INTRODUCTION
Tissue engineered articular cartilage substitutes with functional properties resembling native cartilage tissue are needed to survive the harsh joint-loading environment after implantation into damaged articular cartilage. Engineered cartilage grown in culture for 8 weeks in a clinically-relevant agarose hydrogel scaffold achieves both stiffness and glycosaminoglycan (GAG) content comparable to native tissue. Developing functionally mature tissue within a shorter timeframe may help to increase their clinical use. We have previously reported that the addition of a second population of cells introduced in a co-culture setup with growing engineered cartilage constructs enhances tissue development via a paracrine signaling mechanism. In the current study, we investigate the dependence of this effect on the temporal application of the second population of cells as a means of optimizing their contribution to expediting engineered cartilage tissue formation in culture.

METHODS
Hydrogel Fabrication: Chondrocytes were harvested from immature bovine wrist joints and encapsulated in agarose (2% type VII, Sigma) discs (Φ x 2.33 mm, 30 million cells/mL). Discs were cultured in serum-free, chemically-defined media, and TGF-β3 (10 ng/mL for the first 14 days of culture) either in the absence (control) or presence of a chondrocyte monolayer, as previously described. Feeder Layer: Chondrocytes from the same harvest were plated at a density of 5.6 x 10⁴ cells/cm² beneath a Transwell© setup (Figure 1) and replaced weekly as follows. Feeder cells were introduced either as Study 1: immediately at the start of culture (day 0) or Study 2: after constructs had elaborated matrix and attained approximately half of the modulus and biochemical content as native bovine cartilage (day 42). Mechanical Testing: Constructs were tested in unconfined compression with samples being loaded to 10% strain at a strain rate of 0.005% strain/sec, after an initial 0.02N tare load and allowed to equilibrate (E₀). Dynamic modulus (G*), a functional measure of tissue properties, was measured by superimposing 2% peak-to-peak sinusoidal strain at 0.1Hz. Biochemistry: Constructs were proteinase K-digested, and glycosaminoglycan (GAG), collagen, and DNA content were determined using the DMMB dye assay, Alcian Blue and Picrosirius Red staining. Histology: Samples were fixed in acid formalin ethanol and paraffin embedded. 8 µm thick sections were stained with Alcian Blue and Picrosirius Red for proteoglycan and collagen distribution. Statistics: A one-way ANOVA with Tukey’s HSD post-hoc tests was used to compare groups with α<0.05.

RESULTS
Growth of engineered cartilage constructs was expedited and enhanced only for groups in which the constructs were exposed to the feeder cell population early in culture. As early as 14 days after introduction of a second population of chondrocytes to the culture, Young’s modulus, dynamic modulus and GAG were significantly increased over controls (reaching E₀=40kPa, G* 2.2MPa) with further increases seen by day 28

Figure 1. Experimental design for the feeder layer strategy. The Transwell© insert separates the engineered cartilage disc from the underlying feeder layer while promoting co-culture conditions.

Figure 2. Mechanical properties (Young’s modulus, a; Dynamic modulus, b) and biochemical content (GAG, c; Collagen, d) of constructs exposed to a feeder layer normalized to control samples (absence of feeder cells) with time of exposure. *p<0.05 vs. control, n=5/group.

Figure 3. Alcian Blue (left) and Picrosirius Red (right) stains for representative constructs co-cultured in the absence (top) and presence (bottom) of chondrocyte monolayers 14 days after exposure. Scalebar: 200µm

Figure 4. Histology sections of tissue from constructs cultured in the presence of a second population of cells showed enhanced intensity compared to the control (absence of cells) in both Alcian Blue and Picrosirius Red stains over time in culture. This staining is suggestive of advanced extracellular matrix development and deposition for sections exposed to a transiently changed monolayer (Figure 3). Differences in staining between groups were not observed when so-culture setup was initiated later in culture.

DISCUSSION
These results show that a time-dependence exists for the application of a second population of cells in our experimental setup. Similar to the temporal dependency seen with the addition of growth factors and other chemical factors, the introduction of soluble paracrine factors from a second population of cells may depend on the accessibility of these small molecules to penetrate both the agarose hydrogel and the developing extracellular matrix. As such, samples with a dense matrix and smaller porosity (more similar to native cartilage) may not have been exposed to the available chemical factors present in the media. Alternatively the response may be modulated by the age of the cells in culture, as older cells may not respond to the signaling cues in the same way. Taken together with our previous findings that the effect is only observed when the feeder layer is comprised of freshly harvested cells, these results suggest that optimization of the tissue culture model system depends on the timing of the introduction of the feeder cells, which then further dictate key characteristics of both populations.

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