline clinical phenotype and local biomarkers indicative of reduced angiogenic and inflammatory molecular endotype lost significance when corrected for multiple comparisons, likely due to overcorrection arising from the small sample size. Nonetheless, the multivariate framework of investigating potency features of MSC investigational products combined with clinical and biomarker data to analyze patient phenotypes and endotypes is a useful method to test and stratify OA patients into theratypes, suited for MSC therapies [51]. The absence of a control group in the original clinical trial is another limitation [8], although effects were measured at multiple timepoints including 12-, 24-months and 8-10 years. KOA is a progressive condition [53], with approximately 30% of patients displaying worsening outcomes over two years leading up to joint surgery [54]. While placebo effects cannot be ruled out, less than 30% of patients reported worsening outcomes, potentially speaking to a treatment effect. Indeed, analysis of Non-Responders using the rigorous OMERACT-OARSI criteria [25] showed that even patients within this category did not show any significant worsening in PROMs over 24-month and 8-10 years of follow-up.

CONCLUSIONS

Donor batches of MSC(M) that resulted in positive clinical outcomes differed significantly in specific measurable attributes compared to MSC(M) batches that failed to improve pain or function. These quantitative, multivariate, and functionally relevant attributes can define future CQAs for MSC(M) that can be evaluated to strategically design KOA trials to ensure higher success rates of MSC injection. Additionally, we identified exploratory baseline clinical features and biomarkers in KOA patients responsive to MSC(M) injections that need to be further tested in larger clinical datasets to verify their utility.

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Disclosure of Potential Conflicts of Interest

KR declares current employment status with STEMCELL Technologies. SV declares 60% ownership of Regulatory Cell Therapy Consultants Inc., which does not conflict with this paper in any way. SV, RR and KR are co-inventors on patent applications filed with UHN based on this work. The remaining authors declare they have no competing interests.

Author contributions:

Kevin P. Robb: Conception & design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript
Razieh Rabani: Conception & design, collection and/or assembly of data, data analysis and interpretation
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Shoba Singh: administrative support, collection and/or assembly of data
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Osvaldo Espin-Garia: data analysis
Rajiv Gandhi: financial support, provision of study materials or patients
Sowmya Viswanathan: Conception & design, manuscript writing, final approval of manuscript

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Table 1. Summary of baseline patient characteristics. Association between each covariate was evaluated against responder status (p-values adjusted for multiple comparisons); no significant associations were detected. BMI: body mass index; KL: Kellgren-Lawrence; WORMS: Whole-Organ Magnetic Resonance Imaging Score [23].

Covariate	Full Sample (n=12 total)	F Responder (n=7 total)	Non-Responder (n=5 total)	Adjusted p-value
Cell dose				0.495
1x10^6 [n, (%)]	4 (33)	2 (29)	2 (40)	
10 x10^6 [n, (%)]	4 (33)	1 (14)	3 (60)	
50 x10^6 [n, (%)]	4 (33)	4 (57)	0 (0)	
Gender				0.513
Man x10^6 [n, (%)]	7 (58)	3 (43)	4 (80)	
Woman x10^6 [n, (%)]	5 (42)	4 (57)	1 (20)	
Age				0.601
Mean (SD)	54.8 (6.9)	55.7 (5.6)	53.4 (8.8)	
Median (Min,Max)	53.5 (45.0,65.0)	54 (47,63)	51 (45,65)	
BMI				0.494
Mean (SD)	25.8 (3.5)	24.5 (3.2)	27.5 (3.4)	
Median (Min,Max)	25.5 (19.0,29.9)	24.9 (19.0,29.7)	29.7 (22.1,29.9)	
KL Grade				0.583
3	11 (92)	7 (100)	4 (80)	
4	1 (8)	0 (0)	1 (20)	
MRI Total Synovitis Score [24]				0.494
Mean (sd)	6.8 (2.9)	5.7 (2.1)	9.3 (3.1)	
Median (Min,Max)	6 (3,12)	5 (3,9)	10 (6,12)	
Missing	2	0	2	
MRI WORMS				0.756
Mean (SD)	37.4 (9.3)	36.4 (8.3)	38.8 (11.3)	
Median (Min,Max)	36.2 (20.0,54.0)	36.0 (20.0,45.5)	36.5 (28.0,54.0)	

Figure Legends

Fig. 1. Osteoarthritis patient responders demonstrate improvements in KOOS subscales that are maintained out to 24 months follow-up post-injection. A) Patients categorized as Function Responders displayed significant improvements (indicated by lower score values) in KOOS subscales and mean KOOS at 12- and 24-months follow-up relative to baseline, while Non-Responders displayed no significant changes. KOOS scales were inverted such that higher scores represent greater knee-related issues. B) Flow chart summary of patient responder status at 12-months, 24-months, and 8-10-years follow-up. C) Summary of OARSI-OMERACT Responder classification in 2025 after 8-10 years of follow-up. Responder status is indicated as Function-Pain (F-P), Function (F), or Pain (P) Responders. Changes in patient responder status at 8-10 years are bolded. Patient-reported follow-up notes on additional interventions are included in the right column. N=12 patients. Two-way repeated measures ANOVA, Dunnett's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; asterisks indicate significant differences for responder group relative to baseline at the time points indicated. ADL: function in daily living; Sports/Rec: function in sport and recreation; QOL: knee-related quality of life; KOOS: knee injury and osteoarthritis outcome score; PRP: platelet-rich plasma; TKR: total knee replacement.

Fig. 2. Independent validation that a previously identified gene panel distinguishes clinically effective MSC(M) donors from non-responder MSC(M) donors. A) Principal component (PC) analysis of MSC(M) expression of an immunomodulatory gene panel identified previously for MSC(AT) (*TNFAIP6*, *ACTA2*, *ANGPT1*, *CXCL8*, *EDN1*, *THBS1*, *PDCD1LG2*, *CCN2*; [18]) expressed under licensed conditions. The corresponding loading plot of eigenvectors (right) indicates the relative contribution of each gene to the PC1 and PC2 axes. B) MSC(M) derived from Function Responders displayed significantly lower PC1 scores relative to non-responders, indicating that the gene panel sensitively distinguishes MSC(M) from donors classified as responders. Student's t-test, ***p<0.001, ****p<0.0001. Horizontal lines: group mean; error bars: standard deviation. C) Significant negative correlations between PC1 scores and the percentage change at 24-months relative to baseline for KOOS ADL, pain, symptoms, and mean KOOS. Percent change in KOOS values were made relative to baseline and calculated such that positive values represent improvement. Spearman's correlation. N=9 MSC(M) donors; n=2-3 replicates/donor.

Fig. 3. *In vitro* monocyte/macrophage polarization readouts distinguish donor MSC(M) immunomodulatory fitness for responders versus non-responders. A) Schematic of *in vitro* monocyte/macrophage (MΦ) polarization experiment. Indirect co-cultures of MSC(M) with human peripheral blood-derived MΦ was performed for 48 h prior to removal of MSC(M) and addition of lipopolysaccharide (LPS) for 4 h incubation. B) Summary of MΦ gene expression after co-culture with Function Responders MSC(M). Box-and-whisker plots (left) display changes in gene expression relative to MΦ cultured alone (Solo; dotted line). Horizontal line: median; hinges: first and third quartiles; whiskers: range. Two-way ANOVA, Tukey's post-hoc test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 relative to Solo condition or to groups indicated by brackets. Discriminant canonical plots (right) of -ddCt values relative to Solo condition. Inner ellipses indicate 95% confidence for the mean of each group;

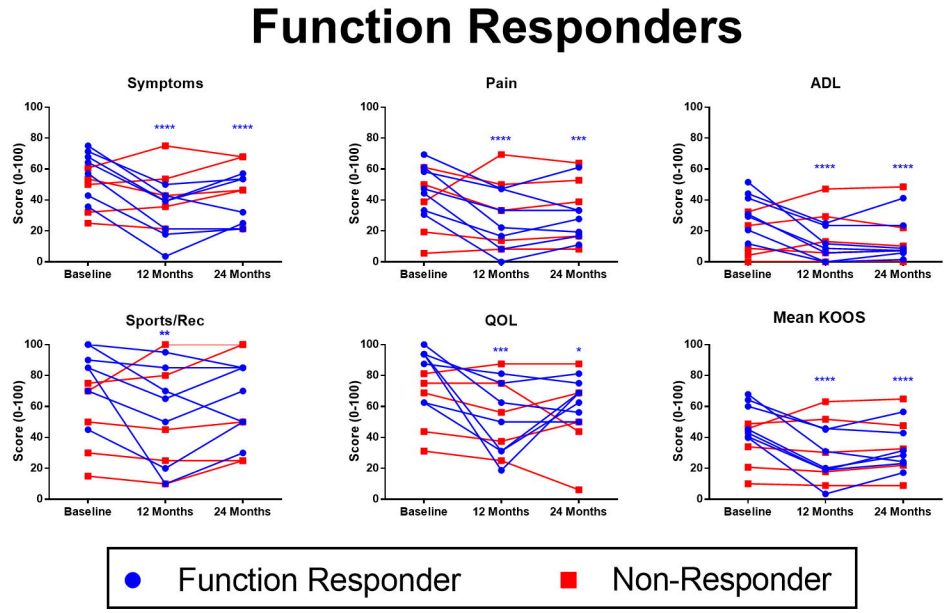
outer ellipses indicate the normal region estimated to contain 50% of the population for each group.

Fig. 4. microRNA-sequencing reveals microRNA signatures in Function Responder donor MSC(M). A) Principal component analysis of microRNA expression profiles demonstrate clustering in Function Responders versus Non-Responders. MSC(M) samples cluster by donor and responder status rather than by synovial fluid (SF) stimulation. B) Volcano plot displays 33 significantly differentially expressed microRNAs in MSC(M) derived from donors classified as Function Responders versus Non-Responders. C&D) Top 30 pathways identified through Reactome analysis of predicted gene targets for the top ten significantly upregulated (C) and downregulated (D) microRNAs. E&F) Top 30 pathways identified through Gene Ontology (Biological Processes) analysis of predicted gene targets for the top ten significantly upregulated (E) and downregulated (F) microRNAs. G) Significant correlations between microRNAs and the delta or percentage change at 12- and 24-months relative to baseline for KOOS ADL, pain, and mean KOOS. Delta and percent change in KOOS values were made relative to baseline and calculated such that positive values represent improvement. Spearman's correlation. N=9 MSC(M) donors.

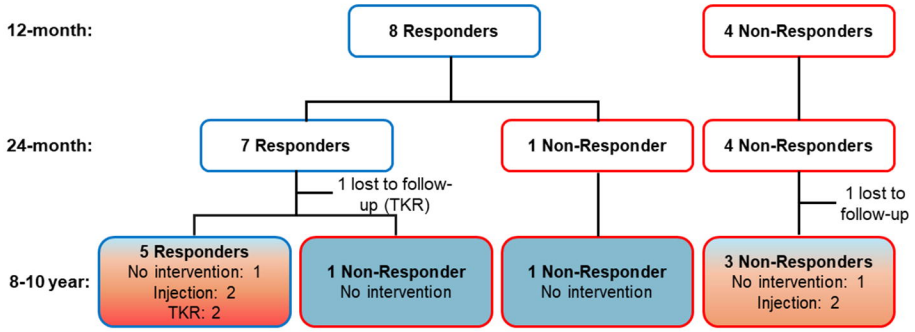
Fig. 5. More severe KOOS and lower MARS physical activity scores correlate to improved patient-reported outcomes at 12- and 24-months follow-up. A) Patients categorized according to Function Responders displayed significantly more severe mean KOOS and Sports/Rec scores at baseline relative to Non-Responders. B&C) Baseline MARS significantly correlated to baseline Sports/Rec, as well as both delta and percent change in KOOS Pain at 12 months relative to baseline. KOOS scales were inverted such that higher scores represent greater knee-related issues; delta and percent change in KOOS values were made relative to baseline and calculated such that positive values represent improvement. N=12 patients. Student's t-test (for baseline Mean KOOS), Two-way ANOVA, Sidak's post-hoc test (for baseline KOOS Subscales), Spearman's correlation. *$p<0.05$, **$p<0.01$. n.s.; non-significant. ADL: function in daily living; Sports/Rec: function in sport and recreation; QOL: knee-related quality of life; Pt.: patient ID; MO: month; KOOS: knee injury and osteoarthritis outcome score; MARS: Marx Activity Rating Scale.

FIG. 1

A



B



C

					8-10 year follow up scores				
					KOOS Pain		KOOS ADL		
Subject ID	Responder Class at 12 Months	Responder Class at 24 Months	Responder Class at 8-10 years	# Years Follow-up in 2025	Delta	% Change	Delta	% Change	Follow-up notes
1	Non-Responder	Non-Responder	Non-Responder	9 years, 10 months	-0.04	-0.21	-1.49	-16.93	Injection of viscosupplementation in 2024 and 2025
2	Non-Responder	Non-Responder	N/A	-	-	-	-	-	N/A
3	Responder (F-P)	Responder (F)	Responder (F-P)	9 years, 6 months	30.52	91.65	27.96	90.49	PRP injection in 2023
4	Non-Responder	Non-Responder	Non-Responder	9 years, 4 months	-44.40	-792.86	-30.88	-3088.00	No surgical intervention
5	Responder (F-P)	Responder (P)	N/A	-	-	-	-	-	TKR in 2025
6	Responder (P)	Pain Responder	Responder (P)	9 years, 0 months	11.11	22.22	-22.07	-501.59	Awaiting bilateral knee replacement
7	Responder (F-P)	Responder (F-P)	**Non-Responder**	8 years, 11 months	0.04	0.13	-1.46	-7.09	No intervention post injection
9	Responder (F-P)	Non-Responder	**Non-Responder**	8 years, 6 months	-2.82	-4.06	-19.14	-43.40	No intervention post injection
10	Responder (F)	Responder (F-P)	Responder (F-P)	8 years, 6 months	19.41	33.29	20.61	50.02	Viscosupplementation - no majory surgery
11	Responder (F-P)	Responder (F-P)	Responder (F-P)	8 years, 6 months	24.96	56.22	16.16	54.97	No knee surgery/scope, no knee injection
12	Non-Responder	Non-Responder	Non-Responder	8 years, 4 months	-30.54	-78.51	-24.95	-77.01	Viscosuppementation in 2019
13	Responder (F-P)	Responder (F-P)	Responder (F-P)	8 years, 2 months	49.99	81.82	39.74	77.17	TKR in 2025

FIG. 2

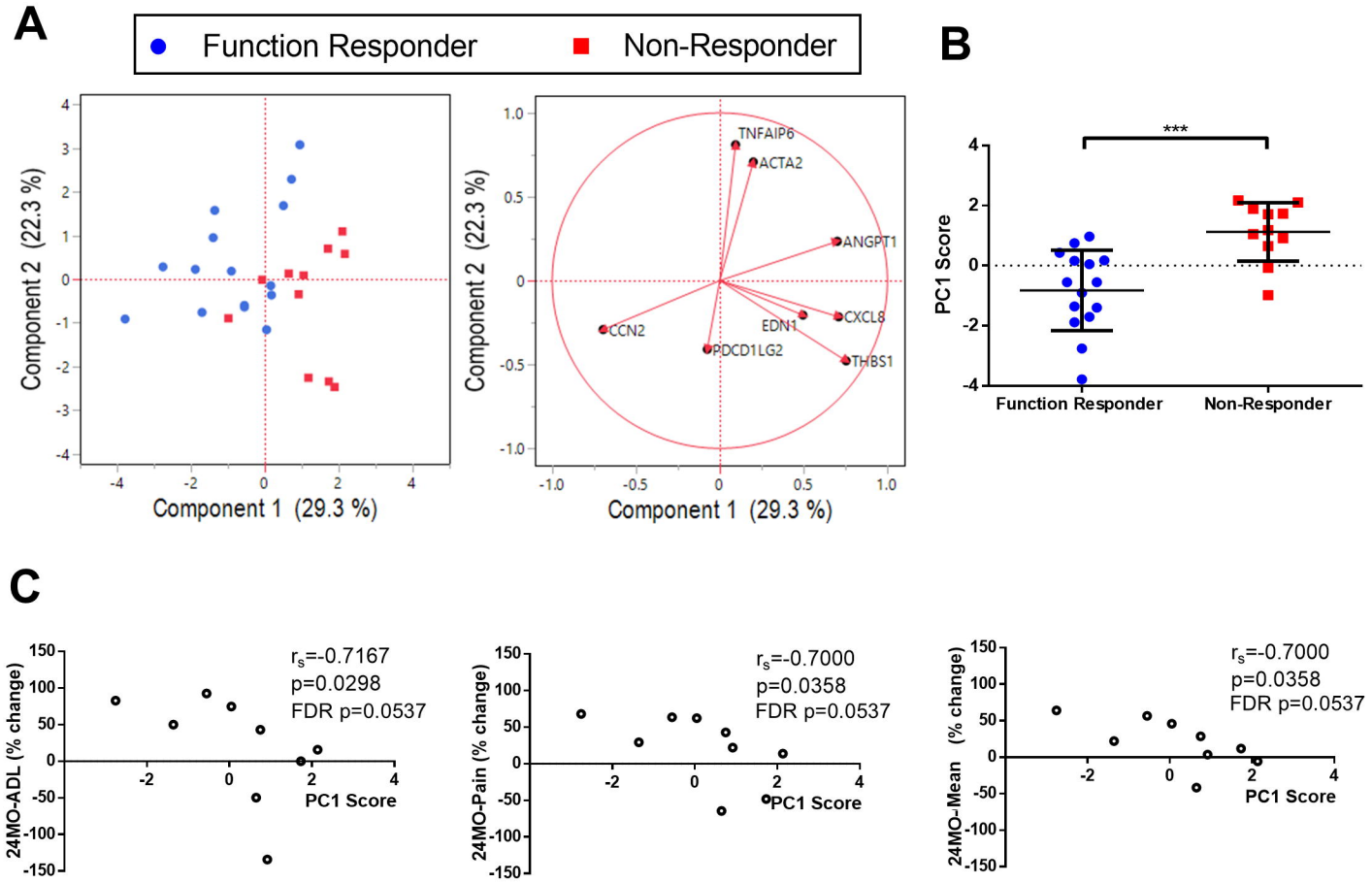
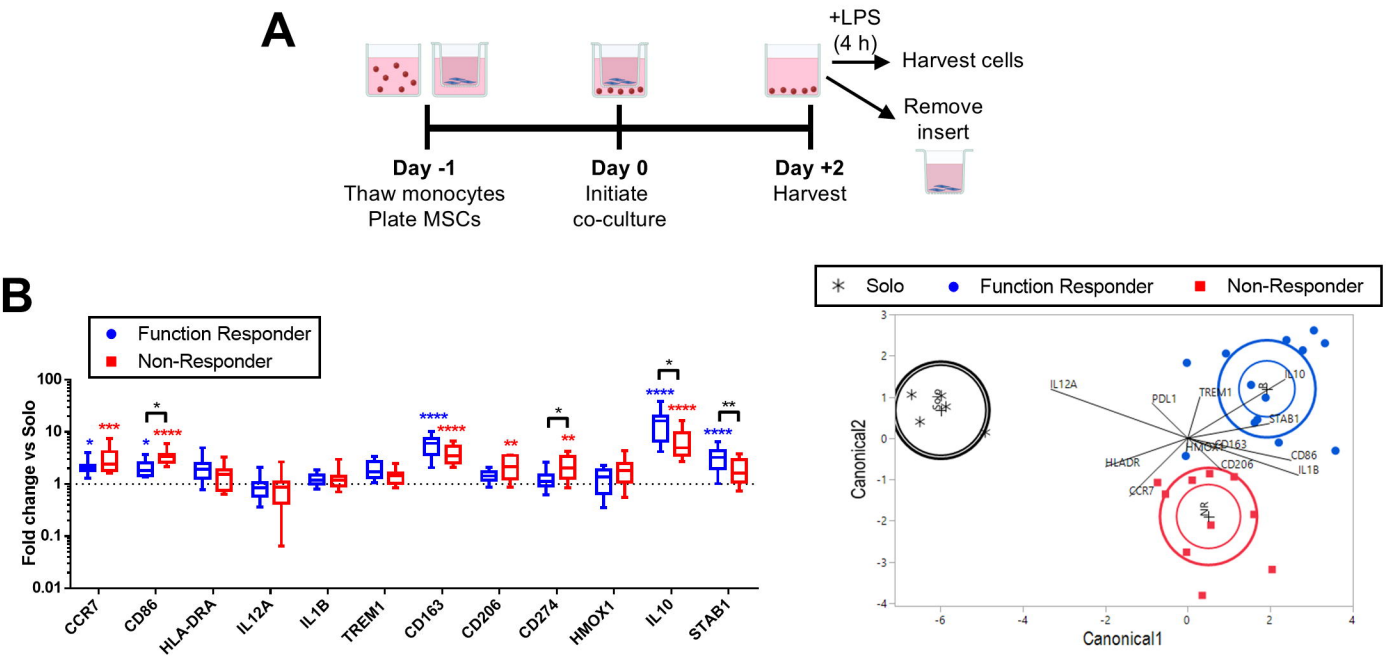

A
Function Responder
Non-Responder
Component 2 (22.3 %)
Component 1 (29.3 %)
TNFAIP6
ACTA2
ANGPT1
CXCL8
EDN1
CCN2
PDCD1LG2
THBS1
B
***
PC1 Score
Function Responder
Non-Responder
C
r_s=-0.7167
p=0.0298
FDR p=0.0537
24MO-ADL (% change)
PC1 Score
r_s=-0.7000
p=0.0358
FDR p=0.0537
24MO-Pain (% change)
PC1 Score
r_s=-0.7000
p=0.0358
FDR p=0.0537
24MO-Mean (% change)
PC1 Score

FIG. 3


A
+LPS
(4 h)
Harvest cells
Remove
insert
Day -1
Thaw monocytes
Plate MSCs
Day 0
Initiate
co-culture
Day +2
Harvest
B
Function Responder
Non-Responder
Fold change vs Solo
100
10
1
0.1
0.01
CCR7
CD86
HLA-DRA
IL12A
IL1B
TREM1
CD163
CD206
CD274
HMOX1
IL10
STAB1
Solo
Function Responder
Non-Responder
Canonical2
Canonical1
IL12A
PDL1
TREM1
IL10
STAB1
HLADR
CD163
CD206
CD86
IL1B
CCR7

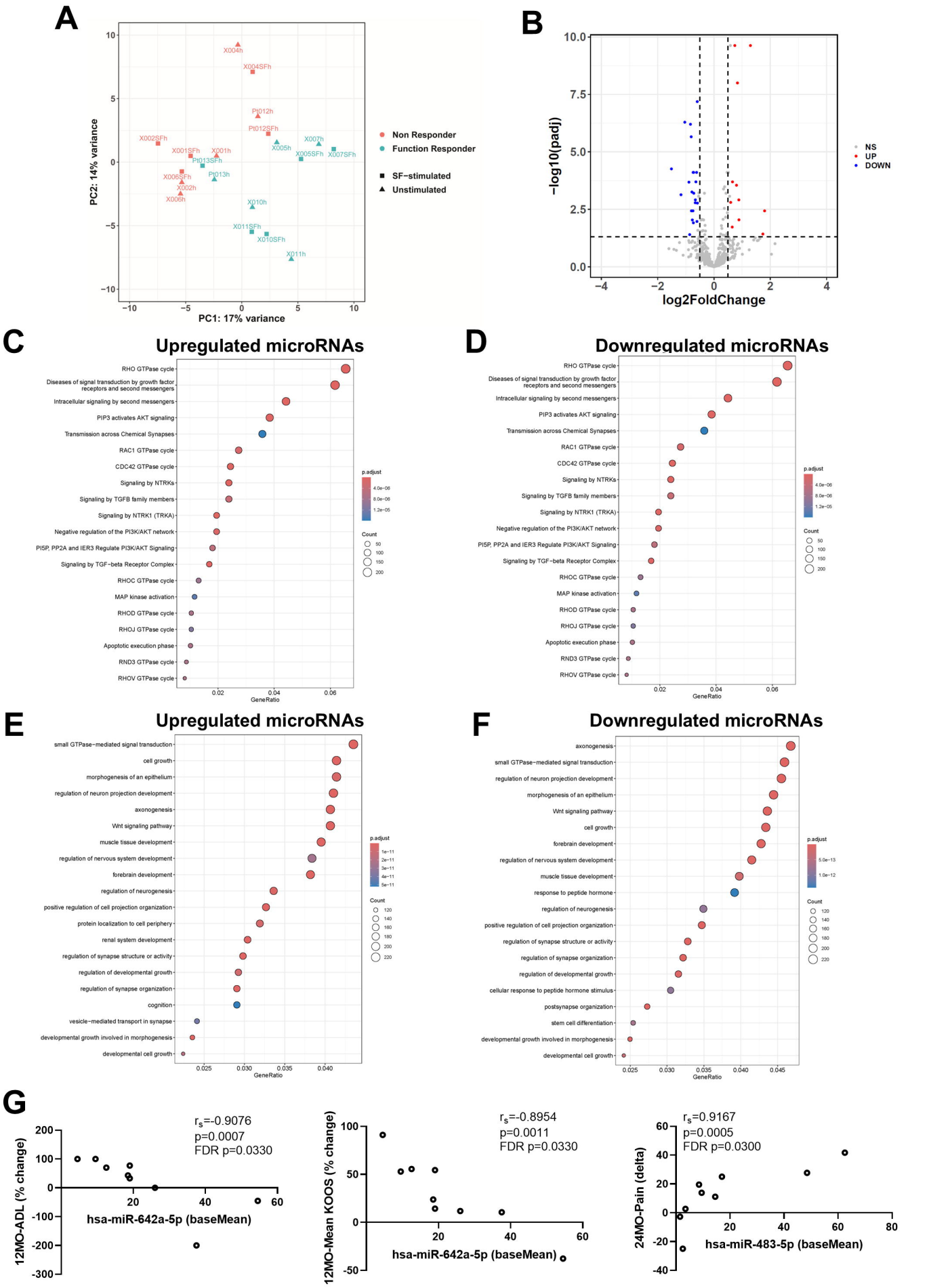
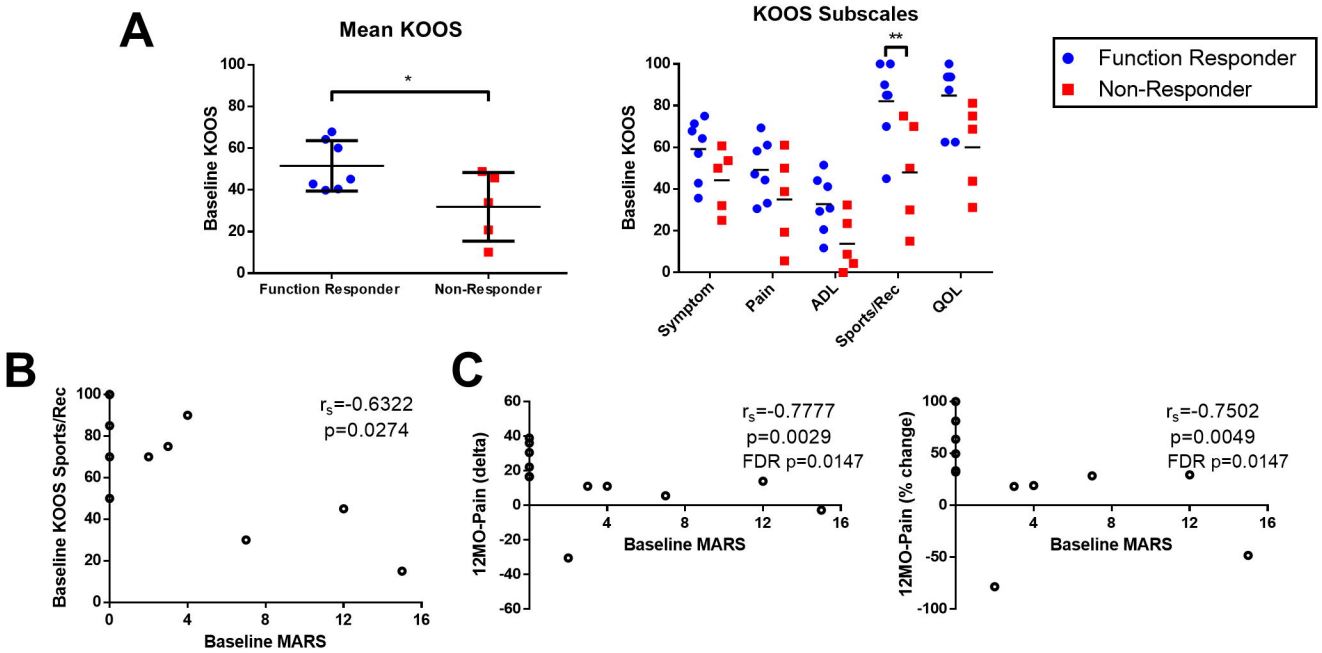
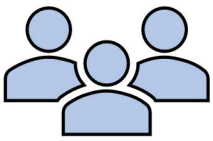

A
PC2: 14% variance
PC1: 17% variance
Non Responder
Function Responder
SF−stimulated
Unstimulated
B
−log10(padj)
log2FoldChange
NS
UP
DOWN
C
Upregulated microRNAs
RHO GTPase cycle
Diseases of signal transduction by growth factor receptors and second messengers
Intracellular signaling by second messengers
PIP3 activates AKT signaling
Transmission across Chemical Synapses
RAC1 GTPase cycle
CDC42 GTPase cycle
Signaling by NTRKs
Signaling by TGFB family members
Signaling by NTRK1 (TRKA)
Negative regulation of the PI3K/AKT network
PI5P, PP2A and IER3 Regulate PI3K/AKT Signaling
Signaling by TGF−beta Receptor Complex
RHOC GTPase cycle
MAP kinase activation
RHOD GTPase cycle
RHOJ GTPase cycle
Apoptotic execution phase
RND3 GTPase cycle
RHOV GTPase cycle
GeneRatio
p.adjust
Count
D
Downregulated microRNAs
E
Upregulated microRNAs
small GTPase−mediated signal transduction
cell growth
morphogenesis of an epithelium
regulation of neuron projection development
axonogenesis
Wnt signaling pathway
muscle tissue development
regulation of nervous system development
forebrain development
regulation of neurogenesis
positive regulation of cell projection organization
protein localization to cell periphery
renal system development
regulation of synapse structure or activity
regulation of developmental growth
regulation of synapse organization
cognition
vesicle−mediated transport in synapse
developmental growth involved in morphogenesis
developmental cell growth
F
Downregulated microRNAs
response to peptide hormone
cellular response to peptide hormone stimulus
postsynapse organization
stem cell differentiation
G
12MO-ADL (% change)
hsa-miR-642a-5p (baseMean)
r_s=-0.9076
p=0.0007
FDR p=0.0330
12MO-Mean KOOS (% change)
hsa-miR-642a-5p (baseMean)
r_s=-0.8954
p=0.0011
FDR p=0.0330
24MO-Pain (delta)
hsa-miR-483-5p (baseMean)
r_s=0.9167
p=0.0005
FDR p=0.0300

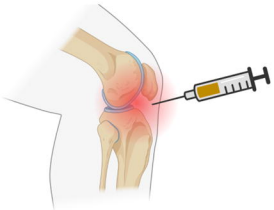
FIG. 5


A
Mean KOOS
Baseline KOOS
Function Responder
Non-Responder
KOOS Subscales
Symptom
Pain
ADL
Sports/Rec
QOL
Function Responder
Non-Responder
B
Baseline KOOS Sports/Rec
Baseline MARS
r_s=-0.6322
p=0.0274
C
12MO-Pain (delta)
Baseline MARS
r_s=-0.7777
p=0.0029
FDR p=0.0147
12MO-Pain (% change)
Baseline MARS
r_s=-0.7502
p=0.0049
FDR p=0.0147

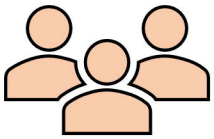
What determines osteoarthritis patient responses to autologous MSC(M) injection?

Responders





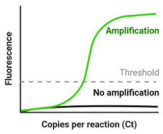
Non-Responders

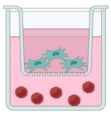


MSC(M) Basal Fitness

Targeted gene expression panel

In vitro MΦ polarization



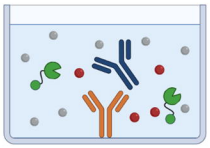


microRNA-Sequencing

Baseline clinical phenotype

Knee Injury & Osteoarthritis Outcome Scores (KOOS)
Marx Physical Activity Scores

Baseline molecular biomarkers



Local biomarkers
Synovial Fluid

Systemic biomarkers
Plasma, Serum, Urine

Responders versus Non-Responders to MSC(M) therapy

Increased MSC(M) Immunomodulatory Basal Fitness

Immunomodulatory CQA transcript expression

Distinct MΦ Polarization

Expression of distinct miR profile

More severe KOOS Sports/Rec

Lower levels of physical activity

Local biomarkers

Lower baseline angiogenic markers (non-significant)

Systemic biomarkers

Non-significant