# 3D Tri-Culture of Mesenchymal Stem Cells with Osteoblasts and Adipocytes Differentially Regulates Gene Expression Towards Each Lineage

Taymour M. Hammoudi,† Nathaniel C. Bloodworth,† Hang Lu,‡ and Johnna S. Temenoff.†
†Coulter Dept. of Biomedical Engineering, and ‡Dept. of Chemical & Biomolecular Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA

**Introduction:** Rational design of mesenchymal stem cell (MSC)-based therapies for clinical application requires understanding the influence of native cells on stem cell fate prior to or following *in vivo* implantation. With time, the initially osteoblastic MSC niche in bone marrow accumulates significant amounts of fat,[1] which may affect differentiation potential or healing properties of MSCs. Current *in vitro* model systems to examine these effects are limited to monolayer (transwell format) or 3D co-culture with only one other cell type. Here, we use a 3D-patterned, readily separable tri-culture platform for evaluating human MSC fate (assessed by MSC gene expression) during culture with osteoblasts, adipocytes, or a combination of both.

**Methods:** PEG-diacrylate (PEG-DA) polymer was synthesized from PEG precursor ($M_n$=3.4kDa).[2] PEG-acrylate derivatized with the adhesive peptide sequence GRGDS or YIGSR was synthesized by reacting with Acrl-PEG-SVA ($M_n$=3.4kDa, LaysanBio; 1:2 molar ratio) in 50 mM $NaHCO_3$ (pH 8.5) for 2.5 h.

Gels were comprised of PEG-DA (10% w/v in phosphate-buffered saline) and D2959 photoinitiator (0.05% w/v), with either 1 μmol/mL GRGDS (MSCs) or YIGSR (adipocytes) or no peptide (osteoblasts). Primary human MSCs (Texas A&M, P6), human adipocytes (Lonza, P2), and human osteoblasts (Lonza, 7 population doublings) were encapsulated at $15\times10^6$ cells/mL and photo-patterned (365-nm light, 10 mW/cm$^2$, 12 min) into 4.5×1.5×1 mm-tri-laminated gel constructs in a serial fashion (Figure 1). Each construct was cultured in a single well of a 6-well plate in 4 mL of basal medium (DMEM with 10% FBS, 2 mM L-glutamine, 50 μM L-ascorbate-2-phosphate, 1 μM insulin, and 1% gentamicin/ amphotericin B) replenished every 2 days.

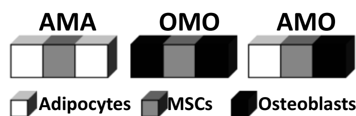


**Figure 1.** Patterns of cells within modules of 3D tri-laminated hydrogels. AMA and OMO are co-culture controls. AMO represents the tri-culture sample.

MSC-containing modules were separated from the constructs on days 1, 7 and 18 and analyzed for gene expression ($n \geq 3$ per sample type) with quantitative reverse-transcription polymerase chain reaction (qPCR, $\Delta\Delta C_T$ method) for genes indicating: osteoblastic commitment, including runt-related transcription factor 2 (*RUNX2*, early master transcriptional regulator), osteocalcin (*BGLAP*, late hormonal marker), or adipocytic commitment, including peroxisome proliferator-activated receptor γ2 (*PPARG*, early master transcriptional regulator), and leptin (*LEP*, late hormonal marker). Ribosomal 18S protein (*RPS18*) and beta-actin (*ACTB*) served as reference genes. Data were analyzed using ANOVA and Tukey's *post-hoc* test ($p \leq 0.05$).

**Results:** Gene expression data revealed that over 18 days in co-culture, MSCs expressed markers suggestive of late-stage differentiation (Fig. 2). Osteoblast-specific transcription factor *RUNX2* was down-regulated in a graded fashion according to relative abundance of osteoblasts in the system. Adipocyte-specific transcription factor *PPARG* was 1.9 fold higher on Day 1 than Day 18 in AMA gels and increased by Day 18 in AMO gels. Markers from opposing lineages were not highly expressed in their respective controls (e.g. MSCs from AMA gels did not express high levels of osteoblastic genes and vice versa). *BGLAP* expression rose in MSCs from OMO gels, while *LEP* expression remained unchanged in MSCs from AMA gels. Curiously, both late hormonal markers of differentiation from different lineages, *LEP* and *BGLAP* (4.18±1.6 and 9.39±5.1 fold up-regulation, respectively), were most upregulated in the MSCs from the tri-culture (AMO) gels rather than in co-culture controls.

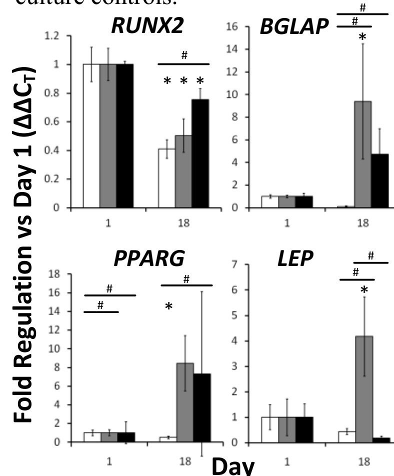


**Figure 2.** Fold regulation changes in mRNA expression in MSCs isolated from co-culture constructs over 18 days ($n \geq 3$). Sample types: AMA (white), AMO (gray), OMO (black). * = Significantly different from Day 1 for same sample type. # = Significantly different from each other, same day. $p \leq 0.05$.

**Conclusions:** We demonstrate a 3D tri-culture system that allows long-term culture of multiple cell types for and subsequent analysis. Expected gene expression results were observed in co-culture controls suggestive MSC differentiation toward the cell type cultured with them. However, non-intuitive changes in MSCs in tri-culture with respect to hormonal gene expression suggest heterogeneous lineage commitment and significant crosstalk effects. Importantly, these results could not have been elucidated with traditional co-culture systems that only examine interactions between two cell types. Consequently, these results support further studies to replicate aspects of the evolving bone marrow niche with our tailorable tri-culture system that may reveal how the cell fate and potential healing functions of MSCs are affected by the types of cells surrounding them.

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**References:** [1]Rosen CJ et al. *Crit Rev Eukaryot Gene Expr*. 2009;19:109-24 [2]Hahn MS et al. *Biomaterials*. 2006;27:2519-2524.

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