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Research Paper

A Clinical Indications Prediction Scale Based on *TWIST1* for Human Mesenchymal Stem Cells

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ABSTRACT

In addition to their stem/progenitor properties, mesenchymal stem cells (MSCs) also exhibit potent effector (angiogenic, antiinflammatory, immuno-modulatory) functions that are largely paracrine in nature. It is widely believed that effector functions underlie most of the therapeutic potential of MSCs and are independent of their stem/progenitor properties. Here we demonstrate that stem/progenitor and effector functions are coordinately regulated at the cellular level by the transcription factor Twist1 and specified within populations according to a hierarchical model. We further show that manipulation of Twist1 levels by genetic approaches or by exposure to widely used culture supplements including fibroblast growth factor 2 (Ffg2) and interferon gamma (IFN-gamma) alters MSC efficacy in cell-based and in vivo assays in a predictable manner. Thus, by mechanistically linking stem/progenitor and effector functions our studies provide a unifying framework in the form of an MSC hierarchy that models the functional complexity of populations. Using this framework, we developed a CLinical Indications Prediction (CLIP) scale that predicts how donor-to-donor heterogeneity and culture conditions impact the therapeutic efficacy of MSC populations for different disease indications.

1. Introduction

Mesenchymal stem cell (MSC)-based therapies are currently being evaluated in more than 500 clinical trials (https://clinicaltrails.gov) for the treatment of various skeletal and non-skeletal related disorders. Although some patients have benefited significantly by these treatments, many MSC-based clinical trials have yielded suboptimal outcomes or failed to meet their primary endpoints of efficacy (Phinney et al., 2013; Lin and Hogan, 2011). A limiting factor in the development of efficacious MSC-based therapies is the fact that MSCs are ill-defined based on physical, phenotypic and functional properties. For example, routinely used methods to isolate MSCs from bone marrow yield populations that exhibit significant heterogeneity in terms of morphologic features, growth rate, life span, differentiation potential, and potency in functional-based assays (Phinney, 2007; Wagner and Ho, 2007; Zhou et al., 2008; Russell et al., 2010; Whitfield et al., 2013). Additionally, few if any established surface markers used to characterize MSCs relate phenotype to function thereby making it difficult to determine whether cell preparations used in translational/clinical studies are of similar composition and biological activity. Finally, the prevailing viewpoint that MSCs modulate the immune system and promote tissue regeneration independent of their stem/progenitor properties (Caplan and Correa, 2011; Bernardo and Fibbe, 2013) has impeded efforts to describe their functional complexity using established principles of stem cell biology.

Here we explored the mechanisms that confer different functional attributes onto MSCs to gain insight at the cellular and molecular level as to how they achieve such broad-based therapeutic effects. Our results establish the transcription factor Twist1 as an intrinsic determinant of intra- and inter-population heterogeneity by regulating multiple pathways affecting cell growth, cell cycle progression, DNA replication, cell surface receptor signaling and survival. Furthermore, we show that Twist1 is a direct target of fibroblast growth factor (Fgf2) and interferon gamma (IFN-gamma) action in MSCs, and that alterations in Twist1 levels mediated by exposure to Fgf2, IFN-gamma or via direct genetic manipulation alters the growth, survival and multi-potency as well as angiogenic, anti-inflammatory and immuno-modulatory activities of cells in predictable ways. These findings demonstrate a direct mechanistic link between stem/progenitor and effector functions in MSCs, and reveal that Twist1-mediated changes in cell function/efficacy can be modeled via a hierarchical process. Using this information we developed a CLinical Indications Prediction (CLIP) scale that predicts the therapeutic efficacy of different human MSC donor populations for a given disease indication based on *TWIST1* expression levels, which specifies the positional identity of cells within the established hierarchy.

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2. Materials and Methods

2.1. Cell Culture

Human MSCs were obtained from bone marrow as previously described (Russell et al., 2010) and seeded at 500–1000 cells/cm^2 in complete culture media (CCM) consisting of α-MEM with 2 mM L-glutamine (GIBCO), 17% FBS (HyClone), 100 U/ml penicillin and 100 μg/ml streptomycin. Colony forming unit-fibroblast (CFU-F) content and multi-lineage differentiation was determined as previously described (Russell et al., 2010). Cell growth was analyzed using the MTT Cell Proliferation Assay Kit (Cayman Chemical Company) or by flow cytometric analysis of carboxyfluorescein succinimidyl ester (CSFE)-labeled cells. Cells were initially seeded at 1000 cells/cm^2 for MTT and CSFE assays and analyses were conducted when cells reached ~50% confluence to ensure log phase growth. MSCs were transfected with small interfering RNAs (siRNAs) using the inverse transfection protocol (Invitrogen) and collected at 3 days post-transfection for viability measures, cell cycle analysis and to harvest RNA and protein, at 7 days post-transfection for cell proliferation assays, and at 14 days post-transfection for CFU-F assays. Stained cells were analyzed using an LSR II Flow Cytometer System (Beckton Dickinson) and FlowJo software (Tree Star, Inc.).

2.2. Microarray Analysis

An equal amount of RNA was pooled from triplicate experiments and cDNA prepared following the manufacturer's protocols from the WT expression kit (4411973, Affymetrix). Biotin-labeled cDNA was fragmented and hybridized to the Human Gene ST 1.0 microarray (901086, Affymetrix) and data analysis was performed using Expression Console analysis software (Affymetrix). These data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE76158 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76158). Functional profiling of microarray data was performed using g:Profiler based on hierarchical sorting using the best parent fit (Reimand et al., 2011). Unbiased gene annotation enrichment analysis for the top five functional clusters of Twist1 targets was performed by DAVID analysis using automated functional annotation clustering based on GO Terms of biological processes (GO_BP_FAT). Gene lists were annotated using the gene accession conversion tool to ensure accuracy. The individual genes of these five categories were then curated for enhanced accuracy by systematic search of the GeneCards Compendium (http://www.genecards.org), which validated almost all of the initial genes.

2.3. Mixed Lymphocyte Reactions

Freshly isolated human peripheral blood mono-nuclear cells (PBMNCs) obtained from OneBlood Florida (http://www.oneblood.org) were suspended in RPMI-10 media supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologics), 1× MEM Vitamin solution, 1× MEM NEAA solution, 1× L-Glut, 0.01 M HEPES, 100 U/ml penicillin, 100 U/ml streptomycin, 1 mM Sodium pyruvate (all from Gibco), and 10 ng/ml IL-2 (Biolegend) and co-cultured with pre-plated MSCs at a MSC:PBMNC ratio of 1:20. Dynabeads® Human T-Activator CD3/CD28 (Gibco) were added to the co-culture at a bead/PBMNC ratio of 2:1. Cultures were then incubated at 37 °C in 5% CO_2 for 5 days after which the non-adherent PBMNCs were collected and stained using the FITC Mouse Anti-Human Ki-67 Set (556026, BD Pharmingen) according to manufacturer's instructions. Alternatively, Th1/Th2/Th17 cytokines were measured in the conditioned media collected 5 days post-co-culture using the Human Th1/Th2/Th17 CBA Kit (560485, BD Biosciences) according to manufacturer's instructions. Briefly, 50 μl of cytokine standards and samples were mixed with 50 μl of mixed capture beads and phycoerthyrin (PE) detection reagent and incubated for 3 h. Beads were washed, suspended in 300 μl wash buffer and analyzed by flow cytometry. Median intensity of PE fluorescence was used to derive concentration vs. fluorescence standard curves to determine the concentration of each cytokine.

2.4. Chip-PCR

MSCs were cultured in media containing 1% formaldehyde to cross-link protein and DNA and the reaction quenched by adding 1.375 M glycine to the culture media. The cell monolayer was harvested by scraping in 1× cold PBS, centrifuged at 1500 rpm for 10 min at 4 °C, incubated in cell lysis buffer and then the DNA sheared by sonication. Twist1 bound chromatin was precipitated using an anti-Twist1 antibody (sc-15393, Santa Cruz Biotechnology) and the Dynabeads® Protein G Immunoprecipitation kit (Life Technologies). Eluted DNA was purified using spin columns (Qiagen) and then used as input in real-time PCR reactions using the primers listed in Table S5.

2.5. TWIST1 Cloning

Genomic DNA was purified from human MCS using the Quick-gDNA MiniPrep Kit (Zymo Research) according to manufacturer's directions. *TWIST1* was amplified by polymerase chain reaction using the KOD HotStart DNA Polymerase (EMD Millipore) with the forward and reverse primers 5′-GCCTGCACGGAGGTATAAG-3′ and 5′-GAGGAAATCG AGGTGGACTG-3′, respectively. Following amplification, the product was blunt ligated into the EcoRV site of pBlueScript II SK+ using T4 DNA Ligase (New England Biolabs). Following sequence and orientation verification, pBlueScript-*TWIST1* was digested with XhoI and EcoRI and the product ligated into the XhoI and EcoRI site of pMSCV-PIG (Addgene) to generate a *TWIST1* retroviral construct (pMSCV-PIG-Twist1). The latter was co-transfected into HEK-293 cells with pCL-Ampho using Lipofectamine 2000 (Life Technologies) according to manufacturer's directions. At 72 h post-transfection supernatant was collected, passed through a .45 μm syringe filter, and 4 ml of filtered supernatant was combined with 1 ml of fresh MSC media and 4 μg/ml polybrene and added to MSCs plated at a density of 1000 cells/cm^2 in a 60 mm dish. MSCs were subsequently sorted for GFP expression by FACS. A pMSCV-PIG vector-only control was generated using the same method.

2.6. In Vivo Treatments

All animal studies conform to the *Guide for the Care and Use of Laboratory Animals* (The Nationals Academic Press, Eight Edition) and protocols approved by the Institutional Care and Use Committee of Scripps Florida. Institutional review board (IRB) approval was obtained for use of human samples. Bleomycin-induced lung injury was performed as previously described (Ortiz et al., 2003; Ortiz et al., 2007) except experiments were conducted using immune-deficient mice (B6.CB17-Prkdcscid/SzJ, The Jackson Labs) and animals were administered 5×10^5 human MSCs. Where indicated human MSCs were cultured in Fgf2 (20 ng/ml) supplemented media for 7 days or transfected with a *TWIST1*-specific siRNA.

2.7. Statistical Analyses

Significance was estimated by unpaired Student's *t* test in experiments where comparisons between two groups was warranted. Alternatively, significance was determined by one-way analysis of variance (ANOVA) and post-hoc Tukey or Tukey–Kramer tests for equal or unequal sample size, respectively. Cell cycle data was fit using the Watson pragmatic model and correlations were determined using the Pearson's moment correlation coefficient. Differences between treatment groups were considered significant with a p value of ≤0.05.