Physiologic TGF-β and Glucose Administration Yield Functional Neocartilage While Maintaining Chondrocyte Metabolic Profile

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INTRODUCTION: A significant challenge for the field of cartilage tissue engineering (TE) is the fabrication of neocartilage grafts that can achieve long-term survival in the mechanochemical environment of the native synovial joint. This requires the recapitulation of a functional cartilaginous extracellular matrix (ECM), to achieve mechanical-load-supporting functionality, while maintaining the chondrocyte metabolic profile, required for continued biosynthesis in the low nutrient environment of the native synovial joint. Notably, in the sparse nutrient environment of synovial joint, native chondrocytes generally favor glycolysis over mitochondrial respiration to achieve requisite energy production[1].

Engineered cartilage growth is accelerated via chondrogenic media formulations comprised of supraphysiologic levels of nutrients—e.g., 4.5 mg/mL glucose—and anabolic growth factors –e.g., transforming growth factor beta (TGF-β) at 10 ng/mL. These levels are in significant excess of the concentrations present in native cartilage (glucose [0.5-1.0 mg/mL][2]; TGF-β [0.1-1.0ng/mL][3]). Supraphysiologic media formulations beneficially accelerate ECM biosynthesis; however, elevated levels of glucose and TGF-β are also associated with the induction of aberrant chondrocyte metabolism[4,5], akin to that observed in osteoarthritis (OA), suggesting that there may be unintended detrimental consequences of administering supraphysiologic media formulations to promote engineered cartilage development.

In our recent work, we demonstrated that the administration of physiologic doses of TGF-β (0.1-1.0ng/mL) in supraphysiologic glucose (4.5 mg/mL) medium accelerated ECM biosynthesis, yielding neocartilage with native-matched mechanical properties, while mitigating detrimental tissue features commonly associated with supraphysiologic TGF-β, such as aberrant collagen subtype expression and hypertrophy[6]. In the current study, we aim to further investigate potential benefits of adopting physiologic-based media formulations for cartilage regeneration by examining the combined effect of physiologic glucose and TGF-β concentrations on the ECM deposition and metabolic profile of tissue engineered constructs. Construct ECM development was evaluated via measures of construct mechanical properties and ECM distribution imaging. The metabolic profile of chondrocytes in constructs was evaluated via measures of glucose consumption rates and characterization of glycolysis and mitochondrial respiration using a Seahorse flux analyzer[7].

METHODS: Tissue constructs: Immature bovine chondrocytes pooled from metacarpal joints (n=8) were seeded in 2% w/v agarose at 3×10^6 cells/mL and prepared as cylindrical constructs (∅3×2mm). Constructs were cultivated in standard chondrogenic DMEM modified with either physiologic glucose (PhysGluc; 1 mg/mL) or supraphysiologic glucose (SupraGluc; 4.5 mg/mL) levels. Further, media was supplemented with TGF-β3 for the initial 2 weeks of culture at a physiologic dose (PhysTGF-β; 0.3ng/mL), supraphysiologic dose (SupraTGF-β; 10ng/mL), or maintained TGF-β-free. SupraGluc and PhysGluc medium was supplied at media-to-tissue volume ratios of 40:1 and 180:1, respectively, in order to avoid glucose depletion during culture, as validated via glucose assay measures. **Mechanical properties**: At day 49, constructs (n=4 per group) were evaluated for Young's modulus (E_Y) in unconfined compression. **ECM heterogeneities**: At day 49, constructs were sectioned and imaged with a Raman confocal microscope to evaluate the spatial distribution of GAG, collagen, and interstitial water constituents[8]. **Construct metabolism**: At day 14, a mitochondrial stress test and glycolytic rate analysis were performed on a Seahorse flux analyzer to evaluate rates of oxygen consumption indictive of mitochondrial respiration and proton efflux rate indictive of glycolysis. **Glucose consumption**: At days 14 and 28, live constructs from each group (n=3 per group per time point) were transferred to 0.6 mL of PhysGluc DMEM. Media glucose levels were measured after 6, 24, and 48 hours via AmplexRed glucose assay kit (Invitrogen). The transient decrease of glucose was curve-fit to a first order reaction kinetic model for determination of the glucose consumption rate constant.

RESULTS: Constructs cultivated with conventional chondrogenic medium (SupraGluc+SupraTGF-β) achieved a 3-fold enhancement in E_Y, relative to TGF-β-free culture, yielding native-matched E_Y levels. Constructs cultivated under the groups—PhysGluc+PhysTGF-β, SupraGluc+PhysTGF-β, or PhysGluc+SupraTGF-β achieved a similar enhancement in E_Y (2.5 to 3.5-fold relative to TGF-β-free) indicating that the combined use of supraphysiologic glucose and TGF-β is not required to achieve constructs with native-matched matched mechanical properties. SupraGluc+SupraTGF-β further induced significant enhancements in the rate of glucose consumption, proton efflux, and oxygen consumption, relative to TGF-β-free, indicating the induction of a hypermetabolic state. In contrast, PhysTGF-β and PhysGluc mitigated the increase in glucose consumption ($p<0.0021$), proton efflux ($p<0.0001$), and oxygen consumption ($p<0.0021$). Under PhysGluc, SupraTGF-β further induced pronounced ECM heterogeneities, as evidenced by depressed GAG and elevated water in the construct central regions, and construct cracking, consistent with heterogeneities in GAG-induced osmotic swelling pressure. In contrast, PhysTGF-β yielded constructs with a more uniform ECM distribution.

DISCUSSION: This study highlights a central paradox for cartilage tissue engineering—while traditional chondrogenic media formulations are effective at accelerating cartilage regeneration, they may also elicit the unintended consequence of triggering chondrocyte hypermetabolism. We observed a metabolic shift from conventional chondrogenic media (SupraGluc+SupraTGF-β) through increases in chondrocyte rates of glucose consumption, glycolysis, and mitochondrial respiration. Such metabolic changes can lead to long-term consequences such as mitochondrial dysfunction and even chondrosenescence[9]. Alternatively, the administration of physiologic levels of glucose and TGF-β maintain chondrocytes at a more physiologic metabolic profile, while still yielding neocartilage with native-matched mechanical properties. Interestingly, previous studies observed sub-optimal ECM biosynthesis and significant chondrocyte viability loss for constructs cultivated with physiologic glucose levels. These differences can likely be attributed to glucose depletion when administered at physiologic levels and low volumes. Here, the adoption of an elevated media-to-tissue volume ratio (180:1 vs 40:1) maintains steady glucose bath concentrations, enabling examination of the intrinsic effect of physiologic glucose levels on construct development. An unexpected outcome of this study was the pronounced ECM heterogeneities observed in constructs cultivated with PhysGluc+SupraTGF-β–heterogeneities for constructs of these relatively small dimensions (∅3×2mm) are typically not observed. These heterogeneities may result from glucose transport gradients created by the hypermetabolic elevated glucose consumption induced by SupraTGF-β. In contrast, constructs cultivated with PhysGluc+PhysTGF-β– achieve a far more uniform ECM, consistent with the lower glucose consumption maintained by PhysTGF-β.

SIGNIFICANCE: In contrast to conventional chondrogenic media, the utilization of media with physiologic glucose and TGF-β yields neocartilage with native-matched mechanical properties while mitigating hypermetabolism, thus increasing the potential of long-term neocartilage survival post-implantation.

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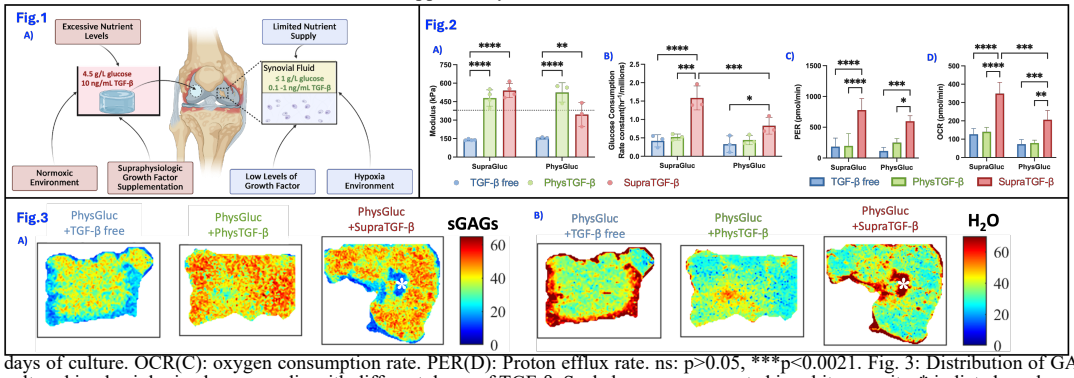

Fig. 1: (A) Differences between in vitro and in vivo environment for neocartilage. Fig. 2: (A) EY of constructs for different doses of TGF-β with supraphysiologic or physiologic glucose media. **p<0.009, ****p<0.0001. Dashed line represented native level modulus. (B) Glucose consumption rate constant normalized by cell density for constructs derived from TGF-β and glucose groups after 28 days of culture. ***p<0.0021. Metabolic rate of constructs for different doses of TGF-β at 14 days of culture. OCR(C): oxygen consumption rate. PER(D): Proton efflux rate. ns: p>0.05, ***p<0.0021. Fig. 3: Distribution of GAG (A) and water (B) inside tissue cultured in physiologic glucose media with different doses of TGF-β. Scale bars were presented in arbitrary units. * indicted cracks.