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Neocartilage integration in temporomandibular joint discs: physical and enzymatic methods

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Integration of engineered musculoskeletal tissues with adjacent native tissues presents a significant challenge to the field. Specifically, the avascularity and low cellularity of cartilage elicit the need for additional efforts in improving integration of neocartilage within native cartilage. Self-assembled neocartilage holds significant potential in replacing degenerated cartilage, though its stabilization and integration in native cartilage require further efforts. Physical and enzymatic stabilization methods were investigated in an *in vitro* model for temporomandibular joint (TMJ) disc degeneration. First, in phase 1, suture, glue and press-fit constructs were compared in TMJ disc intermediate zone defects. In phase 1, suturing enhanced interfacial shear stiffness and strength immediately; after four weeks, a 15-fold increase in stiffness and a ninefold increase in strength persisted over press-fit. Neither suture nor glue significantly altered neocartilage properties. In phase 2, the effects of the enzymatic stabilization regimen composed of lysyl oxidase, $CuSO_4$ and hydroxylysine were investigated. A full factorial design was employed, carrying forward the best physical method from phase 1, suturing. Enzymatic stabilization significantly increased interfacial shear stiffness after eight weeks. Combined enzymatic stabilization and suturing led to a fourfold increase in shear stiffness and threefold increase in strength over press-fit. Histological analysis confirmed the presence of a collagen-rich interface. Enzymatic treatment additionally enhanced neocartilage mechanical properties, yielding a tensile modulus over 6 MPa and compressive instantaneous modulus over 1200 kPa at eight weeks. Suturing enhances stabilization of neocartilage, and enzymatic treatment enhances functional properties and integration of neocartilage in the TMJ disc. Methods developed here are applicable to other orthopaedic soft tissues, including knee meniscus and hyaline articular cartilage.

1. Introduction

Self-assembled neocartilage demonstrates notable potential in replacing a patient's pathological hyaline cartilage or fibrocartilage. The increased cellularity of neocartilage, compared with native autologous or allogeneic cartilage grafts, facilitates cell migration and matrix deposition at the integration boundary. Neocartilage formed through the self-assembling process, in which chondrocytes are seeded at high density in non-adherent wells, demonstrates markers of native cartilage development including upregulation of collagen VI initially, followed thereafter by collagen II synthesis [1]. For use in the self-assembling process, costal cartilage provides a clinically relevant cell source suitable for autologous tissue engineering. Costal cartilage may be isolated resulting in minimal donor site morbidity [2], and costochondral cells may be expanded *in vitro* while maintaining chondrogenic potential [3–5]. Costochondral cell neocartilage shows significant promise in replacing degenerated cartilage. However, challenges remain in establishing methods for stabilizing grafts and achieving integration in native cartilage [6–9].

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Temporomandibular joint (TMJ) disc degeneration represents the most prevalent clinical presentation in TMJ disorders [10]. While degenerative changes usually commence in the disc, often characterized by disc displacement, thinning and perforation, downstream pathology is observed in the surrounding soft tissue and adjacent articulating surfaces, consistent with osteoarthritis [11]. Previous investigation in replacing the TMJ disc with cell-seeded scaffolds resulted in implant displacement, necessitating further efforts in implant stabilization and integration techniques [12]. With the development of methods to stabilize neocartilage within the disc and facilitate integration, neocartilage may hold significant therapeutic potential for TMJ disc degeneration.

Suturing offers one means of improving initial stabilization of neocartilage within defects. Success of suturing depends largely on suture selection and suturing technique, including gauge, material and pattern. Suture gauge must be carefully selected such that the finest size, for example 5-0 or 6-0, is used, commensurate with the mechanical properties of the recipient tissue. Furthermore, synthetic, absorbable sutures may be selected based on *in situ* degradation rates. For example, poliglecaprone-absorbable cord suture, 5-0, retains 26% of original tensile strength after two weeks [13]. Considering suture pattern, interrupted sutures may be advantageous. In the event of suture rupture, in an interrupted pattern, only a single suture may be affected, compared with disrupting the entire closure for a continuous mattress pattern. Previous efforts investigating suture pull-out strength within native articular cartilage and neocartilage found the pull-out strength to be in the range of 4–5 MPa in native cartilage, compared with 1.5 MPa in neocartilage [14]. In an additional investigation of healing capacity in the TMJ discs of rabbits, suturing dermal grafts reapproximated disc structure grossly and histologically in surgically induced 2 × 4 mm focal defects [15]. While further investigation is warranted, suturing may offer a promising method to temporarily stabilize two adjacent tissues to encourage cell migration and matrix synthesis bridging the interface.

Tissue glue offers a second mechanism of maintaining the TMJ disc and neocartilage proximity towards enhancing integration. Randomized controlled trials have demonstrated the rates of infection and dehiscence (rupture along wound edge) are similar with sutures and cyanoacrylate tissue glues [16–18]. There are multiple types of cyanoacrylate glues. For example, 2-octylcyanoacrylate which possesses a longer polymer chain and increased strength, compared with butyl-2-cyanoacrylate. While glue may be used to stabilize neocartilage, if a glue layer results between the two tissues, cell migration may be inhibited diminishing integration potential.

Lysyl oxidase is a copper-dependent amine oxidase that mediates cross-linking of extracellular matrix proteins, collagen and elastin [19,20]. During collagen formation and repair, lysyl oxidase oxidizes amino groups on collagen's lysine residues to form reactive aldehydes, which react with others, condensing to form covalent pyridinoline cross-links [20], a reaction that is dependent upon molecular oxygen and copper ion. Previously, lysyl oxidase has been shown to enhance collagen cross-links towards improving integration of neocartilage and native hyaline cartilage [9]. Following two weeks integration *in vitro*, lysyl oxidase induced 2–2.2 times increase in apparent stiffness across the cartilage interface in both neocartilage-to-native cartilage and native-to-native cartilage interfaces [9]. Additionally, inhibition of lysyl oxidase by β-aminopropionitrile has been shown to inhibit integration between collagen explants [21,22].

Once implanted, the success of engineered cartilage also depends on the neocartilage functional properties. Previous work by our group has demonstrated that transforming growth factor β1 (TGF-β1) and a one-time chondroitinase ABC (C-ABC) treatment synergistically enhances collagen content and tensile properties of self-assembled costochondral cell neocartilage. At four weeks, this resulted in neocartilage demonstrating an average tensile modulus of 2 MPa and compressive instantaneous modulus of 650 kPa [23]. Finally, tensile properties have been shown to increase with lysyl oxidase-mediated cross-linking in articular cartilage, and with $CuSO_4$ and hydroxylysine supplementation in engineered cartilage [24,25]. Enhancing the functional properties of neocartilage will likely improve its success *in vivo*.

Characteristics such as the low cellularity and avascularity of cartilage result in challenges in graft integration [6–8,26,27]. Towards enhancing the potential for neocartilage retention and integration, this work investigated physical and enzymatic stabilization methods in phases 1 and 2, respectively. In phase 1, it was hypothesized that physical stabilization, using either suture or glue, would enhance the shear stiffness and strength at the integration interface initially and after four weeks *in vitro* culture, compared with press-fit neocartilage. In phase 2, it was hypothesized that long-term integration would be enhanced enzymatically through the application of lysyl oxidase, hydroxylysine and $CuSO_4$ following eight weeks *in vitro* culture. Additionally, it was hypothesized that the best stabilization regimen, selected from phase 1, when combined with enzymatic stabilization would further improve interfacial mechanical properties. Regarding neocartilage functional properties, in phase 1, it was hypothesized that neither suture nor glue would negatively affect biochemical content or mechanical properties. In phase 2, it was hypothesized that biochemical stimulation via TGF-β1 and C-ABC would enhance neocartilage properties over no treatment and enzymatic treatment would enhance tensile properties.

2. Material and methods

2.1. Cell isolation, expansion and redifferentiation

Costal cartilage was harvested from the asternal ribs of *Sus scrofa*, six months of age (University of California Davis, Animal Sciences Facility, Davis, CA) within 24 h of death. The perichondrium was excised and discarded, and remaining cartilage was minced and digested in 0.2% type II collagenase (Worthington, Lakewood, NJ) supplemented with 3% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) for 18 h at 37°C in chemically defined chondrogenic medium (CHG) composed of Dulbecco's modified Eagle's medium (DMEM) with 4.5 g l^{-1} glucose and GlutaMAX (Gibco, Grand Island, NY), 1% penicillin–streptomycin–fungizone (BD Biosciences, Bedford, MA), 1% ITS+ premix (BD Biosciences), 1% non-essential amino acids (Gibco), 100 nM dexamethasone, 50 µg ml^{-1} ascorbate-2-phosphate, 40 µg ml^{-1} L-proline and 100 µg ml^{-1} sodium pyruvate. Cells were isolated, counted and frozen in liquid nitrogen until use. Cells from six animals were pooled and expanded to third passage in CHG supplemented with 1 ng ml^{-1} TGF-β1, 10 ng ml^{-1} platelet-derived growth factor and 5 ng ml^{-1} basic fibroblast growth factor (Peprotech, Rocky Hills, NJ) [3,28,29]. Cells were seeded in T-225 flasks at 2.5×10^4 cells cm^{-2} and passaged at 80–90% confluence with 0.5% trypsin–EDTA (Gibco), followed by digestion with 0.2% collagenase solution, as described above, for 45 min. Following expansion, cells were redifferentiated in

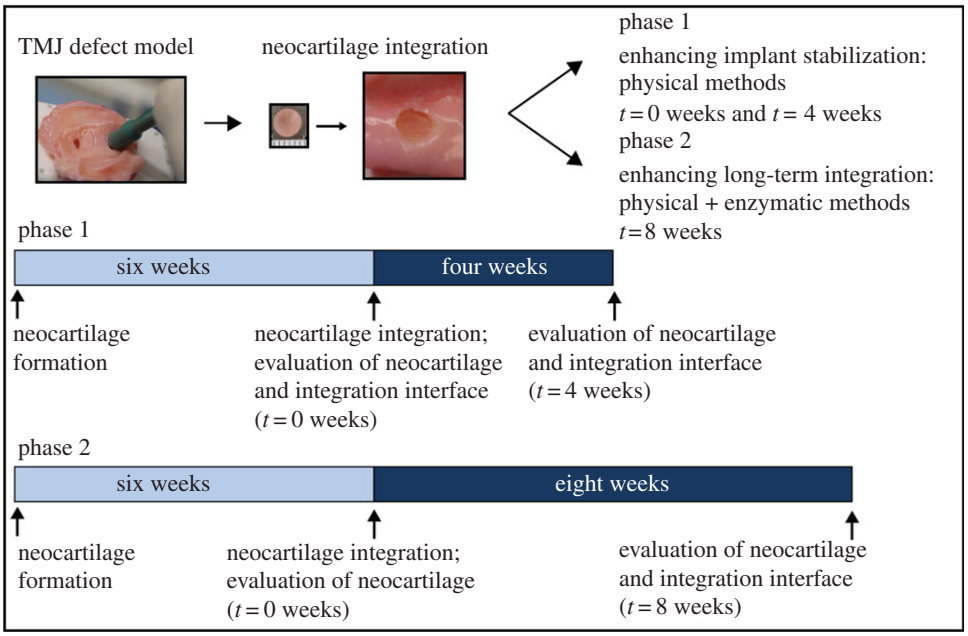

Figure 1. Study design. Self-assembled costochondral cell cartilage was allowed to mature for six weeks prior to integration in phases 1 and 2. Phase 1 examined physical stabilization methods and neocartilage properties. Phase 2 examined physical and enzymatic stabilization methods and neocartilage properties. (Online version in colour.)

aggregate culture to enhance chondrogenic potential [30,31]. During 12 days in aggregate culture, cells were maintained on agarose-coated Petri dishes at 750 000 cells ml^{-1} in CHG supplemented with 10 ng ml^{-1} TGF-β1. Aggregates were maintained in rotation for the first 24 h on an orbital shaker at 50 r.p.m. Following redifferentiation, aggregates were digested in 0.5% trypsin–EDTA (Gibco) for 45 min, followed by digestion with 0.2% collagenase solution for 1 h.

2.2. Neocartilage formation and stimulation

Non-adherent agarose wells, 5 mm in diameter, were generated using custom-built stainless steel moulds. To each well of a 48 well plate, 900 µl molten 2% agarose (Fisher Scientific, Fair Lawn, NJ) in phosphate-buffered saline (Sigma, St Louis, MO) was added, and the mould was submerged. Once the agarose solidified (17 min), the mould was released. Several exchanges of CHG medium were used to saturate the wells prior to cell seeding. To each well, 2×10^6 cells were added in 100 µl CHG, corresponding to a concentration of 2×10^7 cells ml^{-1}. At no point were cells encapsulated within the agarose. After 4 h, 400 µl CHG with or without TGF-β1 was added to each well, and medium was exchanged every 24 h thereafter. After 5 days, neocartilage was released from the agarose wells and maintained for 37 days subsequently in a 48 well plate, each well coated with 100 µl 2% agarose. Once unconfined, 1 ml CHG was exchanged every 48 h. Neocartilage was grown for a total of six weeks.

Catabolic enzyme, C-ABC, and anabolic protein, TGF-β1, were employed to enhance the mechanical properties of engineered cartilage [23]. Neocartilage was treated with 2 unit ml^{-1} C-ABC in CHG for 4 h on day 14. C-ABC was activated with 0.05 M sodium acetate (Sigma) and inactivated with 2 mM Zn^{2+} (Sigma). TGF-β1 was employed at 10 ng ml^{-1} continuously throughout culture.

2.3. Physical stabilization: phase 1

TMJ discs were isolated sterilely from *S. scrofa*, 6 months of age (Yosemite Meat Company, Modesto, CA). Discs were washed in four exchanges of DMEM containing 1% penicillin–streptomycin–fungizone and maintained for 4 days. Excess tissue was dissected from the periphery of the discs, and each disc was cut in half anteroposteriorly, yielding two disc samples. A 5 mm biopsy punch was used to generate a defect in the central portion of each sample. Neocartilage was either (i) press-fit, (ii) glued (surgi-lock 2oc, Meridian, Omaha, NE) or (iii) sutured (Monocryl 5-0 Violet Monofilament, Ethicon, Somerville, NJ) into the defect (figure 1), using $n = 3–4$ samples per group. To simulate immediate post-operative loading, the initial stabilization of the neocartilage within the disc defect was tested following 4 h of culture (figure 1). Additionally, the stabilization of the neocartilage was assessed following four weeks of *in vitro* culture. Throughout *in vitro* culture, 8 ml of CHG medium was exchanged once weekly.

2.4. Enzymatic stabilization: phase 2

The best physical stabilization method identified in phase 1 was carried forward to phase 2. Using a full factorial design, the effects of physical and enzymatic stabilization were assessed (figure 1). The enzymatic stabilization method employed was developed based on previously demonstrated beneficial effects of copper sulfate and hydroxylysine [24] on collagen cross-linking in neocartilage and in neocartilage–articular cartilage integration [9]. In neocartilage receiving enzymatic stabilization, CHG medium was supplemented with 0.15 µg ml^{-1} lysyl oxidase homologue 2 (SignalChem, Richmond, British Columbia, Canada), 1.6 µg ml^{-1} copper sulfate (Sigma) and 0.146 mg ml^{-1} hydroxylysine (Sigma) during weeks 2–6 of culture. Furthermore, following integration, weekly medium exchanges were likewise supplemented.

2.5. Mechanical and histological analysis of disc–neocartilage interface

The shear modulus and strength of the disc–neocartilage interface were assessed using an Instron 5565 (Instron, Norwood, MA) via a push-through compressive test (American Society for Testing Materials standard D732). The native TMJ disc tissue was mounted, and a cylindrical platen (4 mm diameter) was passed through the defect at a constant strain rate of 1% neocartilage thickness per second. The cross-sectional area of the construct perimeter was used to calculate stress, and stress–strain curves were generated from the load–displacement curve. Shear modulus and strength were quantified. The TMJ disc–neocartilage interface

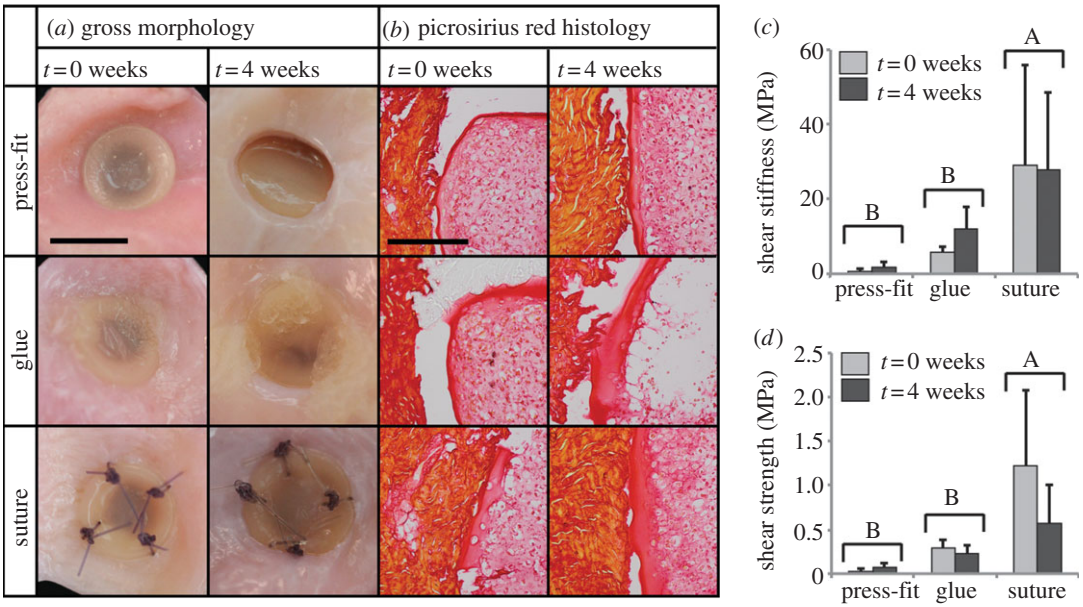

Figure 2. Phase 1 integration interface properties. Costochondral cell neocartilage was placed in defects in the intermediate zone of TMJ discs. (*a*) Gross morphology, (*b*) histology and (*c*) interface shear stiffness and (*d*) strength were evaluated prior to ($t = 0$ weeks) and following integration *in vitro* ($t = 4$ weeks), for press-fit, glue and suture groups. Gross morphology scale bar, 5 mm. Histology scale bar, 250 µm. Data are presented as mean ± standard deviation. Factors not connected by a common letter are significantly different ($p < 0.05$). (Online version in colour.)

was assessed histologically. Disc–neocartilage complexes were frozen in Histoprep tissue embedding media (Fisher Scientific), sectioned at 14 µm, fixed in formalin and stained with picrosirius red for collagen.

2.6. Neocartilage biochemical and mechanical analysis

Neocartilage that was implanted into the TMJ discs was also evaluated biochemically and mechanically. Collagen and sulfated glycosaminoglycan (sGAG) content was quantified following digestion in 125 µg ml^{-1} papain (Sigma) in phosphate buffer (pH 6.5). A modified hydroxyproline assay was used to quantify collagen [32] using Sircol collagen standard (Biocolor, Westbury, NY). Blyscan GAG assay kit was used to quantify sGAG (Biocolor). The mechanical properties of neocartilage were quantified in compression and tension. A 2 mm biopsy punch was used to generate a compression sample. The sample was compressed in stress-relaxation. The tissue was pre-stressed with 15 cycles of 5% strain followed by sequential stress-relaxation under 10 and 20% strain, using a rate of 10% tissue thickness per second. A load–displacement curve was generated and fitted using a Kelvin-solid viscoelastic model, from which the instantaneous and relaxation modulus values were derived [33]. A second 2 mm biopsy punch was made adjacent to the first, resulting in a dog-bone-shaped tensile specimen in the neocartilage. The tensile sample was fixed to paper tabs, establishing a consistent gauge length of 1.45 mm. The sample was strained at 1% gauge length per second to failure. Young's modulus and ultimate tensile strength (UTS) were evaluated from the load–displacement curve.

2.7. Statistical evaluation

In phase 1, a two-way analysis of variance (ANOVA) was used to detect significant differences in shear stiffness and strength with physical stabilization method and time as factors. Fisher's protected least significant difference (PLSD) and Student's *t*-test *post hoc* tests were used to detect significant differences between physical stabilization methods and time, respectively, where warranted. In phase 2, a two-way ANOVA was used to detect significant differences in shear stiffness and strength with enzymatic and physical stabilization methods as factors, followed by Student's *t*-test *post hoc*, where warranted. In both phases, a one-way ANOVA was used to detect significant differences in neocartilage properties, followed by Fisher's PLSD *post hoc* test where warranted.

3. Results

3.1. Phase 1 disc–neocartilage interface: gross morphology and histology

Gross morphology was assessed at $t = 0$ weeks and $t = 4$ weeks (figure 2*a*). Press-fit neocartilage, initially within the disc, could easily be disturbed with manoeuvring at $t = 0$ and $t = 4$ weeks (figure 2*a*). Glue at the disc–neocartilage interface was visible initially and after four weeks. Glued neocartilage was grossly stabilized at $t = 0$ weeks and at $t = 4$ weeks. Sutured neocartilage demonstrated firm attachment at the suture locations. Following four weeks, stabilization persisted and sutures showed degradation, as evidenced by suture fragmenting.

Histologically, suturing resulted in greater interface contact between the two surfaces compared with other stabilization methods at both time points (figure 2*b*). Press-fit resulted in some direct contact between the neocartilage and disc, but gaps were apparent. Glue yielded a persistent gap between the tissues. Additionally, a separation was apparent in some glued samples with the glued surface separating from the tissue bulk (figure 2*b*).

3.2. Phase 1 disc–neocartilage interface: mechanical properties

Mechanical properties at $t = 0$ weeks and $t = 4$ weeks are shown in figure 2*c*,*d*. Suturing was a significant factor for both shear modulus ($p = 0.01$) and shear strength ($p = 0.01$), independent of time. Suturing increased the shear modulus compared with press-fit, at $t = 0$ weeks (29.1 ± 26.8 versus 0.7 ± 0.5 MPa) and at $t = 4$ weeks (27.8 ± 20.9 versus 1.9 ± 1.3 MPa). Glue yielded shear moduli of 5.7 ± 1.7 MPa at

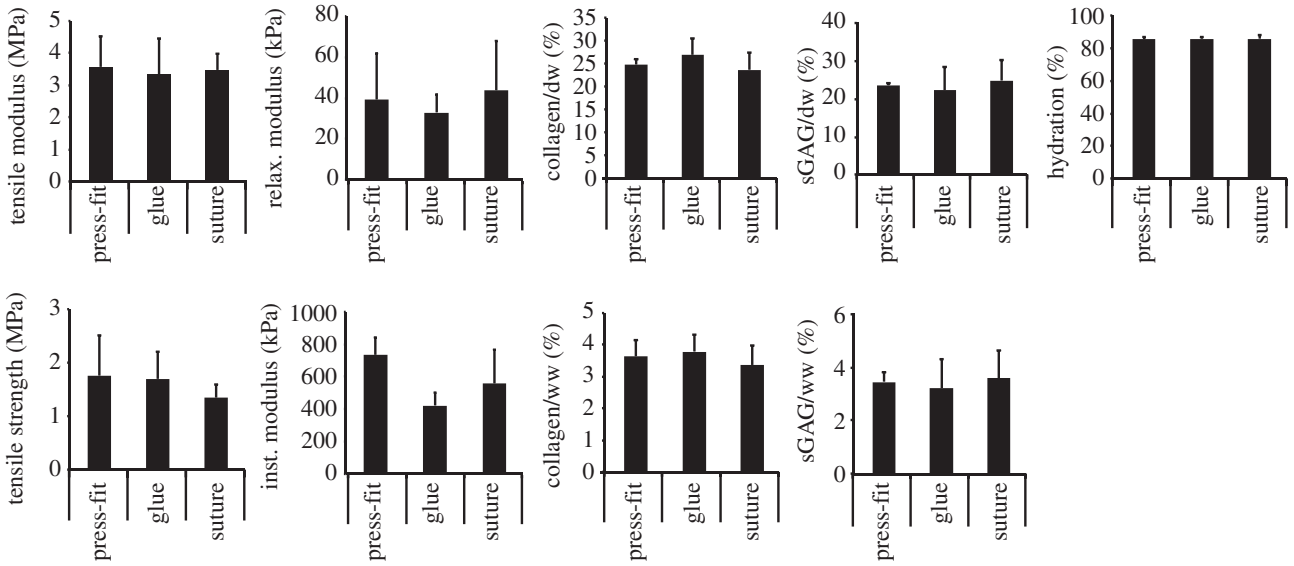

Figure 3. Phase 1 neocartilage mechanical and biochemical properties. Neocartilage properties were evaluated following integration *in vitro*, for press-fit, glue and suture groups. All properties were found to be independent of physical stabilization method. Data are presented as mean ± standard deviation. Groups not connected by a common letter are significantly different ($p < 0.05$).

$t = 0$ weeks and 12.0 ± 5.9 MPa at $t = 4$ weeks, which was not significantly different from press-fit.

3.3. Phase 1 neocartilage: mechanical properties and biochemical content

Following integration ($t = 4$ weeks), no significant differences were detected in mechanical properties or biochemical content of press-fit, glued or sutured neocartilage (figure 3). Regarding tensile properties, tensile moduli of 3.6 ± 1.0, 3.3 ± 1.1 and 3.5 ± 0.5 and UTS of 1.8 ± 0.8, 1.7 ± 0.5 and 1.3 ± 0.2 MPa were demonstrated in press-fit, glued and sutured neocartilage, respectively. Compressive properties presented were obtained under 20% strain. Compressive instantaneous moduli of 734.8 ± 110.0, 422.7 ± 76.2 and 558.4 ± 209.4 kPa and relaxation moduli of 39.3 ± 22.8, 33.0 ± 8.5 and 43.9 ± 24.1 kPa were measured in press-fit, glued and sutured neocartilage, respectively. Regarding biochemical content, press-fit neocartilage was composed of 24.8 ± 1.3% collagen and 23.6 ± 0.5% sGAG, glued neocartilage was composed of 26.9 ± 3.7% collagen and 22.4 ± 6.1% sGAG, and sutured neocartilage was composed of 23.7 ± 3.7% collagen and 24.9 ± 5.5% sGAG per dry weight.

3.4. Phase 2 disc–neocartilage interface: gross morphology and histological evaluation

Based upon superior integration and no deleterious effects on neocartilage biochemical content or mechanical properties, suturing was carried forward to phase 2. Gross morphology and histology are depicted in figure 4*a*,*b*. Enzymatically treated neocartilage and sutured neocartilage remained fixed within the defect after eight weeks. However, press-fit neocartilage was easily disrupted with minimal manipulation (figure 4*a*). Both in the presence and absence of enzymatic treatment, sutures had disintegrated. Histologically, a band of intense collagen staining was apparent at the interface of sutured, enzymatically treated and combined treatment neocartilage. Press-fit neocartilage demonstrated a small amount of separation from the disc remaining at eight weeks.

3.5. Phase 2 disc–neocartilage interface: mechanical properties

Mechanical properties of the disc–neocartilage interface, assessed at $t = 8$ weeks, are presented in figure 4*c*,*d*. Enzymatic stabilization significantly increased shear modulus, independent of physical stabilization method ($p = 0.03$). Additionally, suturing led to a trending increase in shear strength over press-fit ($p = 0.06$), independent of enzymatic stabilization method. The interaction term between main effects (physical stabilization and enzymatic stabilization) was not found to be significant in the ANOVA. Enzymatic stabilization alone yielded a shear stiffness of 17.1 ± 9.2 MPa and strength of 0.2 ± 0.02 MPa, whereas suturing alone yielded a stiffness of 11.4 ± 6.9 MPa and strength of 0.3 ± 0.05 MPa. Combined enzymatic stabilization and suturing led to a shear stiffness of 16.8 ± 7.7 MPa, compared with that of press-fit, 4.3 ± 3.0 MPa, and shear strength of 0.5 ± 0.3 MPa, compared with that of press-fit, 0.2 ± 0.1 MPa.

3.6. Phase 2 neocartilage: mechanical properties and biochemical content

Neocartilage in the absence of treatment was evaluated and compared with biochemically treated or combined biochemically and enzymatically treated neocartilage (figure 5). At $t = 0$ weeks, biochemical stimulation significantly decreased hydration, and increased collagen content and tensile properties over no treatment (hydration: 83.6 ± 0.9 versus 85.6 ± 1.1%, Col/dw: 18.5 ± 0.8 versus 8.4 ± 0.9%, E_Y: 3.7 ± 1.0 versus 1.8 ± 0.3 MPa, UTS: 1.5 ± 0.3 versus 0.6 ± 0.2 MPa). Combined biochemical and enzymatic treatment yielded tensile stiffness of 6.0 ± 1.3 MPa and strength of 2.2 ± 0.3 MPa, which were significantly increased over biochemical stimulation alone. No significant differences were detected in biochemical content or compressive properties (combined treatment properties: E_R: 98.4 ± 21.5 kPa, E_i: 758.5 ± 335.7 kPa, Col/dw of 20.4 ± 2.2%, sGAG/dw of 34.0 ± 3.9% and hydration of 84.0 ± 1.0%). Compressive properties presented were obtained under 20% strain.

At $t = 8$ weeks, combined biochemically and enzymatically treated neocartilage retained significantly greater tensile

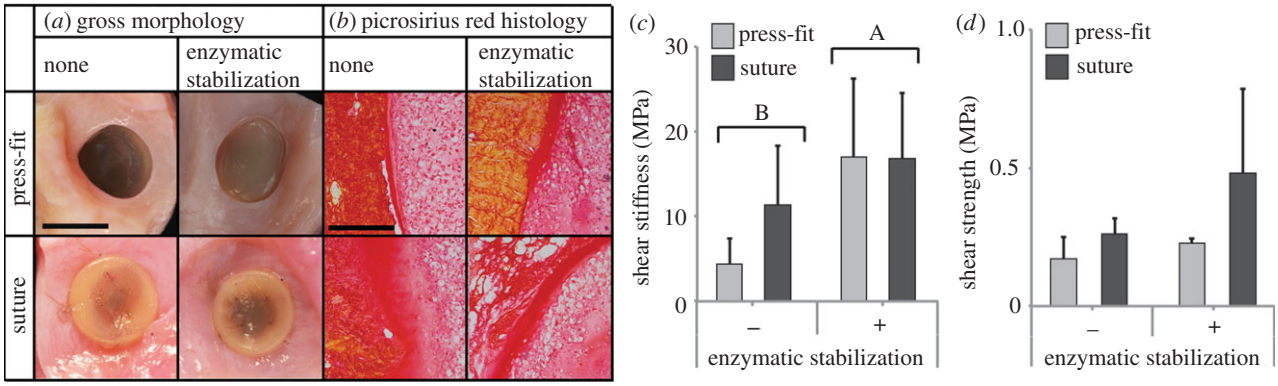

Figure 4. Phase 2 integration interface properties. (*a*) Gross morphology, (*b*) histology and (*c*) interface shear stiffness and (*d*) strength were evaluated following eight weeks *in vitro* culture for press-fit, enzymatic stabilization, suturing and combined enzymatic stabilization and suturing. Enzymatic stabilization represents treatment with 0.15 μg ml^{-1} lysyl oxidase, 1.6 μg ml^{-1} copper sulfate and 0.146 mg ml^{-1} hydroxylysine. Gross morphology scale bar, 5 mm. Histology scale bar, 250 μm. Data are presented as mean ± standard deviation. Factors not connected by a common letter are significantly different ($p < 0.05$). (Online version in colour.)

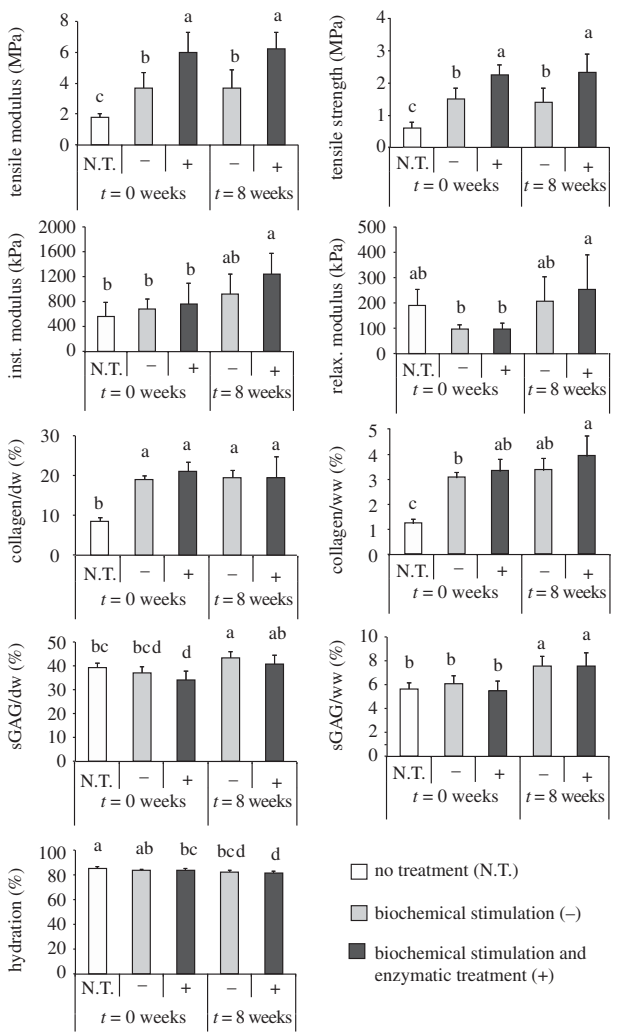

Figure 5. Phase 2 neocartilage mechanical and biochemical properties. Neocartilage properties were evaluated prior to ($t = 0$ weeks) and following integration *in vitro* ($t = 8$ weeks) for neocartilage without stimulation (N.T.), neocartilage stimulated with TGF-β1 and C-ABC (−), and neocartilage stimulated with TGF-β1 and C-ABC and enzymatic treatment (+). Data are presented as mean ± standard deviation. Groups not connected by a common letter are significantly different ($p < 0.05$).

properties compared with biochemical treatment alone (E_Y: 6.2 ± 1.1 versus 3.7 ± 1.2 MPa, UTS: 2.3 ± 0.6 versus 1.4 ± 0.4 MPa). Again, no significant differences were detected in hydration, biochemical content or compressive properties between combined and biochemical treatment alone (hydration: 81.4 ± 2.0 versus 82.5 ± 1.5%, Col/dw: 18.9 ± 1.7 versus 18.9 ± 1.8%, sGAG/dw of 40.6 ± 3.8 versus 43.3 ± 2.7%, E_R: 255.2 ± 135.6 kPa versus 208.5 ± 95.7 kPa, E_i: 1243.7 ± 336.4 versus 917.4.2 ± 327.5 kPa). Both the combined treatment and biochemical treatment demonstrated significantly greater sGAG content at $t = 8$ weeks, compared with $t = 0$ weeks, respectively. Combined treatment additionally significantly increased compressive properties at $t = 8$ weeks, compared with $t = 0$ weeks.

4. Discussion

The inherent low cellularity and avascularity of cartilage yields challenges in graft integration [6]. Self-assembled cartilage demonstrates enhanced potential for integration owing to its high cellularity and matrix production compared with native cartilage. This study sought to further enhance the integration potential of engineered cartilage in TMJ disc defects through physical and enzymatic stabilization methods. In phase 1, the hypothesis that physical stabilization (suture or glue) would enhance shear stiffness and strength at the integration interface initially and after four weeks *in vitro*, compared with press-fit neocartilage, was confirmed but only for suture. Glue did not significantly alter interfacial mechanical properties, compared with press-fit. Regarding tissue properties, following four weeks integration, no significant differences were detected in biochemical or mechanical properties between sutured, glued or press-fit neocartilage. In phase 2, the hypothesis that the mechanical integrity of the integration interface would be enhanced enzymatically by cross-linking mediators following eight weeks *in vitro* culture was confirmed. Enzymatic stabilization significantly increased shear stiffness. Furthermore, prior to integration, biochemical stimulation significantly enhanced the mechanical and biochemical properties of neocartilage, compared with no treatment. At both time points, enzymatic treatment significantly increased neocartilage mechanical properties. Towards improving *in vivo* success of engineered cartilage, in this study, suturing enhanced initial stabilization of the neocartilage in the TMJ disc, biochemical stimulation enhanced neocartilage functional properties, and enzymatic treatment enhanced both neocartilage properties and integration properties.

Suturing enhanced stabilization and integration of neocartilage in TMJ disc defects initially and following *in vitro* culture. As a factor, suturing significantly increased the shear stiffness and strength of the integration interface independent of time, compared with press-fit and glued neocartilage. Suturing yielded a 40-fold increase in both shear stiffness and strength over press-fit at $t = 0$ weeks. After four weeks, a 15-fold increase in shear stiffness and a ninefold increase in strength over press-fit were detected. *In vivo*, the half-life of 5-0 monocryl (poliglecaprone 25) sutures is approximately 7 days, and histology confirms complete resorption in 90–110 days [13]. After two weeks of subcutaneous implantation, 5-0 monocryl sutures maintain 26% breaking strength [13]. Owing to their mechanical properties and degradation time, monocryl sutures maintain tissue proximity to allow for cell-based integration between neocartilage and native TMJ disc. Histologically, it was apparent that greater surface area of neocartilage remained in contact with native cartilage with suturing. Furthermore, from four to eight weeks, increased collagen deposition is apparent in the interface of sutured neocartilage. At eight weeks, this yielded an interfacial stiffness over 11 MPa. This suggests persistence of the sutures and increased tissue contact contribute to cell-based integration.

Tissue glue grossly stabilized neocartilage in disc defects, though a significant difference was not detected in interfacial mechanical properties, compared with press-fit. Glue achieved a shear strength over 280 kPa initially and 220 kPa after four weeks integration. Visible histologically, once the glue solidified, a gap was apparent between the native tissue–neocartilage interface. Furthermore, separation of the neocartilage bulk from the glued periphery was apparent in some samples. Previous work bonding hydrogels to articular cartilage with chondroitin sulfate adhesive achieved a shear strength of 46 kPa, exceeding the hydrogel's bulk strength (40 kPa) [26]. Cellularization of the hydrogel induced matrix production in the hydrogel and interface [26]. Indeed, in the present study, the impermeable nature of the cyanoacrylate used may have inhibited cells from migrating across the interface thus not allowing matrix production. Allowing for cell migration across the interface by employing a bioactive agent such as chondroitin sulfate adhesive and achieving a more consistent glued boundary may enhance the success of glues in neocartilage integration.

The enzymatic stabilization regimen, composed of lysyl oxidase, $CuSO_4$ and hydroxylysine, significantly increased shear stiffness of the integration interface, independent of physical stabilization method. Previously, in articular cartilage–neocartilage integration, a tensile test demonstrated lysyl oxidase induced an apparent interfacial stiffness of 1.5 MPa when treated two weeks prior to and during one week of integration [9]. In this study, detected by a push-through test, which assesses primarily shear mechanics, an interfacial stiffness of 17 MPa was measured with lysyl oxidase, $CuSO_4$ and hydroxylysine treatment, following eight weeks integration. Lysyl oxidase mediates the formation of pyridinoline cross-links between collagen fibrils through the generation of reactive aldehydes on collagen's lysine groups [20]. Importantly, collagen cross-linking correlates positively with tensile properties in articular cartilage [25]. In the presence of enzymatic stabilization, the interface between neocartilage and native TMJ disc stained intensely for picrosirius red, confirming collagen joining the two tissues. Additionally, the notable mechanical properties in this study validate the benefits of continuous delivery of the enzyme during integration. As such, future *in vivo* efforts may also explore a system for controlled release. A lysyl-oxidase-based enzymatic treatment significantly increased the shear stiffness of the integration interface of neocartilage in a TMJ disc defect.

Combined enzymatic stabilization plus suturing did not significantly increase the interfacial mechanical properties over single treatments at eight weeks. Our hypothesis that combined treatment would beneficially enhance mechanical properties of the interface over single treatments was not confirmed at eight weeks. A time course analysis is needed to assess the interfacial properties as sutures degrade and as collagen is synthesized and cross-linked at the interface. *In vitro*, it appears the tissues were in sufficient proximity for collagen deposition and cross-linking across the interface without suturing. However, *in vivo* mobility will likely require suturing to achieve and maintain proximity for cross-link-mediated integration. Furthermore, it was demonstrated in phase 1 that sutures result in no deleterious effects on neocartilage properties compared with press-fit. While combined physical and enzymatic stabilization did not yield significant increases in mechanical properties of the interface compared with single treatments, mobility post-implantation will likely necessitate suturing for successful integration *in vivo*. One limitation of this study was the absence of native tissue controls in integration investigations. Subsequent studies may investigate integration of native TMJ disc punches within the defect model established here. *In vivo* studies will additionally aid in establishing target interface mechanical properties indicative of restoring joint function.

Functionality of engineered cartilage *in vivo* additionally depends on the cartilage's ability to sustain loads. Both biochemical and enzymatic treatment enhanced neocartilage functional properties. Stimulation with TGF-β1 and C-ABC significantly increased collagen content and tensile stiffness and strength in neocartilage, evaluated prior to integration. Additionally, enzymatic treatment combined with biochemical treatment further increased the tensile stiffness and strength of neocartilage, over biochemical treatment alone. Neocartilage demonstrated tensile moduli of 6.0 and 3.7 MPa with and without enzymatic treatment, respectively, prior to integration. This is within range of the modulus of the TMJ disc mediolaterally (approx. 4 MPa) [34]. Following eight weeks integration *in vitro*, in the presence of enzymatic treatment, sGAG content and compressive properties increased, and no significant differences were detected in collagen content or tensile properties, compared with prior to integration. Compressive instantaneous and relaxation moduli over 1200 and 250 kPa, respectively, with enzymatic treatment and 915 and 200 kPa, respectively, without enzymatic treatment were achieved. The TMJ disc demonstrates instantaneous and relaxation moduli of approximately 500 and 120 kPa, respectively [33]. Prior to and following integration, biochemical and enzymatic treatment beneficially enhance the biochemical content and mechanical properties of neocartilage, achieving or exceeding the range of TMJ disc properties.

5. Conclusion

Owing to the avascularity and low cellularity of cartilage, methods for enhancing graft stabilization and integration are necessitated. Costochondral cell neocartilage offers significant potential in addressing cartilage pathologies. Costochondral

cells may be isolated resulting in minimal donor site morbidity, expanded *in vitro*, and self-assembled to form mechanically robust neocartilage. In this study, costochondral cell neocartilage stabilization and integration were enhanced in a TMJ disc defect model. Suturing significantly increased the shear stiffness and strength of the integration interface, whereas glue did not significantly alter interface properties, compared with press-fit. The enzymatic stabilization regimen, composed of lysyl oxidase, $CuSO_4$ and hydroxylysine, significantly increased the shear stiffness of the integration interface. In combining enzymatic stabilization with suturing, no significant differences were detected in the mechanical properties of the interface compared with single treatments. However, *in vivo* mobility will likely require physical stabilization to maintain tissue proximity for enzymatically driven collagen cross-linking. Additionally, biochemical stimulation as well as enzymatic treatment significantly increased the biochemical content and mechanical properties of engineered cartilage. Self-assembled costochondral cell cartilage demonstrates significant potential for functionality and integration in the TMJ disc. Previous efforts have confirmed pre-treatment with this cross-linking regimen enhances interfacial tensile stiffness and strength by 730% and 745% in fibrocartilage grafts implanted subcutaneously [35]. Future work will explore translation of this pre-treatment approach in the TMJ. Furthermore, the methods developed here may also be applicable to the knee meniscus and hyaline articular cartilage.

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References

1. Ofek G, Revell CM, Hu JC, Allison DD, Grande-Allen KJ, Athanasiou KA. 2008 Matrix development in self-assembly of articular cartilage. *PLoS ONE* **3**, e2795. (doi:10.1371/journal.pone.0002795)
2. Kawanabe Y, Nagata S. 2006 A new method of costal cartilage harvest for total auricular reconstruction: part I. Avoidance and prevention of intraoperative and postoperative complications and problems. *Plast. Reconstr. Surg.* **117**, 2011–2018. (doi:10.1097/01.prs.0000210015.28620.1c)
3. Murphy MK, Huey DJ, Reimer AJ, Hu JC, Athanasiou KA. 2013 Enhancing post-expansion chondrogenic potential of costochondral cells in self-assembled neocartilage. *PLoS ONE* **8**, e56983. (doi:10.1371/journal.pone.0056983)
4. Tay AG, Farhadi J, Suetterlin R, Pierer G, Heberer M, Martin I. 2004 Cell yield, proliferation, and postexpansion differentiation capacity of human ear, nasal, and rib chondrocytes. *Tissue Eng.* **10**, 762–770. (doi:10.1089/1076327041348572)
5. Lee JD, Hwang O, Kim SW, Han S. 1997 Primary cultured chondrocytes of different origins respond differently to bFGF and TGF-β. *Life Sci.* **61**, 293–299. (doi:10.1016/S0024-3205(97)00385-8)
6. Zhang Z, McCaffery JM, Spencer RG, Francomano CA. 2005 Growth and integration of neocartilage with native cartilage *in vitro*. *J. Orthop. Res.* **23**, 433–439. (doi:10.1016/j.orthres.2004.08.028)
7. Obradovic B, Martin I, Padera RF, Treppo S, Freed LE, Vunjak-Novakovic G. 2001 Integration of engineered cartilage. *J. Orthop. Res.* **19**, 1089–1097. (doi:10.1016/S0736-0266(01)00030-4)
8. DiMicco MA, Sah RL. 2001 Integrative cartilage repair: adhesive strength is correlated with collagen deposition. *J. Orthop. Res.* **19**, 1105–1112. (doi:10.1016/S0736-0266(01)00037-7)
9. Athens AA, Makris EA, Hu JC. 2013 Induced collagen cross-links enhance cartilage integration. *PLoS ONE* **8**, e60719. (doi:10.1371/journal.pone.0060719)
10. Farrar WB, McCarty Jr WL. 1979 The TMJ dilemma. *J. Ala Dent. Assoc.* **63**, 19–26.
11. Ballesteros LE, Leon SF. 1999 Anatomical and pathological study of the temporomandibular joint disk in Colombian individuals. *Rev. Med. Chile* **127**, 1469–1474.
12. Ahtiainen K, Mauno J, Ella V, Hagstrom J, Lindqvist C, Miettinen S, Ylikomi T, Kellomaki M, Seppanen R. 2013 Autologous adipose stem cells and polylactide discs in the replacement of the rabbit temporomandibular joint disc. *J. R. Soc. Interface* **10**, 20130287. (doi:10.1098/rsif.2013.0287)
13. Bezwada RS *et al*. 1995 Monocryl suture, a new ultra-pliable absorbable monofilament suture. *Biomaterials* **16**, 1141–1148. (doi:10.1016/0142-9612(95)93577-Z)
14. DuRaine GD, Arzi B, Lee JK, Lee CA, Responte DJ, Hu JC, Athanasiou KA. In press. Biomechanical evaluation of suture-holding properties of native and tissue-engineered articular cartilage. *Biomech. Model. Mechanobiol.* (doi:10.1007/s10237-014-0589-1)
15. Wallace DW, Laskin DM. 1986 Healing of surgical incisions in the disc and retrodiscal tissue of the rabbit temporomandibular joint. *J. Oral Maxillofac. Surg.* **44**, 965–971. (doi:10.1016/S0278-2391(86)80050-7)
16. Singer AJ, Quinn JV, Clark RE, Hollander JE. 2002 Closure of lacerations and incisions with octylcyanoacrylate: a multicenter randomized controlled trial. *Surgery* **131**, 270–276. (doi:10.1067/msy.2002.121377)
17. Quinn J, Wells G, Sutcliffe T, Jarmuske M, Maw J, Stiell I, Johns P. 1997 A randomized trial comparing octylcyanoacrylate tissue adhesive and sutures in the management of lacerations. *JAMA* **277**, 1527–1530. (doi:10.1001/jama.1997.03540430039030)
18. Quinn JV, Drzewiecki A, Li MM, Stiell IG, Sutcliffe T, Elmslie TJ, Wood WE. 1993 A randomized, controlled trial comparing a tissue adhesive with suturing in the repair of pediatric facial lacerations. *Ann. Emerg. Med.* **22**, 1130–1135. (doi:10.1016/S0196-0644(05)80977-1)
19. Smith-Mungo LI, Kagan HM. 1998 Lysyl oxidase: properties, regulation and multiple functions in biology. *Matrix Biol.* **16**, 387–398. (doi:10.1016/S0945-053X(98)90012-9)
20. Kagan HM, Li W. 2003 Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J. Cell. Biochem.* **88**, 660–672. (doi:10.1002/jcb.10413)
21. Ahsan T, Lottman LM, Harwood F, Amiel D, Sah RL. 1999 Integrative cartilage repair: inhibition by β-aminopropionitrile. *J. Orthop. Res.* **17**, 850–857. (doi:10.1002/jor.1100170610)
22. DiMicco MA, Waters SN, Akeson WH, Sah RL. 2002 Integrative articular cartilage repair: dependence on developmental stage and collagen metabolism. *Osteoarthritis Cartilage* **10**, 218–225. (doi:10.1053/joca.2001.0502)
23. Murphy MK, Duraine GD, Reddi AH, Hu JC, Athanasiou KA. 2013 Inducing articular cartilage phenotype in costochondral cells. *Arthritis Res. Ther.* **15**, R214. (doi:10.1186/ar4409)
24. Makris EA, MacBarb RF, Responte DJ, Hu JC, Athanasiou KA. 2013 A copper sulfate and hydroxylysine treatment regimen for enhancing collagen cross-linking and biomechanical properties in engineered neocartilage. *FASEB J.* **27**, 2421–2430. (doi:10.1096/fj.12-224030)
25. Williamson AK, Chen AC, Masuda K, Thonar EJ, Sah RL. 2003 Tensile mechanical properties of bovine articular cartilage: variations with growth and relationships to collagen network components. *J. Orthop. Res.* **21**, 872–880. (doi:10.1016/S0736-0266(03)00030-5)
26. Wang DA, Varghese S, Sharma B, Strehin I, Fermanian S, Gorham J, Fairbrother DH, Cascio B, Elisseeff JH. 2007 Multifunctional chondroitin sulphate for cartilage tissue–biomaterial integration. *Nat. Mater.* **6**, 385–392. (doi:10.1038/nmat1890)
27. Ahsan T, Sah RL. 1999 Biomechanics of integrative cartilage repair. *Osteoarthritis Cartilage* **7**, 29–40. (doi:10.1053/joca.1998.0160)

28. Barbero A, Ploegert S, Heberer M, Martin I. 2003 Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. *Arthritis Rheum.* **48**, 1315–1325. (doi:10.1002/art.10950)
29. Barbero A, Grogan S, Schafer D, Heberer M, Mainil-Varlet P, Martin I. 2004 Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. *Osteoarthritis Cartilage* **12**, 476–484. (doi:10.1016/j.joca.2004.02.010)
30. Murphy MK, Masters TE, Hu JC, Athanasiou KA. In press. Engineering a fibrocartilage spectrum through modulation of aggregate redifferentiation. *Cell Transplant*. (doi:10.3727/096368913X676204)
31. Huey DJ, Athanasiou KA. 2013 Alteration of the fibrocartilaginous nature of scaffoldless constructs formed from leporine meniscus cells and chondrocytes through manipulation of culture and processing conditions. *Cells Tissues Organs* **197**, 360–371. (doi:10.1159/000346252)
32. Reddy GK, Enwemeka CS. 1996 A simplified method for the analysis of hydroxyproline in biological tissues. *Clin. Biochem.* **29**, 225–229. (doi:10.1016/0009-9120(96)00003-6)
33. Allen KD, Athanasiou KA. 2006 Viscoelastic characterization of the porcine temporomandibular joint disc under unconfined compression. *J. Biomech.* **39**, 312–322. (doi:10.1016/j.jbiomech.2004.11.012)
34. Murphy MK, Arzi B, Hu JC, Athanasiou KA. 2013 Tensile characterization of porcine temporomandibular joint disc attachments. *J. Dent. Res.* **92**, 753–758. (doi:10.1177/0022034513494817)
35. Makris EA, MacBarb RF, Paschos NK, Hu JC, Athanasiou KA. 2014 Combined use of chondroitinase-ABC, TGF-β1, and collagen crosslinking agent lysyl oxidase to engineer functional neotissues for fibrocartilage repair. *Biomaterials* **35**, 6787–6796. (doi:10.1016/j.biomaterials.2014.04.083)