HARNESSING A BIOACTIVE MODIFIED CHITOSAN TO HASTEN CHONDROGENESIS IN THREE-DIMENSIONAL CELL CULTURE SYSTEMS

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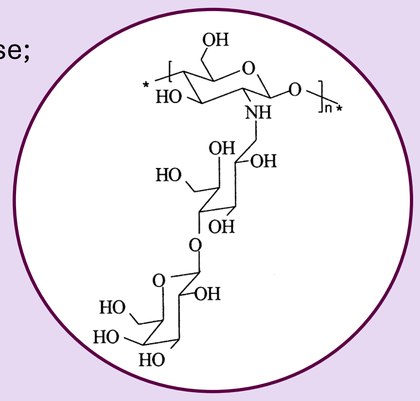
Introduction

Combining the regenerative potential of adipose tissue-derived mesenchymal stem cells (**ASCs**) and the multi-faceted bioactivity of **ECM-mimicking biopolymers** represents a research frontier for the **restoration of bone and cartilage**. While two-dimensional cell expansion and differentiation systems remain largely ineffective as a permanent solution, **spheroid cultures**, despite bearing significant limitations, represent a turning point in research. Our research group studies **3D cell culture systems** for cartilage regeneration, and to this end we employ **CTL**, a patented bioactive polysaccharide.

Key features of CTL:

- Produced through chitosan N-alkylation with lactose;
- Promotes cell aggregation and spheroid formation;
- Promotes early COL2A1 gene expression;
- Acts irrespectively of its initial physical form;
- Forms a self-healing hydrogel when interacting with boric acid;
- Cell-polymer interaction still to be elucidated.

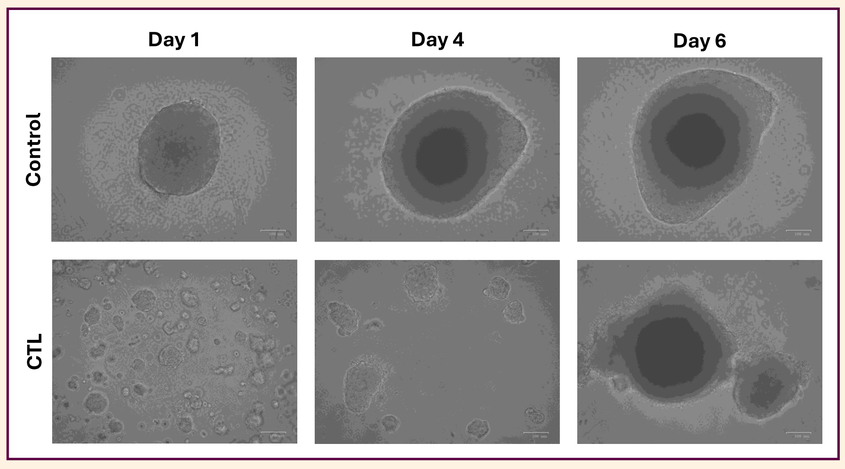
Sources: Donati et al. (2005); Sacco et al. (2017); Scognamiglio et al. (2024); Conz et al. (2025).

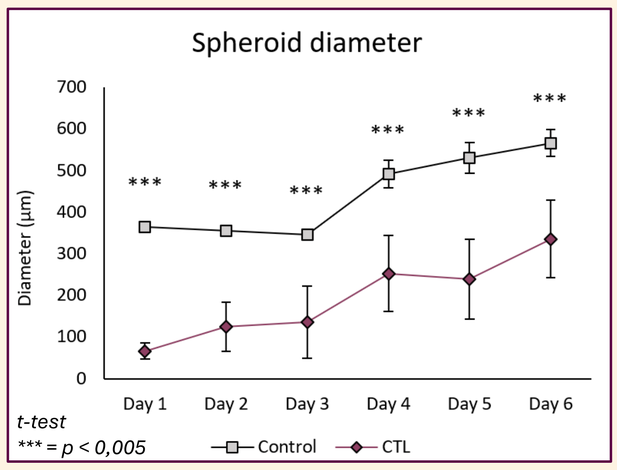


Aim of the project

- To uncover the molecular signaling pathways involved in cell-CTL interaction;
- To develop a cellularized hydrogel system to test for cartilage restoration potential.

Results



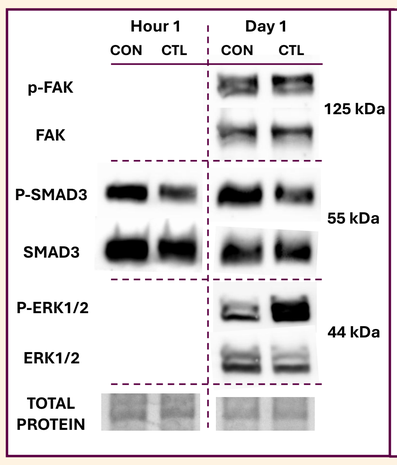


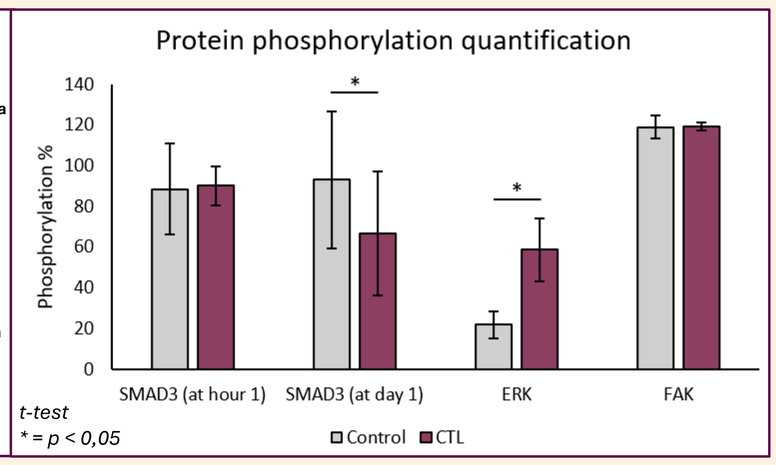
Spheroid dimensional analysis

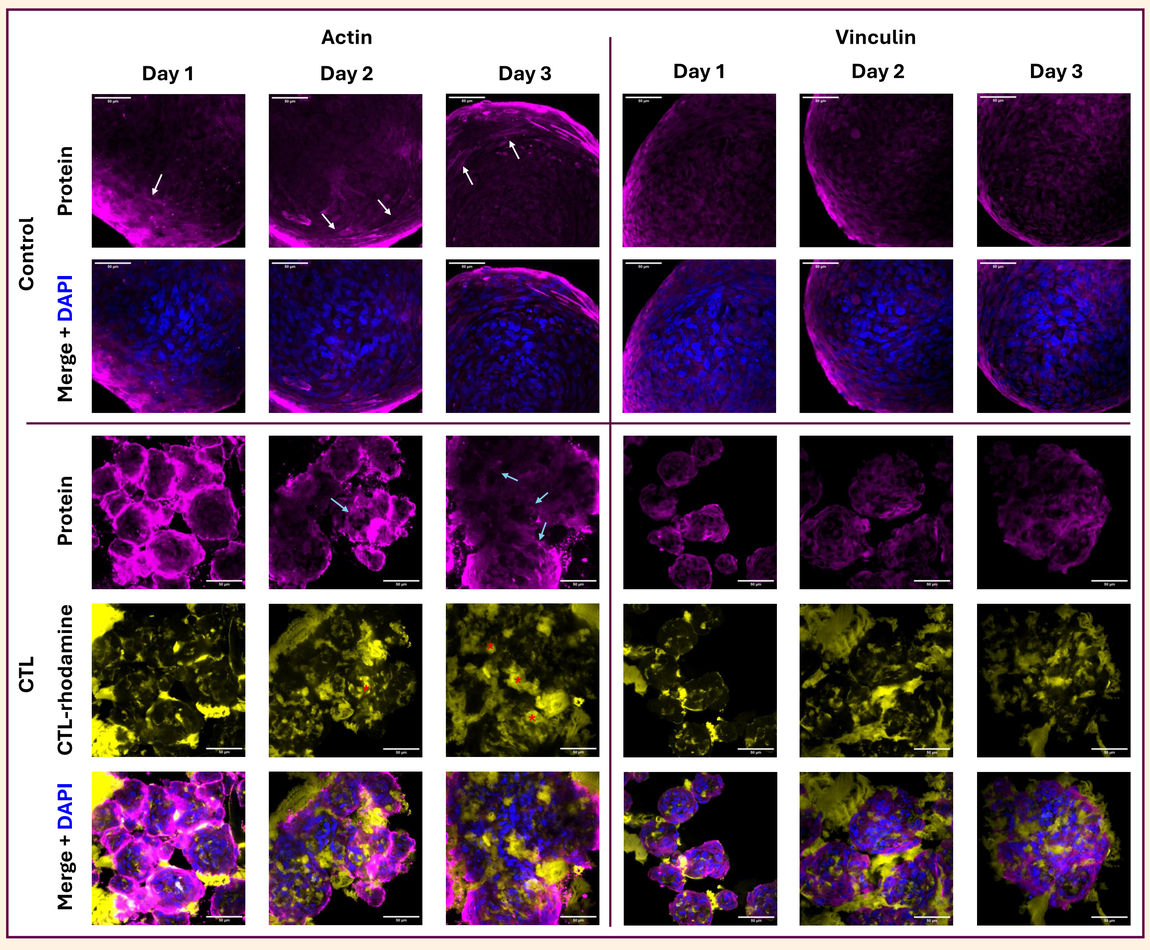
ASCs seeded with (CTL spheroids) or without (Control spheroids) polymer on ULA multiwells follow distinct growth dynamics. Control spheroids rapidly aggregate into a single body, while CTL spheroids use polymer chains as clustering platforms, resulting in the formation of many small aggregates, progressively coalescing and growing. With spheroids, lesser dimensions improve both nutrient perfusion and cell vitality. *Scale bar: 100µm.*

Western blot

Data analysis displays a significant increase in ERK1/2 signaling in CTL samples at day one, paired with a reduction of p-SMAD3.





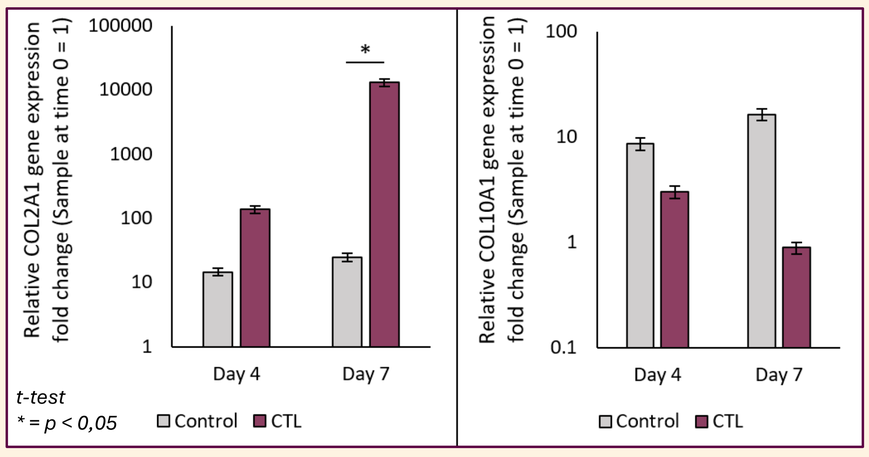


Immunofluorescence

While in control spheroids actin polymerization (white arrows) is exclusive to the outer cell layers, any significant actin polymerization present in CTL spheroids (teal arrows) is localized in parallel to polymer clumps (red asterisks). Vinculin staining highlights cellular organization is specular to the spatial disposition of CTL, which localizes both inside and outside spheroids. *Scale bar: 50µm.*

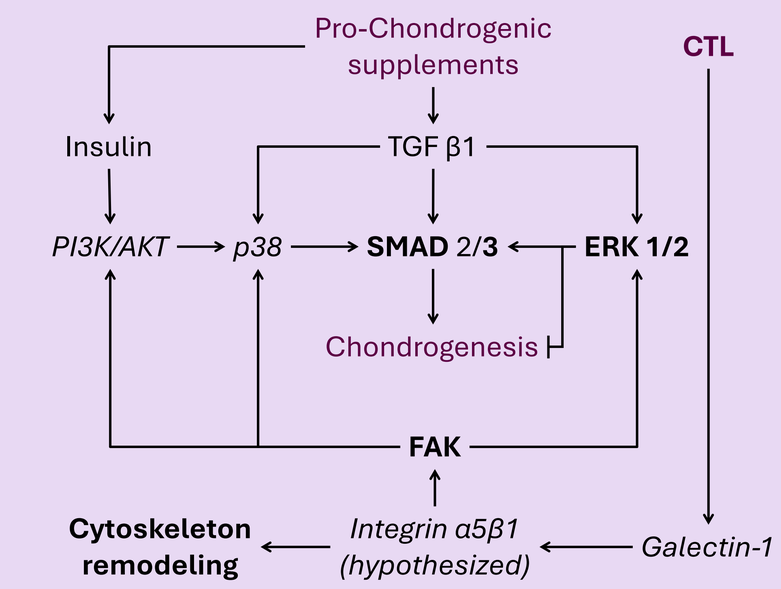
RT-qPCR

In standard chondrogenesis protocols, COL2A1 gene expression increases only at day 21. CTL induces an early increase in the transcription of this gene, which reaches significant levels by day 7. Chondrogenesis master regulator SOX9 showed no transcription increase at days 4 and 7 (not shown), suggesting an earlier increase for CTL samples. Low COL10A1 gene expression in CTL spheroids excludes the possibility spheroids reached hypertrophy.



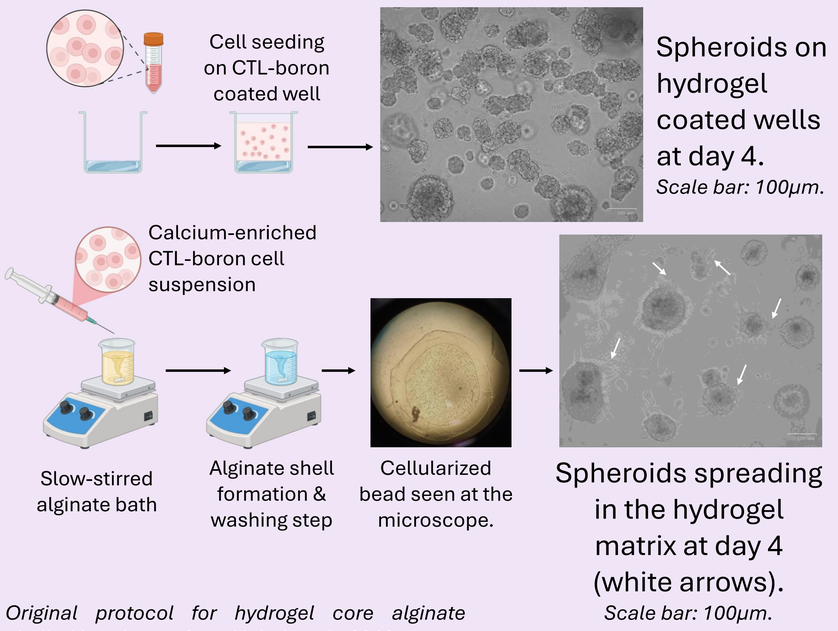
Choosing our molecular targets

Our chondrogenesis induction protocol relies on the supplementation of TGF β1 and insulin, which activate intertwined signaling pathways. Meanwhile CTL, as stated in the literature (Marcon et al., 2005) clusters on the cell surface together with galectin-1, likely promoting integrin cross-linking and activation. Below, a simplified summary of the pathways involved. In bold text, the mediators presented in this work. In italics, those which will be included in future research.



CTL-boron formulations

Exploiting the versatility of CTL-boron formulations, two cell culture systems have been developed for ASC chondrogenesis.



Original protocol for hydrogel core alginate shelled beads was from Nebel et al., 2012.

Conclusion and future perspectives

- CTL retains its pro-aggregative capability in two new cell culture systems; these will be studied further for their ability to promote ASC chondrogenesis;
- In the first days of cell culture, CTL clusters mainly outside of spheroids and potentially guides spheroid coalescence;
- The hypothesis of an early completion of chondrogenic differentiation is supported by gene expression analysis; we aim to validate this hypothesis by assessing over all days of cell culture the activity of all previously mentioned cell signaling components involved in chondrogenesis signaling for their potential role in the promotion of differentiation prompted by CTL;
- As cytoskeletal reorganization heavily influences ASC fate (Saidova et al., 2012), lower actin polymerization is compatible with chondrogenesis and hints at a softer CTL spheroid structure; we intend to quantify this in the future;
- Galectin-1 and integrin α5β1 will be studied for their involvement the interaction between cells and CTL.

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