Peptide-functionalized 3D-printed biomaterial scaffolds direct *in situ* osteochondral tissue regeneration

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INTRODUCTION: Osteoarthritis (OA) is a debilitating disease characterized by progressive osteochondral (OC) tissue degeneration that affects over 30 million adults in the U.S.[1] The gold-standard treatment for end-stage OA is total joint replacement, which is not suitable for younger patients that may outlive their implant.[2] Early-stage interventions for cartilage repair, such as microfracture and autologous chondrocyte implantation, can delay disease progression.[2] However, the newly formed tissue is mechanically inferior to native cartilage and poorly integrated with the subchondral bone, leading to unsatisfactory long-term clinical outcomes.[1,2] Functional OC regeneration is particularly challenging because the transition between bone and cartilage contains complex biochemical and physical gradients necessary for joint function. Biomaterials offer promising strategies to direct OC tissue formation and organization to augment early-stage treatments. To achieve this, we developed a 3D printing strategy to fabricate biodegradable polymer scaffolds spatially functionalized with multiple bioactive peptides.[3,4] Peptide-poly(caprolactone) (PCL) conjugates bearing hyaluronic acid (HA)-binding (HAbind-PCL) or mineralizing (E3-PCL) peptides were designed to promote human mesenchymal stromal cell (hMSC) chondrogenesis or osteogenesis, respectively.[4] We hypothesized that scaffolds presenting both peptide in discrete regions will direct OC matrix organization. We investigated how the spatial presentation of both peptides in dual-peptide scaffolds influenced hMSC differentiation *in vitro* compared to PCL and single-peptide scaffold controls. Furthermore, we implanted PCL and dual-peptide scaffolds in a critically sized osteochondral defect model in rabbits to evaluate scaffold-directed tissue formation and integration *in vivo*.

METHODS: *Scaffold fabrication*: HAbind-PCL and E3-PCL conjugates were synthesized using methods previously described.[3,4] Conjugates were dissolved with unmodified PCL in hexafluoro-2-propanol (HFIP) to fabricate 5 scaffold groups: (1) PCL; (2) HAbind; (3) E3; (4) dual spatial (HAbind and E3 in discrete zones); and (5) dual mixed (both peptides presented homogeneously). Scaffolds were printed for *in vitro* (0.5-mm thick, 3-mm diameter) and *in vivo* (3-mm thick, 4-mm diameter) studies. *In vitro*: hMSCs (50,000 cells/mm^3) were seeded into scaffolds and cultured in growth media without added differentiation factors for up to 42 days. Three distinct hMSC donors (male, male, female; 20-26 years old) were evaluated by RT-qPCR and biochemical assays for GAG content and alkaline phosphatase (ALP) activity. Collagen I, II, and X production was observed with immunohistochemistry. Mixed ANOVA with Sidak post-hoc was performed with scaffold type as the within-group variable and donor as the between-group variable. *In vivo*: Under IACUC approval, full-thickness (3-mm depth, 4-mm diameter) critically sized osteochondral defect sites in 4 rabbits (female only for pilot study) were prepared in the trochlear groove of both hindlimbs for two observations per animal comparing dual-peptide scaffolds to PCL. Scaffolds were incubated in autologous bone marrow aspirate concentrate obtained during the same procedure before implantation. Samples harvested at 3 months were analyzed using microCT and histology.

RESULTS: *In vitro*: Gene expression and biochemical assays showed dual spatial scaffolds significantly increased hMSC chondrogenic (*SOX9*, GAG, Col II) and osteogenic (*RUNX2*, ALP, Col X) markers compared to PCL and single-peptide scaffold controls. At Day 28, dual spatial scaffolds induced collagen II and X deposition in HAbind and E3 regions, respectively. At Day 42, dual mixed scaffolds promoted upregulation of cartilage matrix genes, including lubricin (*PRG4*), aggrecan (*ACAN*), and cartilage oligomeric matrix protein (*COMP*) (Fig. 1A) and significantly higher GAG deposition compared to PCL and dual spatial scaffolds (Fig. 1B). In contrast, dual spatial scaffolds upregulated osteogenic genes osterix (*SP7*) and osteocalcin (*OC*) (Fig. 1A) and significantly higher ALP activity compared to PCL and dual mixed scaffolds (Fig. 1C). *In vivo*: Macroscopic images correlated with microCT (Fig. 2A) and histology (Fig. 2B) confirmed scaffolds remained in the defects in all groups for the entire 3-month study with minimal lymphohistiocytic inflammation. Spatial cartilage and bone formation and adjacent tissue integration was observed in respective superficial and deep zones of both dual spatial and dual mixed implants while unfunctionalized PCL scaffolds resulted in displaced cartilage matrix formed at the base of the defect (Fig. 2B left).

DISCUSSION: The *in vitro* results indicated that hMSC response was directed by dual-peptide presentation. The *in vivo* data correlated with dual-peptide scaffolds enhancing spatial tissue formation and integration compared to PCL scaffolds. These exciting results underscore how our peptide-functionalized scaffolds drive spatial tissue formation with endogenous cells and tissue.

SIGNIFICANCE/CLINICAL RELEVANCE: This work advances a novel biomaterial-based approach for functional osteochondral tissue regeneration towards clinical relevance as an early-stage treatment for osteoarthritis.

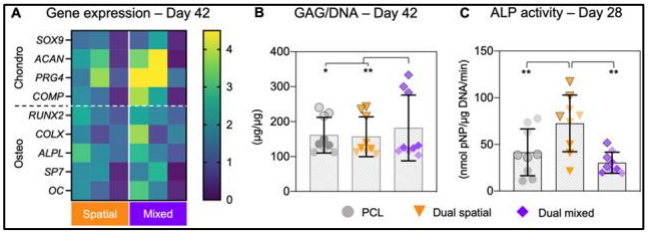

Fig. 1: Evaluation of (A) chondrogenic and osteogenic gene expression, (B) GAG deposition, and (C) ALP activity of hMSCs (three donors) cultured in 3D-printed scaffolds (*p<0.1, **p<0.05). Gene expression reported as fold-difference relative to PCL (N = 5/donor/group).[4]

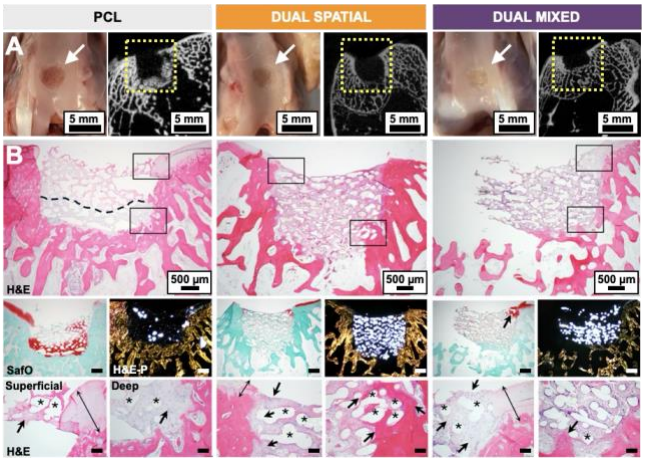

Fig. 2: Macroscopic images correlated with microCT (A) of scaffolds harvested at 3-months from critically sized osteochondral rabbit defects (left: arrow; right: box). (B) Histology showed proteoglycan-rich (chondroid) matrix at the base of PCL scaffolds while dual spatial and dual mixed scaffolds showed proteoglycan-rich matrix within superficial regions (upper box) and osteogenesis at the base (lower box). Corresponding high-magnification images showed PCL scaffold (asterisks) surrounded by loose primitive mesenchyme (arrow) at the interface of the flanking articular cartilage (double-headed arrows) compared to chondrogenesis and osteogenesis found in respective superficial and deep regions of both dual-peptide scaffolds. Central regions of all implants showed scaffold surrounded by primitive mesenchymal cells admixed with bland adipose islands, multinucleate giant cells, and few lymphoplasmacytic infiltrates. (rows 1 and 2: scale bar = 500 μm; row 3: scale bar = 100 μm)

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