Development of a Hydrogel-Based Cellular Model of Vocal Fold Lamina Propria

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Introduction: The human vocal folds (VFs) are responsible for sound production and can become damaged or dysfunctional due to biochemical, mechanical, and pathological factors. The VF consists of a stratified squamous epithelium, a matrix-rich lamina propria (LP), and the underlying vocalis muscle[1]. The LP of a newborn VF is a single homogeneous soft layer, and throughout adolescence, the LP slowly matures into a trilayered structure with varying composition of stiffness[2]. To establish a tissue engineered model of the VF LP, a bioorthognal hydrogel platform (Fig 1) is developed to enable user directed temporal evolvement of a laminated structure from a homogeneous cellular construct. Time and stiffness-dependent cellular responses, in terms of the expression of genes and proteins related to the matrix remodeling, is analyzed and compared to homogenous soft constructs.

Materials and Methods: Tetrazine (Tz) and *trans*-cyclooctene (TCO)-modified hyaluronic acid (HA-Tz, HA-TCO) as well as TCO-modified RGDSP (RGD-TCO) were prepared following our reported procedures (Fig 1A)[3]. Norbornene (Nb)-functionalized, matrix metalloprotease (MMP)-cleavable peptide crosslinker, SMR-bisNb (Fig 1A), was synthesized by reacting Nb-NHS with lysine-terminated SMR peptide[4]. To produce the initial soft cell-laden gel construct, human bone marrow-derived mesenchymal stem cells (hMSCs) were suspended in a HA-Tz/SMR-bisNb/RGD-TCO mixture with a Tz/Nb ratio of 5/2. Addition of HA-TCO on top of the soft gel construct led to matrix stiffening without adversely affecting the resident hMSCs (Fig 1B-C). Cell-gel constructs were cultured for 14 days and cellular responses to the changing microenvironment were analyzed by qPCR and immunofluorescence.

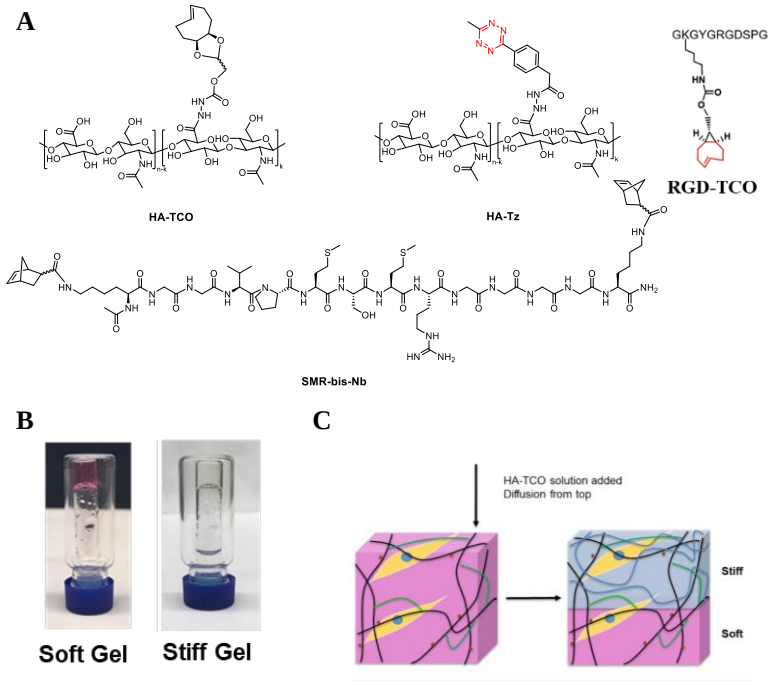

Fig. 1. Fabrication of a VF LP-mimetic bilayer hydrogel construct. (**A**) Chemical structures of HA-TCO, HA-Tz, SMR-bisNb and RGD-TCO. (**B**) The disappearance of the pink tetrazine chromophore indicates the consumption of Tz during interfacial crosslinking. (**C**) Termination of interfacial crosslinking halfway through the gel construct gives rise to a bilayered construct.

Results and Discussion: The soft gel construct was created via the slow Tz/Nb reaction exhibited an elastic shear modulus (G') of ~400 Pa. The matrix was stiffened by diffusion-controlled interfacial crosslinking through rapid Tz/TCO reaction 0- or 7-days post cell encapsulation, resulting in densely crosslinked hydrogels with a G' of ~2000 Pa. By controlling the interfacial reaction part-way through the gel, a bilayer construct was fabricated, with a stiff gel seamlessly superimposed on top of the soft gel (Fig 1C). First, matrix was stiffened immediately after cell encapsulation at day 0. hMSCs in both the original soft gels and the soft portion of the bilayer gels could spread out and adopted a spindle-shaped morphology, while those in the stiff gel or the stiffer region remained smaller and rounded after 14 days of culture. With a 7-day delayed matrix stiffening, cells were able to spread moderately. Day-7 stiffening were found to upregulate the expression of fibrotic ECM proteins (Col I, DCN, and FN EDA) , classic fibroblastic markers (TNC, FAP and FSP1) and ECM remodeling enzymes (MMP2, TIMP1 and HAS3), compared to the corresponding soft controls. Day-7 stiffening also upregulated catabolic activities, enhanced ECM turnover, and promoted YAP expression at both the transcript and the protein levels compared to the soft gel controls.

Conclusions: Employing bioorthogonal tetrazine ligation with slow and fast dienophiles, we have successfully fabricated a physiologically relevant *in vitro* tissue model of the VF LP. In-situ delayed matrix stiffening promoted a fibroblastic transition from hMSCs and enhanced YAP regulated mechanosensing expression.

Acknowledgement. This work was supported NIH (NIDCD, R01DC014461) and NSF (DMR 1809612). **Reference**: (1). Kutty, J. K., et al., *Tissue Eng. Part B*, **2009**, *15*, 249. (2). Hartnick, C.J. et al, *The Laryngoscope*. 2005. 115(1): 4. (3) Hao, Y. et al., *ACS Appl. Mater & Interfaces*, **2018**, *10*, 26016. (4) Patterson, J. et al., *Biomaterials*, **2010**, *31*, 7836.