prior surgery with change in each outcome was evaluated using multiple linear regression with stepwise selection for each country.

Results: Across countries mean ages were 64-65 years, three quarters were women, three quarters had the knee joint as primary complain and one of three in Denmark and four of ten in Canada and Australia were obese (BMI $\geq$ 30 kg/m^2). There were no clinically meaningful differences in baseline status between those with complete or those without follow-up data. Change in outcome measurements are shown in table 1. Patients from the three countries improved 26-33% in mean pain intensity, 7-13% in walking speed, 18-30% in chair stand ability and 12-26% in joint related quality of life from baseline to immediately after treatment. Patients from Canada and Australia seem to have slightly larger improvement in pain intensity and chair stand test than patients from Denmark. Patients in Australia have the largest improvement in KOOS/HOOS QOL followed by patients from Canada and with patients from Denmark having the lowest mean improvement. The sensitivity analysis imputing the missing values at follow up support the complete cases analyses. There was no consistency in factors associated with outcomes across the three countries, and factors associated with the outcome explained only up to 5% of the variance in the different change scores (R^2 0.0036 to 0.0528).

Conclusions: Patients with symptomatic knee and hip OA attending a structured supervised patient education and exercise program across three countries on three continents improve overall in pain intensity, functional performance and joint-related quality of life. The results show minor differences in improvement patterns among the three countries studied, although these differences are not likely clinically meaningful. Among a range of baseline characteristics there was no consistency in factors associated with outcome change across countries and the included factors had low explanatory value. Similar results across different countries indicate successful implementation of the GLA:D®-program in the three countries and shows that positive results can be obtained when health care practitioners nationwide take a standardized approach to implementing clinical guidelines for knee and hip OA into practice.

Table 1. Crude mean changes in outcomes following GLA:D® in Denmark, Canada and Australia for knee and hip OA patients

		Baseline Mean (SD)	After treatment Mean (SD) MI mean (SD)	Change Mean (SD)	95 % CI for mean change score
Pain intensity (NRS 0-10 best to worst)	**Denmark** (n: 26,354; FMI: 0.21)	4.7 (2.2)	3.5 (2.3) 3.6 (2.3)	-1.2 (2.3)	(-1.2; -1.2)
	Canada (n: 1,167; FMI: 0.37)	5.1 (2.1)	3.6 (2.2) 3.6 (2.2)	-1.5 (2.3)	(-1.6, -1.3)
	Australia (n: 849; FMI: 0.43)	4.2 (2.1)	2.7 (2.2) 2.8 (2.2)	-1.4 (2.3)	(-1.6, -1.3)
40 m Walk test (Sec.)	**Denmark** (n: 24,143; FMI: 0.15)	28.5 (8.0)	26.2 (6.9) 26.6 (7.3)	-2.3 (5.3)	(-2.4, -2.2)
	Canada (n: 658; FMI: 0.09)	34.1 (15.7)	31.1 (15.3) 30.1 (14.6)	-2.4 (8.4)	(-3.7, -2.4)
	Australia (n: 715; FMI: 0.47)	27.7 (10.7)	24.2 (5.8) 24.3 (5.8)	-3.5 (9.6)	(-4.2, -2.8)
30 sec chair stand test (no of stands)	**Denmark** (n: 24,729; FMI: 0.23)	12.0 (3.8)	14.1 (4.4) 14.0 (4.4)	2.1 (3.2)	(2.1, 2.2)
	Canada (n: 919; FMI: 0.11)	12.2 (5.1)	15.9 (6.1) 15.8 (6.1)	3.7 (4.5)	(3.4, 4.0)
	Australia (n: 738; FMI: 0.37)	10.6 (3.2)	13.8 (3.6) 13.8 (3.6)	3.2 (2.9)	(3.0, 3.4)
K/HOOS QOL (0-100 worst to best)	**Denmark** (n: 26,320; FMI: 0.17)	46.1 (15.2)	51.8 (17.0) 51.3 (17.1)	5.7 (14.8)	(5.5, 5.8)
	Canada (n: 1,174; FMI: 0.29)	38.8 (17.3)	46.6 (19.4) 45.9 (19.7)	7.8 (15.6)	(6.9, 8.7)
	Australia (n: 865; FMI: 0.31)	44.0 (14.9)	55.2 (17.1) 54.6 (17.2)	11.3 (15.7)	(10.2-12.3)

MI mean: Mean included missing values at follow up imputed with multiple imputations; FMI: Fraction of missing values

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EVALUATION OF ENGRAFTABLE HUMAN PLURIPOTENT STEM CELL-DERIVED CHONDROCYTES IN A LONG-TERM PRECLINICAL PIG MODEL OF FOCAL CARTILAGE INJURY

F. Petrigliano, N. Liu, J. Bogdanov, S. Lee, A. Sarkar, D. Evseenko. *Univ. of Southern California, Los Angeles, CA, USA*

Purpose: The pathogenesis of OA often begins from an injury to articular cartilage, which establishes chronic, low-grade inflammation that eventually promotes matrix degradation leading to the destruction of cartilage. Currently, there are no agents that efficiently slow or inhibit this process. Pluripotent stem cell-derived chondrocytes (PDC) represent a promising new tool for cartilage repair, but *in vivo,* specification of these cells remains unclear.

Methods: Detailed molecular analysis of human primary articular chondrocytes was carried out at embryonic, fetal, adolescent and adult stages. Human pluripotent feeder-free stem cell line (hES17) was propagated and differentiated using protocols previously established in our lab. Generated PDCs were evaluated using single-cell RNA-sequencing to assess cellular heterogeneity and purity. Full thickness, 6-mm in diameter, critical size cartilage lesions were generated in articular cartilage of male Yucatan minipigs. In pig model, PDC were transplanted using 2 formulations: cell aggregates (chondrospheres) at a high dose (group 1), and a low dose (1 group 2), and cells on collagen 1/3 membrane at a high dose (group 3), and a low dose (group 4). Control groups included vehicle only (fibrin glue) and clinical grade collagen 1/3 membrane transplanted with no cells on it. Analysis was performed 1 and 6 months after transplantation using Mach 1 bio-indenter (Biomomentum, Canada), immunohistochemistry (IHC), flow cytometry, PCR and routine histology. Detection of human cells was carried out using human specific TERT PCR assay, CD29 antibody (flow cytometry) or Ku80 (IHC). Statistical methods: Student t-test, one-way ANOVA followed by the Newman-Keuls test.

Results: Our studies have shown that transcriptional, epigenetic and functional signatures of PDC closely mimic their primary counterparts, but PDC remain immature *in vitro*. Single cell sequencing showed that more than 90% of PDCs expressed high levels of cartilage specific markers. Our data also demonstrated that implanted human PDC engrafted and generated human articular cartilage tissue in pig joints. After 1 month (N=4 per group), analysis of engrafted tissue clearly showed the presence of human cells post transplantation as detected by both FACS and IHC staining using human specific antibodies. High dose membrane formulation demonstrated the best repair potential as shown by both biomechanical and histological assessment. Defects repaired with low dose formulations and high dose chondrospheres showed some islands of cartilage tissue formation in the defect area mixed with fibrotic tissue and also highly vascularized loose connective tissue. At 6 months after transplantation (n=5 per group) lesions treated with high dose membrane formulation showed significantly better repair compared to control groups. IHC analysis using antibodies against PRG4/lubricin and other markers showed the presence of all 4 populations of zonally organized human chondrocytes in pig cartilage lesions. Safranin O staining and IHC for cartilage markers collagen type 2 and SOX9 showed the presence of human cartilage tissue in pig defects minimally expressing collagens type 1 and 10. In contrast, control animals healed cartilage defects with a highly disorganized fibrotic tissue marked by collagen type 1. No signs of neoplastic growth were detected in any of the transplanted pig joints as well as the liver, kidney, or lung tissues.

Conclusions: Transplanted human PDC enhance repair in Yucatan minipig model of cartilage injury and demonstrate clear ability to form human cartilage cells in vivo. The study showed that although some human cells were present in the site of injury 6 months post-transplantation the vast majority of repaired tissue was composed of pig cells. These findings indicate a prominent paracrine effect of implanted PDC constructs in addition to direct integration of implanted human chondrocytes into pig tissue. Overall, current study is the first to demonstrate long-term safety and efficacy of engraftable allogenic mass-produced pluripotent stem cell-derived chondrocytes in preclinical large animal model of articular cartilage repair.

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DEVELOPMENT OF A MURINE MODEL TO STUDY MITOCHONDRIAL TRANSFER BETWEEN MESENCHYMAL STROMAL CELLS AND INJURED CHONDROCYTES

M.P. Bennett, R. Vivancos-Koopman, L.A. Seewald, T. Robinette, M.L. Delco;. *Cornell Univ., Ithaca, NY, USA*

Purpose: Mesenchymal stromal cells (MSCs) have been an increasingly common therapy for orthopedic diseases for decades.While the mechanisms governing the beneficial effects of MSCs remain unclear, existing evidence supports two theories: direct repair via engraftment at sites of tissue damage, and facilitation of endogenous healing via immunomodulation. One or both of these paradigms are likely true under given circumstances, however neither fully explain the observed therapeutic effects. Recent evidence suggests an alternate paradigm; failing cells can recruit help from MSCs in the form of whole-organelle donation of

mitochondria (MT). MT transfer has been documented in several cell types *in vitro* including corneal epithelium and myocytes, and *in vivo* in alveolar epithelial cells.Preliminary work from our group revealed the first evidence of MT transfer between MSCs and chondrocytes *in vitro*, using equine cells stained with fluorescent probes to track transfer events. Given the limitations associated with live-cell staining techniques (including short-term fluorescence, rapid photobleaching, and the possibility of non-specific staining), our goal was to develop an *in vivo* model, utilizing transgenic mice expressing endogenous MT-targeted fluorescent proteins, in order to: 1) validate previous findings suggesting MSC-chondrocytes MT transfer *in vitro*, 2) quantify and characterize MT transfer events between these cell types over time and under various environmental conditions, and 3) allow future study of MT transfer in an *in vivo* model of post-traumatic osteoarthritis.

Methods: Chondrocytes were harvested from the acetabulofemoral joints of 5-day-old mCherry mice and cultured under either normal (1g/L) or low (0.45g/L) glucose conditions, then stressed by the addition of either a general inflammatory stimulus, IL-1β (1ng/ml), or one of three mitochondria-specific stressors 1) oligomycin (1μM), 2) rotenone/antimycin (0.5μM/0.5μM), or 3) FCCP (1μM). Stimulated and control chondrocytes were cultured with or without SS-31, a mitoprotective peptide. Bone marrow-derived MSCs (bMSCs) were harvested from 5-week-old mitoDendra2 mice (endogenous green MT fluorescence) and cultured. After 12 hours, chondrocytes were rinsed, and mitoDendra2 MSCs were added to chondrocyte cultures in 1:10 ratio. Cells were co-cultured for 12 hours, lifted, and fixed for flow cytometry. Experiments were duplicated on chambered cover-glass slides, and live confocal 3-dimensional (z-stacked) imaging was performed longitudinally for 10 hours after initiation of co-culture. Experiments were performed under both 5% O_2(hypoxic) and 21% O_2 (normoxic) conditions.

Results: Time lapse confocal imaging and flow cytometry data support MT transfer between bMSCs and chondrocytes in murine cells expressing endogenous fluorescent proteins. Stimulated mCherry chondrocytes gained green fluorescence and mitoDendra2 MSCs gained red fluorescence following co-culture. The percentage of green+ chondrocytes, red+ MSCs, and total double positive (red+ green+) cells were significantly higher in normoxic than hypoxic conditions. The percentage of transfer events was significantly different between stimulants under low glucose conditions. Total transfer events increased over the 10-hour co-culture and was not significantly affected by treatment. The transgenic mouse lines enabled live-cell longitudinal confocal imaging for 10 hours to follow specific MT transfer events, as well as 3-dimensional localization of transferred MT within chondrocytes.

Conclusions: Consistent with data in other cell types and species, our findings suggest that chondrocyte MT dysfunction initiates MT donation by MSCs *in vitro*. The difference in MT transfer events in 21% O_2 and 0.45g/L glucose may be a result of non-physiologic culture conditions, and may have important implications in experimental design and therapeutic development going forwards. Intercellular MT transfer is a possible mechanism underlying the beneficial effects of therapeutically implanted MSCs. These findings confirming MT delivery by bMSCs to stressed chondrocytes may represent a new strategy for stem cell therapy, and has the possibility to be engineered for disease-modifying and regenerative medicine applications to treat a broad range of diseases.

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SIMPLE COMBINATION OF A WNT ACTIVATOR AND A RETINOIC ACID RECEPTOR AGONIST ROBUSTLY INDUCES HUMAN PLURIPOTENT STEM CELLS INTO CHONDROCYTES

M. Kawata, T. Saito, F. Yano, S. Tanaka. *The Univ. of Tokyo, Tokyo, Japan*

Purpose: Human pluripotent stem cells (hPSCs), such as human embryonic stem cells and human induced pluripotent stem cells (hiPSCs), are a promising cell source for treating degenerative diseases such as osteoarthritis, because of their unlimited expansion potential. They are also valuable experimental tools for *in vitro* studies of cell differentiation including chondrogenesis. While some efficient methods using several kinds of cytokines have been developed for chondrocyte induction from hPSCs, a simple induction protocol to differentiate chondrocytes from hPSCs using small-molecule compounds is beneficial both for cartilage regenerative medicine and for mechanistic studies of chondrogenesis. Because canonical Wnt signaling regulates mesendoderm induction of PSCs, treatment of PSCs with Wnt activator such as CHIR99021, a glycogen synthase kinase 3 inhibitor, will be useful in the initial step of chondrogenesis. Moreover, while retinoic acid (RA) and retinoids are reported to suppress collagen production or cartilage matrix synthesis in cultured chondrocytes, they are also known to play critical roles in limb bud formation and subsequent chondrogenesis during skeletal development. Therefore, RA and retinoids may be useful for inducing chondrocytes from PSCs under a certain condition. The purpose of the present study is to demonstrate that chondrocytes are robustly induced from hPSCs by simple combination of two compounds (2C), CHIR99021 and TTNPB, a retinoic acid receptor (RAR) agonist, under serum- and feeder-free conditions within 5-9 days. We also aim to reveal molecular functions of RA and Wnt/β-catenin signaling in the process of chondrogenesis by genome-wide analysis of RAR and β-catenin association.

Methods: To confirm reproducibility of results, multiple clones of hiPSCs adapted to feeder-free defined culture conditions were used for experiments. At least three independent experiments were performed for gene expression analyses, and P-values < 0.05 by Dunnett's test were considered as significant for multiple group comparisons. FACS analysis was performed with a FACSAria Fusion cell sorter, and data were analyzed by BD FACSDiva software. To form a hiPSC-derived particle for transplantation, 1.7×10^6 hiPSC-derived cells that had undergone the present protocol for 9 days were seeded into cloning rings set on a permeable membrane insert. After 1 week of culture, the formed particles were transplanted into subcutaneous spaces or knee cartilage defects of 8-week-old male NOD/SCID mice. The mice were sacrificed after 8 weeks for subcutaneous transplantation, and after 6 months for transplantation in knees. Microarray analysis was performed using $n = 3$ sequential samples from independent experiments during the 2C differentiation, and gene ontology (GO) terms with corrected P-values of < 0.01 were considered as significant in GO analysis. For open chromatin analysis, assay for transposase-accessible chromatin using sequencing (ATAC-seq) was performed using two biological replicates of hiPSC-derived cells at each stage of the 2C protocol. ChIP-seq of RARα and Wnt/β-catenin were also performed at each differentiation stage with two biological replicates per condition. A P-value cut-off of 1×10^{-5} compared with input controls was used for peak calling, and peaks with a false discovery rate of < 0.01 were incorporated into further analysis.

Results: The optimized standard protocol was combined treatment with 10 μM CHIR99021 for the initial 2 days, and 100 nM TTNPB for the whole period. Under this protocol, expression of mesendoderm markers *T* and *MIXL1* was upregulated from day 1 to 2, and expression levels of mesoderm markers, such as *TBX6*, *MEOX1* and *HAND1*, were elevated from day 2 to 4. Then, chondrogenic markers including *SOX9*, *SOX5*, *SOX6*, *COL2A1* and *COL11A2* were upregulated after about day 5. FACS analysis of hiPSC-derived differentiated cells after 9 days of culture under the present protocol showed that 97.0% of cells were positive for SOX9 while NANOG- or OCT4-positive cells were not totally detected. We compared marker gene expression between the hiPSC-derived cells on day 9 of the 2C protocol and those after differentiation by two other reported protocols using cytokines for about 2 weeks; expression levels of chondrogenic markers were significantly increased by 2C differentiation compared with the other protocols, while those of pluripotent and other lineage markers were significantly decreased. Particles prepared from hPSC-derived cells differentiated by the 2C protocol formed hyaline cartilaginous tissues when transplanted into subcutaneous spaces and articular cartilage defects in NOD/SCID mouse knee joints, and no signs of teratoma or other tumor formations were seen. GO analysis using upregulated genes in day 9 samples compared with those of day 0 in microarray datasets showed that terms related to skeletal and cartilage formation were enriched at highest ranks. ATAC-seq of sequential samples under the present protocol demonstrated that enhancer regions of key marker genes for mesendoderm, mesoderm, and chondrocyte were activated at each differentiation stage. Finally, ChIP-seq analysis for RAR and β-catenin detected peaks in the enhancer regions of the key marker genes for the respective stages, and some of which were common between the signals.

Conclusions: The 2C protocol induces chondrocytes more efficiently compared with reported protocols using cytokines. The present method provides a promising cell source for cartilage regenerative medicine and may facilitate elucidation of molecular mechanisms underlying