

# Epigenetic Dynamics in Meniscus Cell Migration and Inflammation: Implications for Regeneration Strategies

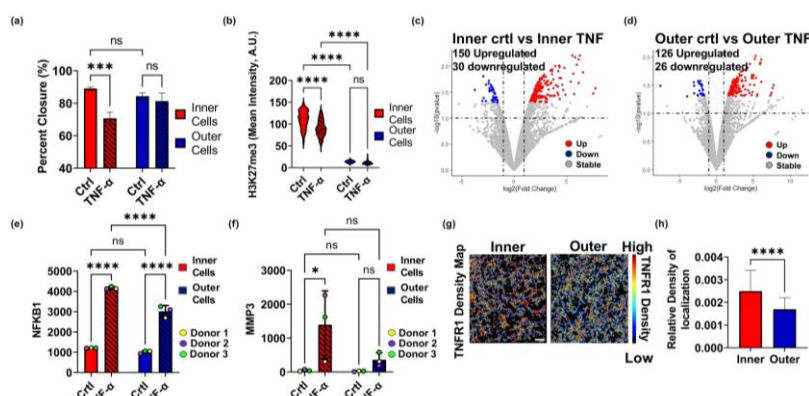
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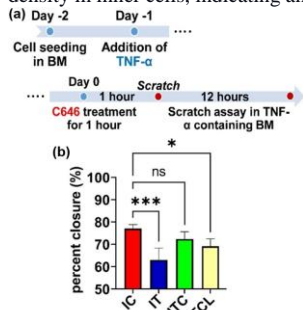
**INTRODUCTION:** Tears in the meniscus represent the most prevalent knee injuries, necessitating proficient cell migration to facilitate effective wound healing. The knee meniscus, characterized by its heterogeneous nature, exhibits zone-dependent properties. Although differences in cellular phenotypes between the inner and outer zones of the meniscus are well-documented, a comprehensive understanding of cell migration behaviors and their underlying mechanisms remains elusive [1-3]. Furthermore, it has been demonstrated that inflammatory conditions within the knee substantially influence meniscus cell behaviors, impeding the regenerative process [4,5]. However, the precise mechanisms through which inflammatory conditions alter these behaviors and mechanisms remain unclear. Thus, this study delves into the impact of inflammatory conditions on meniscus cell behaviors, concurrently probing the underlying epigenetic mechanisms to pave the way for potential epigenetic-targeted therapies to address meniscus injuries.

**METHODS:** Inner or outer zone meniscus cells were isolated from juvenile (< 3 months) bovine menisci (Fig. 1a). To explore alterations in trimethylation of lysine 27 on histone H3 (H3K27me3, a marker of transcriptional suppression and pivotal in the migration of various cell types [6]) levels were examined in inner or outer meniscus cells positioned at the migration “front” or “back” (10 cell-layers behind the “front”) (Fig. 1b). Wound closure scratch assays (WCA) were performed, and at each time point, cells were fixed, followed by immunofluorescence (IF) staining for H3K27me3 (Cell Signaling) and quantified using Image J. To elucidate the contribution of histone methyltransferase EZH2 (known for catalyzing H3K27me3) in meniscus cell migration, WCA were performed on inner and outer zone cells pre-treated with GSK343 (a selective EZH2 inhibitor, 14 μM). Next, to examine the impact of inflammatory conditions on cell migration and H3K27me3 levels, TNF-α (50nM) treatment was employed. After an overnight TNF-α treatment, WCA was conducted in the presence of TNF-α. Cells were fixed at 0 and 12-hour intervals post-scratch, followed by H3K27me3 IF. Additionally, the effect of TNF-α treatment on gene expression profiles in inner and outer cells was investigated. Cells received overnight TNF-α treatment, followed by mRNA extraction for subsequent RNA sequencing (Azenta). All experiments were performed in triplicates with 3 different donors. To discern variations in TNF-α Receptor 1 (TNFR1) presence between inner and outer cells, IF and super-resolution STORM imaging (Nanoimager, ONI) for TNFR1 were carried out following our established protocols [7]. Lastly, for the potential development of an epigenetic targeted therapy aimed, inner cells exposed to overnight TNF-α treatment were subsequently treated with the histone acetyltransferase p300 inhibitor C646 for 1 hour (Sigma, well known to decrease the H3K27-acetylation level [8] antagonizing for H3K27me3 [8,9]), and WCA was then performed for 12 hours (Fig. 3a).

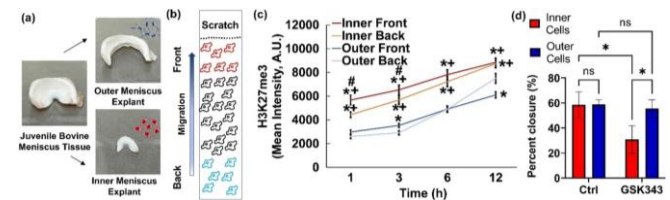


**Figure 2:** (a) WCA results for cells treated with TNF-α (n=6/group, \*\*\*: p<0.005 vs. Ctrl). (b) IF results for H3K27me3 after TNF-α treatment (n>100/group, \*\*\*\*: p<0.001 vs. Ctrl). (c) RNA-seq volcano plots comparing Inner ctrl vs Inner cell with TNF-α treatment and (d) Outer ctrl vs Outer cell with TNF-α treatment (n=3 donors). Expression level of NFKB1 (e) and MMP3 (f) in inner and outer cells treated with TNF-α, as determined by RNA-seq (n=3 donors, \*: p<0.05 vs. Ctrl, \*\*\*\*: p<0.001 vs. Ctrl). (g) STORM image, scale bar = 300 nm, and (h) quantitative density for TNFR1 (n>10 cells/group, \*\*\*\*: p<0.001).

migration, the average baseline H3K27me3 level was higher in inner cells than in outer cells (Fig. 1c). Inner cell migration exclusively necessitated H3K27me3, unlike outer cells (Fig. 1d). Upon exposure to TNF-α, a reduction in inner cell migration speed was observed (Fig. 2a), whereas outer cell migration speed remained unaffected. Notably, baseline H3K27me3 levels were higher in inner cells compared to outer cells, with TNF-α treatment selectively decreasing H3K27me3 solely in inner cells (Fig. 2b). Transcriptomic analysis revealed a more pronounced response to TNF-α in inner cells, with a higher number of genes exhibiting up/down regulation (150 up/30 down) than in outer cells (126 up/26 down) (Fig. 2c,d). Remarkably, TNF-α-associated gene expressions, such as NFKB1 and MMP3 displayed more pronounced elevation in inner cells than in outer cells (Fig. 2e,f), underscoring the heightened sensitivity of inner cells to TNF-α treatment. The analysis of TNFR1 distribution via immunofluorescence (not shown) and TNFR1 STORM images revealed a higher nanoscale density in inner cells, indicating an increased TNFR1 presence in inner cells (Fig. 2g,h). Lastly, the application of the C646 led to reduced histone acetylation



**Figure 3:** (a) Timeline illustrating the experimental design for C646 treatment. (b) WCA results for inner cells treated with C646 at high (30μM, ITC) and low (10μM, ITCL) doses. (n=6/group, \*: p<0.05 vs. Control, \*\*\*\*: p<0.005 vs. Control). IC: Inner cell ctrl, IT: Inner cells with only TNF-α treatment).



**Figure 1:** (a) Schematic showing isolation of inner and outer zone meniscus cells (3 different donors). (b) Schematic illustrating the quantification methodology for immunofluorescence in the migration study. (c) Intensity of H3K27me3 IF after the initiation of migration (n>100/group, \*: p<0.05 vs. Outer Cell-Back, +: p<0.05 vs. Outer Cell-front, #: p<0.05 vs. Inner Cell-Back). (d) WCA results treated with GSK343 (n=6/group, \*: p<0.05 vs. Ctrl).

and H3K27me3 levels, TNF-α (50nM) treatment was employed. After an overnight TNF-α treatment, WCA was conducted in the presence of TNF-α. Cells were fixed at 0 and 12-hour intervals post-scratch, followed by H3K27me3 IF. Additionally, the effect of TNF-α treatment on gene expression profiles in inner and outer cells was investigated. Cells received overnight TNF-α treatment, followed by mRNA extraction for subsequent RNA sequencing (Azenta). All experiments were performed in triplicates with 3 different donors. To discern variations in TNF-α Receptor 1 (TNFR1) presence between inner and outer cells, IF and super-resolution STORM imaging (Nanoimager, ONI) for TNFR1 were carried out following our established protocols [7]. Lastly, for the potential development of an epigenetic targeted therapy aimed, inner cells exposed to overnight TNF-α treatment were subsequently treated with the histone acetyltransferase p300 inhibitor C646 for 1 hour (Sigma, well known to decrease the H3K27-acetylation level [8] antagonizing for H3K27me3 [8,9]), and WCA was then performed for 12 hours (Fig. 3a).

**RESULTS:** Consistent with a previous study [10], H3K27me3 levels exhibited an incremental trend as meniscus cells migrated (Fig. 1c). Interestingly, during migration, the average baseline H3K27me3 level was higher in inner cells than in outer cells (Fig. 1c). Inner cell migration exclusively necessitated H3K27me3, unlike outer cells (Fig. 1d). Upon exposure to TNF-α, a reduction in inner cell migration speed was observed (Fig. 2a), whereas outer cell migration speed remained unaffected. Notably, baseline H3K27me3 levels were higher in inner cells compared to outer cells, with TNF-α treatment selectively decreasing H3K27me3 solely in inner cells (Fig. 2b). Transcriptomic analysis revealed a more pronounced response to TNF-α in inner cells, with a higher number of genes exhibiting up/down regulation (150 up/30 down) than in outer cells (126 up/26 down) (Fig. 2c,d). Remarkably, TNF-α-associated gene expressions, such as NFKB1 and MMP3 displayed more pronounced elevation in inner cells than in outer cells (Fig. 2e,f), underscoring the heightened sensitivity of inner cells to TNF-α treatment. The analysis of TNFR1 distribution via immunofluorescence (not shown) and TNFR1 STORM images revealed a higher nanoscale density in inner cells, indicating an increased TNFR1 presence in inner cells (Fig. 2g,h). Lastly, the application of the C646 led to reduced histone acetylation

and increased histone methylation levels (not shown) and this intervention successfully restored the migration speed of inner meniscus cells that had been decelerated under TNF-α treatment conditions (Fig. 3b).

**DISCUSSION:** The study provides valuable insights into the intricate interplay among epigenetic factors, inflammation, and meniscus cell migration, elucidating pivotal mechanisms that influence meniscus healing. Notably, it underscores the significance of H3K27me3 in mediating meniscus cell migration, alongside revealing discernible zonal variations in baseline levels of this marker. Intriguingly, distinct histone modification patterns were observed between inner and outer cells during migration, with inner cells exhibiting heightened responsiveness to TNF-α treatment, substantiated by gene expression profiles and TNFR1 distribution. Moreover, the study introduces the potential of leveraging epigenetic manipulation for therapeutic purposes, exemplified by the restoration of cell migration speed through histone acetyltransferase inhibition. In the pursuit of an epigenetic-driven approach to meniscus tissue engineering, we are presently evaluating alternative epigenetic drug candidates such as GSKJ4 and TSA. Ongoing investigations involve testing our findings within a 3D migration model and integrating epigenetic agents with tissue engineered meniscus scaffolds for future in vivo experimentation.

**SIGNIFICANCE:** This study possesses the potential to revolutionize treatment strategies for zone-dependent meniscus injuries and regenerative approaches by unraveling the intricate interplay among epigenetic regulation, inflammatory response, and cell migration dynamic.

**REFERENCES:** [1] Furumatsu+, Connect. Tissue Res. 2011; [2] Kambic +, J. Orthop. Res., 2005.; [3] Englund +, Nat. Rev. Rheumatol., 2012; [4] Swärd +, Osteoarthr. Cartil., 2012; [5] Hennerbichler +, Osteoarthr. Cartil. 2007; [6] Gerlitz +, Front. Cell Dev. Biol., 2020; [7] Heo+, Sci Rep. 2015; [8] Ono +, Sci Rep. 2021; [9] Tie+, Development. 2009.; [10] Zhang +, ORS, 2023.

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