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RESEARCH ARTICLE

Fetal dermal fibroblasts exhibit enhanced growth and collagen production in two- and three-dimensional culture in comparison to adult fibroblasts

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Abstract

The high morbidity of tendon injuries and the poor outcomes observed following repair or replacement have stimulated interest in regenerative approaches to treatment and, in particular, the use of cell-based analogues as alternatives to autologous and allogeneic graft repair. Given the known regenerative properties of fetal tissues, the objective of this study was to assess the biological and mechanical properties of tissue-engineered three-dimensional (3D) composites seeded with fetal skin cells. Dermal fibroblasts were isolated from pregnant rats and their fetuses and characterized in monolayer culture and on 3D resorbable polyester scaffolds. To determine the differences between fetal and adult fibroblasts, DNA, total protein and types I and III collagen production were measured. In addition, morphology and mechanical properties of the 3D constructs were examined. In monolayer culture, fetal fibroblasts produced significantly more types I and III collagen and displayed serum-independent growth, while adult fibroblasts elaborated less collagen and exhibited reduced cell spreading and attachment under low-serum conditions. In 3D culture, fetal constructs appeared more developed based on gross examination, with significantly more total DNA, total protein and normalized type I collagen production compared to adult specimens. Finally, after 35 days, fetal fibroblast-seeded constructs possessed superior mechanical properties compared to adult samples. Taken together, these findings indicate that fetal dermal fibroblasts may be an effective source of cells for fabricating tissue equivalents to regenerate injured tendons.

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Keywords **fetal; dermis; tendon; proliferation; collagen; scaffold; wound healing; tissue engineering**

1. Introduction

Soft tissue injuries, such as those affecting tendons and ligaments, are common in the USA (Praemer *et al.*, 1992). Each year, >150 000 reconstructive surgeries are performed annually to repair tendon defects (Woo *et al.*, 2000). Unfortunately, the tissue is often too damaged to salvage, and in other instances even robust-appearing tissue does not successfully heal. Thus, there is a need for graft replacement tissue. Graft replacement itself is limited by several factors, including the limited supply, donor site morbidity in the case of autografts and delayed remodelling and integration of the implanted material when allografts are used (Jackson *et al.*, 1996). Tissue engineering may offer an alternative source of graft material for tendon repair.

The performance of tissue-engineered constructs is influenced by the scaffold material, which serves as a

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template for tissue regeneration. A wide array of natural and synthetic biomaterials has been explored as scaffolds for tendon tissue engineering. Poly(glycolic acid) (PGA) meshes are synthetic, FDA-approved scaffolds commonly used because of their fibrous structure onto which the cells align. A study investigating the use of parallel aligned and non-aligned PGA fibres as a scaffold found that tenocytes on the aligned matrix exhibited greater collagen fibril organization after 6 weeks than on the non-aligned matrix, although the constructs only had one-third the mechanical strength of normal tendons (Cao *et al*., 1994). Several other studies have examined the combination of PGA with other scaffolding materials, including Dacron (Rodkey *et al*., 1985), collagen (Long *et al*., 2005), small intestinal submucosa (SIS) (Cao *et al*., 2002; Liu *et al*., 2006) and poly(L-lactic acid) (PLLA) (Kim *et al*., 1999). For example, Cao *et al*. encapsulated PGA fibres with SIS, and while these cell-seeded scaffolds were histologically similar to native tendons, by 12 weeks of culture, their ultimate tensile strength was only 78% of that of native tendon (Cao *et al*., 2002). In another study, PGA fibres were shown to withstand tensional strains up to 7% without damage when reinforced with PLLA (Kim *et al*., 1999). This type of composite scaffold is appealing for use in tendon reconstruction, since tendons typically experience strains in the range of 1–4% (Abrahams, 1967; West *et al*., 2004).

Previous investigations have also evaluated a variety of cell sources for tendon tissue engineering, including tenocytes (Cao *et al*., 1994; Cao *et al*., 2002), bone marrow-derived mesenchymal stem cells (Young *et al*., 1998; Awad *et al*., 1999, 2000, 2003; Butler and Awad, 1999; Ouyang *et al*., 2003; Kall *et al*., 2004) and dermal fibroblasts (Bellincampi *et al*., 1998; Chen *et al*., 2002). Cells derived from fetal tissues may represent a uniquely ideal source for regenerative therapies. Fetal skin and tendon heal scarlessly with recapitulation of the tissue architecture (Longaker *et al*., 1990; Lorenz *et al*., 1995; Lovvorn *et al*., 1999; Beredjiklian *et al*., 2003; Bullard *et al*., 2003; Colwell *et al*., 2003). Previous studies in monolayer culture have demonstrated that fetal dermal fibroblasts exhibit serum-independent behaviour with respect to both adhesion (Brink *et al*., 2005) and migration (Kondo *et al*., 1993; Kondo and Yonezawa, 1995), and elevated collagen production compared to adult fibroblasts (Thomas *et al*., 1988; Takeda *et al*., 1992; Lorenz and Adzick, 1993; Bullard *et al*., 2003; Goodman *et al*., 2004; Brink *et al*., 2005, 2006).

Although the properties of fetal and adult fibroblasts have been characterized for several different species (i.e. human, ovine and equine), those of fetal and adult rat fibroblasts have not been reported. In addition, few studies have attempted to engineer fetal cell-based tissue equivalents for regeneration of musculoskeletal tissues. Therefore, the objectives of this investigation were: (a) to evaluate the differences between fetal and adult rat dermal fibroblasts in monolayer culture in terms of serum dependence and collagen production; and (b) to assess proliferation, total protein, collagen content and mechanical properties of three-dimensional (3D) fibroblast-seeded scaffolds. These fundamental studies are important for further elucidating the differences between fetal and adult cells, but could also lay the foundation for future *in vivo* studies, using such constructs in a clinically relevant rat supraspinatus tendon injury model (Soslowsky *et al*., 1996). It was hypothesized that fetal dermal fibroblasts would exhibit enhanced growth and produce significantly more types I and III collagen compared to adult dermal fibroblasts in both monolayer and 3D culture.

2. Materials and methods

2.1. Primary cell culture

Skin specimens from the dorsa of five time-dated pregnant rats (16 days gestation; Ihara *et al*., 1990) and their fetuses were used to isolate dermal fibroblasts in accordance with the IACUC protocol No. 708 233. Rat dermal fibroblasts were characterized due to their ease of isolation and similarities to tenocytes. Tissue samples were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen: Gibco BRL, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 2.5 U/ml fungizone (Invitrogen, Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Gibco) to allow dermal fibroblasts to grow out from the explants. Medium with 20% serum was used during the initial isolation since it has been shown to enhance cell viability (compared to medium with 10% serum) when establishing fetal cell lines (Watanabe *et al*., 1997). Once confluent, the cells were passaged and maintained in DMEM supplemented with 10% FBS and antibiotics. Cells from passage 3 were used for all experiments, and the experiments were repeated for multiple dame/fetus pairs. Serum from the same lot was used for all studies.

2.2. Two-dimensional studies

2.2.1. Serum-dependent attachment and morphology

Dermal fibroblasts were plated in monolayer culture at a concentration of 12 500 cells/cm^2 and maintained in reduced serum medium for 3 days to assess serum-dependent effects on cell adhesion and growth. Three different low-serum medium formulations were used to culture the cells: DMEM with 0.5% FBS, antibiotics and 1% insulin-transferrin-selenium (ITS) (0.5% serum); DMEM with 1% FBS, antibiotics and 1% ITS (1% serum); and DMEM with 2% FBS, antibiotics and 1% ITS (2% serum). The ITS supplement was included to assist in maintaining cell viability under the severe culture conditions. Cells were allowed to attach to the tissue culture dishes for 24 h, after which 0.05 mg/ml ascorbic

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acid was added to facilitate collagen formation. At day 3, images of the different cell cultures were taken using a Zeiss Axiovert 200 inverted-phase microscope (Carl Zeiss Inc., Thornwood, NY, USA). Axiovision software was used to quantify the surface area of individual cells attached to the tissue culture plate and an average cell surface area was calculated for each medium and cell type. Approximately 45 different cells were measured for each group to determine the average cell surface area.

2.2.2. Collagen quantification

Dermal fibroblasts were plated in monolayer culture at a concentration of 12 500 cells/cm^2 and maintained in DMEM with 10% FBS and antibiotics for a period of 1, 3 or 7 days. Cells were allowed to attach to the tissue culture dishes for 24 h, after which 0.05 mg/ml ascorbic acid was added. Medium for all 7 day time points was changed at day 3 and fresh ascorbic acid was added on days 3 and 5. At each time point, fibroblasts from six samples for each cell type were isolated in 0.05 N acetic acid, pH 2.8. Samples were frozen at −20 °C prior to digestion for protein and DNA quantification.

At the designated time points, an enzyme-linked immunosorbant assay (ELISA) was performed to determine the production of types I and III collagen. Samples were removed from acetic acid and extracted with 500 µl 3 M guanidine HCl/0.05 M Tris–HCl, pH 7.5 (GuHCl), at 4 °C for 16 h, followed by digestion in 525 µl 1.9 mg/ml pepsin in 0.05 N acetic acid at 4 °C for 48 h. At the end of the incubation, pepsin was neutralized by the addition of 100 µl 10× Tris-buffered saline (1.0 M Tris, 2 M NaCl, 50 mM $CaCl_2$), pH 8.0. Acetic acid, GuHCl, and pepsin digest samples were assayed for collagen content. Serial dilutions of rat collagen stock solutions (type I collagen from Southern Biotech, Birmingham, AL, USA, and type III collagen from AbD Serotec, Kidlington, Oxford, UK) were used to make standards. A 50 : 50 dilution of each of the standards and samples in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ and 3 mM NaN_3) was prepared and plated in a 96-well flat bottom immunosorbant plate (Nalge Nunc International, Rochester, NY, USA). Samples were incubated overnight at 4 °C and then non-specific binding was blocked using 2% bovine serum albumin fraction V (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS). Primary antibodies specific to types I and III collagen (Sigma) at a concentration of 0.5 µg/ml were applied and the samples were incubated at 4 °C overnight. A peroxidase-based detection system, using a biotinylated secondary antibody (anti-mouse IgG/anti-rabbit IgG H+L; Vector Laboratories, Burlingame, CA, USA), a streptavidin-conjugated horseradish peroxidase enzyme conjugate (R&D Systems, Minneapolis, MN, USA) and 3,3′,5,5′-tetramethylbenzidine (Vector Laboratories) as the substrate chromagen, was used. Samples were treated with 1 N sulphuric acid to terminate the reaction, and absorbance was read at 450 nm on a Synergy-HT microplate reader (Bio-Tek Instruments, Winooski, VT, USA). All samples and standards were run in triplicate and reported collagen values were normalized to total protein content.

2.2.3. Protein quantification

Total protein was quantified for the acetic acid, GuHCl and pepsin digest samples processed for the ELISA, using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Briefly, samples and albumin standards were incubated at 37 °C for 30 min with standard working solution, according to the manufacturer's instructions, and allowed to cool to room temperature. Absorbance was read at 562 nm, using a Synergy-HT microplate reader (Bio-Tek Instruments).

2.2.4. DNA quantification

Total DNA was quantified for the acetic acid, GuHCl and pepsin digest samples processed for the ELISA using a Picogreen DNA assay, as previously described (Singer *et al*., 1997; McGowan *et al*., 2002). Prior to running the Picogreen assay, 10× Tris-buffered saline was added to the pepsin and acetic acid samples to raise the pH to 8.0. Next, 5% Triton X-100 in 0.05 N acetic acid was added to the samples, resulting in a 1% Triton X-100 solution. Specimens were subjected to repeated freeze–thaw cycles and vortexed prior to being assayed. Briefly, samples and calf thymus DNA standards dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA), pH 7.5, were incubated in a 1 : 200 dilution of Picogreen reagent (Molecular Probes, Eugene, OR, USA) and read at excitation and emission wavelengths of 480 nm and 520 nm, respectively, on a Synergy-HT microplate reader (Bio-Tek Instruments).

2.2.5. Immunohistochemistry

Dermal fibroblasts were plated in monolayer culture at a concentration of 12 500 cells/cm^2 and maintained in serum-containing medium with ascorbic acid for a period of 1, 3 or 7 days, at which point cells were fixed in 4% paraformaldehyde. The cells were stained with monoclonal antibodies to types I (1 : 200 dilution) and III (1 : 100 dilution) collagen (Sigma) and visualized using a Vectastain ABC kit with 3,3′-diaminobenzidine as the substrate (Vector Laboratories). Haematoxylin QS counterstain (Vector Laboratories) was applied to stain the cell nuclei. Non-immune controls without primary antibody were included for all immunohistochemical analyses. Images were captured using a Zeiss Axiovert 200 inverted phase contrast microscope with Axiovision software (Zeiss).

2.3. Three-dimensional studies

2.3.1. Scaffold fabrication

A non-woven PGA mesh (1.2 mm thickness; Biomedical Structures LLC, Slatersville, RI, USA) was reinforced

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with a 3% solution of 300 kDa PLLA (Polysciences Inc., Warrington, PA, USA) in chloroform, using a modified solvent evaporation procedure as previously described (Vacanti and Upton, 1994). The resulting fibre-bonded scaffolds were then cut into 0.5×2.5 cm strips and treated with 1 N NaOH for 2 min, distilled water for 4 min and 100% ethanol for 4 min. The scaffolds were blotted dry using filter paper and stored in a dessicator for up to 1 week. Prior to cell seeding, the materials were immersed in 70% ethanol overnight, air-dried and incubated in medium overnight. All samples were secured in custom-made polysulphone clamps that allowed access to the top and bottom faces of the scaffolds. The available seeding dimensions were 1.5×0.5 cm on each face.

2.3.2. Preparation of cell-seeded constructs

Fetal and adult dermal fibroblasts were seeded at a density of 1×10^6 cells/polymer at a concentration of 25 000 cells/μl in a total volume of 40 μl. Controls without cells were seeded with 40 μl medium. Cell suspension (or medium) (20 μl) was uniformly seeded on each face of the polymer and 5 ml medium was placed in the bottom of the culture dish to prevent evaporation. After 1 h of incubation at 37 °C to allow for cell attachment, the culture dish was flooded with 45 ml medium to cover the polymers. The seeded polymers were cultured in an incubator at 37 °C and 5% CO_2 for a period of 1, 7, 21 or 35 days and maintained in DMEM with 10% FBS and antibiotics. Although fetal cells are capable of surviving in low-serum environments, medium with 10% FBS was used in the 3D studies, since higher serum content is more conducive to long-term maintenance of cultures, particularly for adult cells. The constructs were acclimated for 24 h prior to adding 0.05 mg/ml ascorbic acid to the culture medium. The medium was changed every other day and fresh ascorbic acid was added.

2.3.3. Collagen, protein and DNA quantification

At the designated time points, types I and III collagen, total protein and DNA were quantified as described above for the two-dimensional (2D) studies.

2.3.4. Gross, histological and immunohistochemical characterization

Polymers were removed from the polysulphone clamps and rinsed with PBS. Gross images of the fibroblast-seeded constructs were captured using a Stemi 2000-C stereomicroscope with Axiovision software (Zeiss). Constructs were then fixed with 4% paraformaldehyde, rinsed three times with PBS, dehydrated in a series of alcohols and embedded in paraffin. The samples were serially sectioned at 8 μm thickness, using a Leica microtome (Leica Microsystems Inc., Bannockburn, IL, USA) and mounted on slides. The sections were stained with haematoxylin Gill No. 2 and eosin Y alcoholic (Sigma) to assess cellular structure and organization. In addition, sections were stained with monoclonal antibodies to types I (1 : 200 dilution) and III (1 : 100 dilution) collagen (Sigma) and visualized using a Vectastain ABC kit with 3,3′-diaminobenzidine as the substrate (Vector Laboratories) to evaluate collagen localization. Blocking solution consisted of either 10% horse serum (type I collagen) or 1% horse serum (type III collagen) in PBS with 2% bovine serum albumin (Sigma). Non-immune controls without primary antibody were included for all immunohistochemical analyses. Images of the stained sections were captured using a Zeiss Axioskop 40 optical microscope with Axiovision software (Zeiss).

2.3.5. Mechanical testing

Controls without cells and polymer scaffolds seeded with either fetal or adult dermal fibroblasts were mechanically tested for maximum load, maximum stress and modulus. Day 35 constructs were rinsed with PBS, wrapped in PBS-soaked gauze and frozen at −20 °C prior to testing. Polymers were cored into a dumbbell shape for mechanical testing, using a custom-made punch. The cross-sectional area, thickness and width of the samples were measured as previously described (Soslowsky *et al.*, 2000). The polymer specimens were fixed in the testing device grips using a cyanoacrylate adhesive and sandpaper, immersed in a 37 °C PBS bath, preloaded to 0.01 N and preconditioned for 15 cycles. Structural (maximum load) and material (maximum stress and modulus) properties were determined using a constant strain rate to failure test (0.01%/s) for each sample.

2.3.6. Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Statistics were performed using the JMP-IN (SAS Institute Inc., Cary, NC, USA) software package. An analysis of variance with Tukey's HSD *post-hoc* test was used to determine the significant differences ($\alpha = 0.05$) between fetal and adult samples. Measurement variables included average cell surface area, types I and III collagen content, total protein content, total DNA content and mechanical properties.

3. Results

3.1. Two-dimensional studies

3.1.1. Serum-dependent attachment and morphology

Fetal fibroblasts in low-serum monolayer culture displayed few rounded cells and were more spread, as indicated by a significantly larger average cell surface area compared to adult fibroblasts (Figure 1). In addition,

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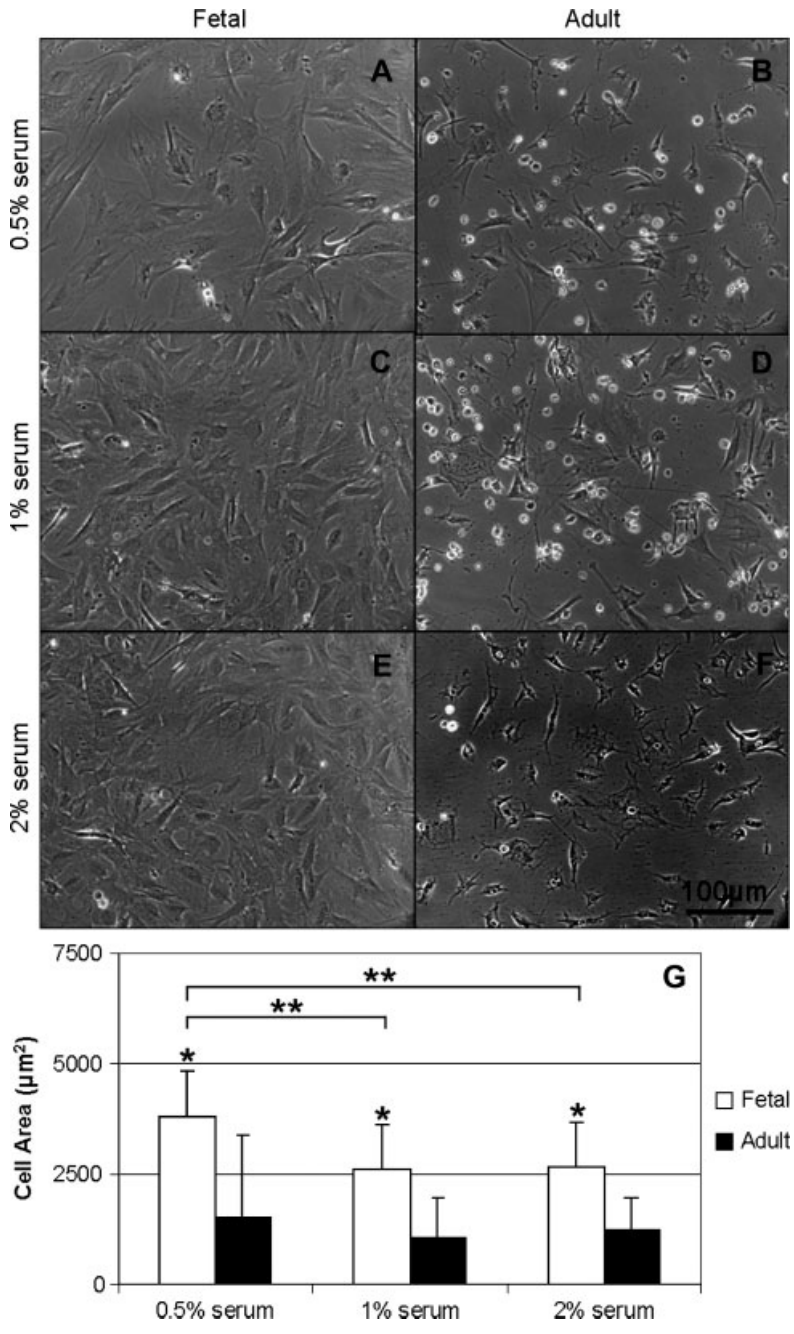

Figure 1. Fetal and adult fibroblast growth in low-serum monolayer culture. (A, C, E) Fetal and (B, D, F) adult rat dermal fibroblasts were grown for 3 days in (A, B) 0.5% serum medium, (C, D) 1.0% serum medium and (E, F) 2.0% serum medium. The average cell surface area was calculated for the different cell and medium types as shown in (G) *Significant difference ($p < 0.05$) between fetal and adult fibroblasts. **Significant difference ($p < 0.05$) between the different medium formulations

there was no significant difference in the average cell surface area of adult fibroblasts under the different culture conditions; however, fetal fibroblasts in 0.5% serum medium had a significantly larger average cell surface area compared to fetal fibroblasts cultured in either the 1% or the 2% serum medium.

3.1.2. Collagen production

Adult fibroblasts produced significantly more type I collagen (normalized to total protein) compared to fetal fibroblasts after the first 3 days in monolayer culture. However, fetal fibroblasts elaborated significantly more type I collagen compared to adult fibroblasts after 7 days, as evidenced by both immunohistochemistry and ELISA quantification (Figure 2). By day 7, both fetal and adult fibroblasts had produced significantly more type I collagen compared to day 3. Additionally, fetal fibroblasts elaborated significantly more type III collagen compared to adult fibroblasts at days 3 and 7, as determined by both immunohistochemistry and ELISA quantification (Figure 3). Normalized type III collagen production was significantly increased from day 3 to day 7 for both fetal and adult fibroblast cultures.

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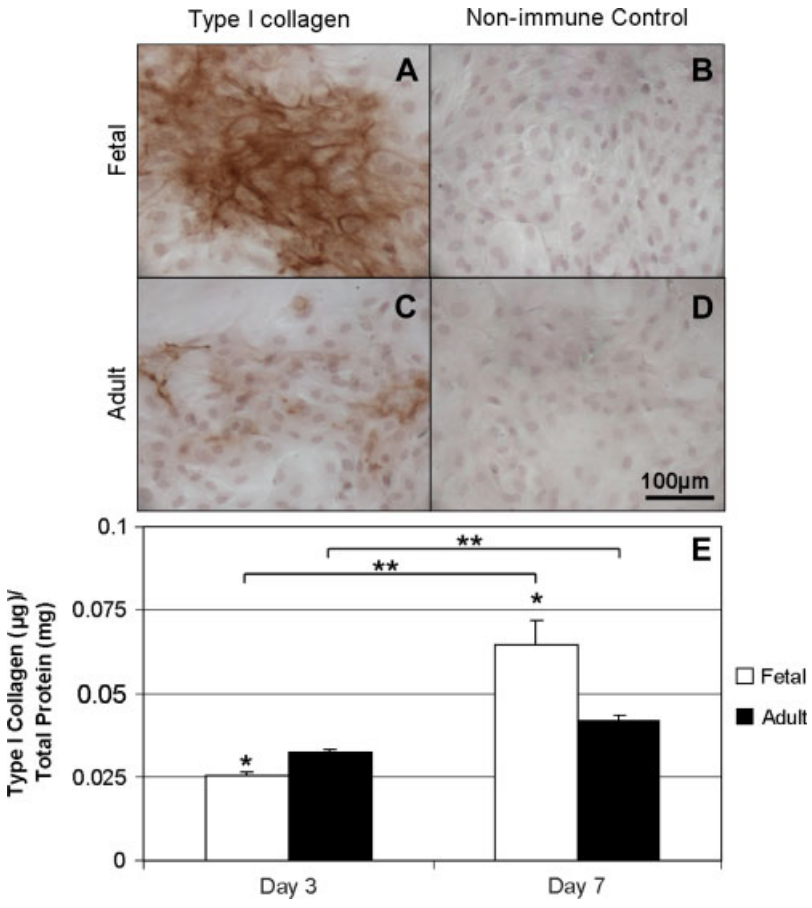

Figure 2. Immunohistochemical localization and quantification of type I collagen. Representative images of (A) fetal dermal fibroblasts, (C) adult dermal fibroblasts, and non-immune controls stained with haematoxylin QS counterstain for (B) fetal dermal fibroblasts and (D) adult dermal fibroblasts after 7 days in culture. (E) Type I collagen ELISA data for fetal and adult dermal fibroblasts grown in monolayer culture. *Significant difference ($p < 0.05$) between fetal and adult fibroblasts. **Significant difference ($p < 0.05$) between the various time points

3.2. Three-dimensional studies

3.2.1. DNA content

Fetal fibroblast-seeded constructs had significantly more DNA per construct than adult specimens at all time points (Figure 4A). In addition, the total DNA per construct significantly increased for the fetal fibroblast samples from day 1 to day 21, with no significant difference in total DNA between days 21 and 35. Total DNA significantly increased from day 1 to day 7 for the adult fibroblast-seeded constructs, with no significant difference in total DNA between days 7, 21 and 35.

3.2.2. Protein content

There was no significant difference in total protein between fetal and adult fibroblast-seeded constructs at day 1 (Figure 4B). However, fetal samples produced significantly more protein than adult constructs at days 7, 21 and 35. In addition, fetal specimens produced significantly more protein over time, whereas there was no significant difference in total protein for adult constructs at the different time points.

3.2.3. Collagen production

Fetal fibroblast-seeded constructs produced significantly more type I collagen (normalized to total protein) compared to adult constructs (Figure 4C). There was no significant difference in the amount of type I collagen produced by fetal specimens at the different time points; however, adult constructs at day 35 expressed significantly less type I collagen compared to days 7 and 21. In addition, adult fibroblast-seeded constructs had significantly more type III collagen (normalized to total protein) compared to fetal specimens at day 7 (Figure 4D). However, by days 21 and 35, there was no significant difference in type III collagen produced by fetal and adult samples. Additionally, type III collagen elaboration was significantly decreased with time for adult fibroblast-seeded constructs.

3.2.4. Gross, histological and immunohistochemical characterization

After 35 days, fetal constructs appeared opaque, with extensive extracellular matrix (ECM) elaboration, based on visual inspection (Figure 5). By contrast, adult specimens were less developed, with sparse ECM

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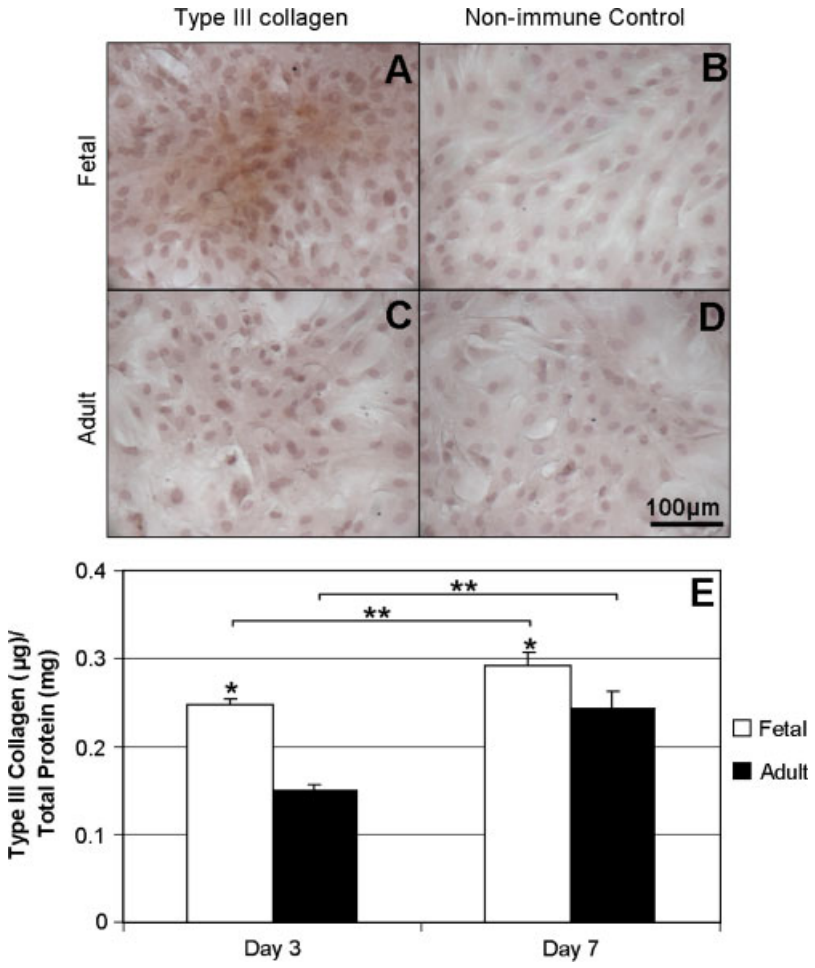

Figure 3. Immunohistochemical localization and quantification of type III collagen. Representative images of (A) fetal dermal fibroblasts, (C) adult dermal fibroblasts, and non-immune controls stained with haematoxylin QS counterstain for (B) fetal dermal fibroblasts and (D) adult dermal fibroblasts after 7 days in culture. (E) Type III collagen ELISA data for fetal and adult dermal fibroblasts grown in monolayer culture. *Significant difference ($p < 0.05$) between fetal and adult fibroblasts. **Significant difference ($p < 0.05$) between the various time points

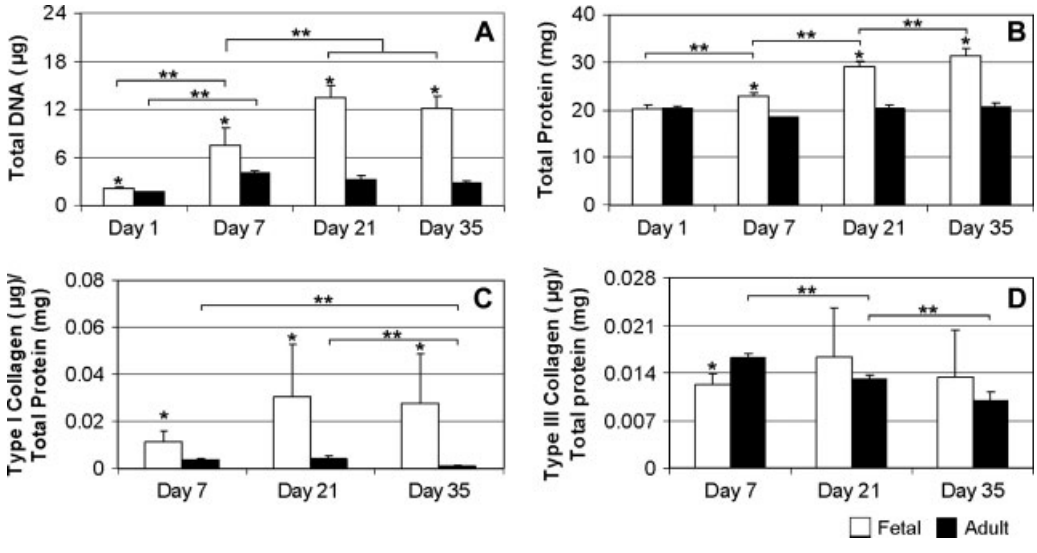

Figure 4. Biochemical characterization of cell-seeded constructs. (A) Total DNA content, (B) total protein content, (C) normalized type I collagen content and (D) normalized type III collagen content for fetal and adult dermal fibroblast-seeded analogues. *Significant difference ($p < 0.05$) between fetal and adult fibroblasts. **Significant difference ($p < 0.05$) between the various time points

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throughout the construct. Representative histological sections of the cell-seeded scaffolds confirmed that there were more fetal fibroblasts attached to the polymers than adult fibroblasts, and that the fetal fibroblasts exhibited greater alignment along the construct surface and more infiltration into the interior of the scaffold (Figure 5). In addition, immunohistochemical characterization revealed that fetal constructs displayed robust elaboration of types I and III collagen (Figure 6), while only trace amounts localized to discrete cell clusters were detected in adult constructs (not shown).

3.2.5. Mechanical properties

After 5 weeks in culture, fetal fibroblast-seeded constructs had an average maximum load of 0.18 ± 0.04 N, an average maximum stress of 0.10 ± 0.03 MPa and an average modulus of 0.62 ± 0.19 MPa. This was in contrast to adult samples and cell-free controls, in which the specimens were too weak to measure the mechanical properties, due to failure while handling and placement in the testing grips.

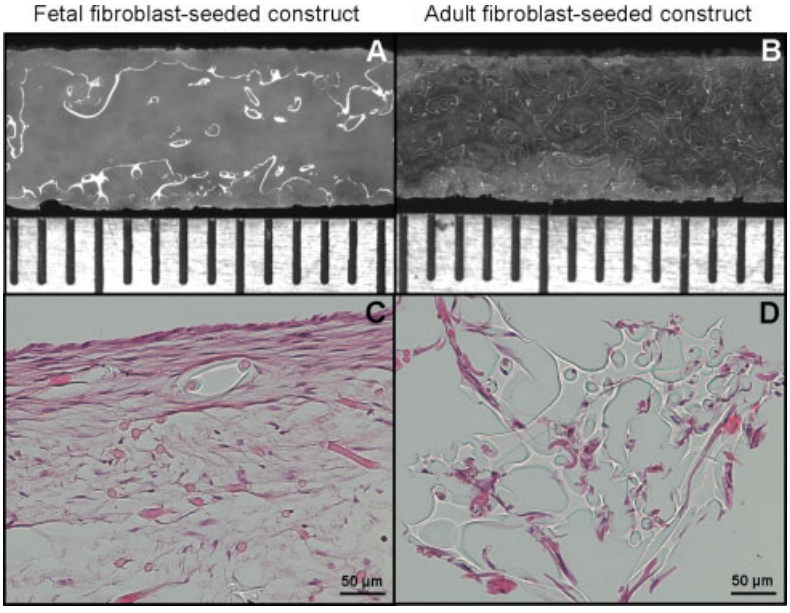

Figure 5. Gross and histological characterization of cell-seeded constructs. Stereomicrographs of (A) fetal and (B) adult dermal fibroblast-seeded constructs after 35 days in culture. Scale in millimeters. H&E-stained sections of (C) fetal and (D) adult specimens at day 35

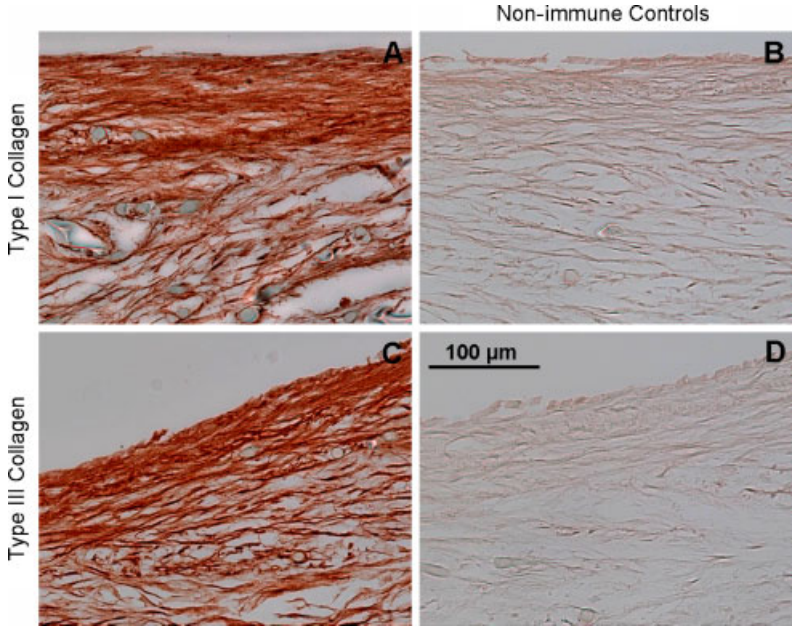

Figure 6. Immunohistochemical evaluation of fetal rat dermal fibroblast-seeded scaffolds. Representative images of fetal constructs stained for (A) type I collagen and (C) type III collagen after 35 days in culture. Non-immune controls for (B) type I collagen and (D) type III collagen showed faint background staining

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4. Discussion

This investigation was the first to evaluate the differences between fetal and adult rat dermal fibroblasts in 2D and 3D culture. Fetal dermal fibroblasts exhibited enhanced growth and ECM production compared to adult fibroblasts, suggesting that the superior wound healing associated with fetal tissue may be due to intrinsic cellular characteristics. These findings confirm previous reports describing the properties of fetal fibroblasts from other species, and lay the foundation for future work employing 3D constructs for tissue regeneration.

In this study, fetal dermal fibroblasts cultured in monolayer in a low-serum environment appeared healthier, as they exhibited a characteristic spindle-shaped morphology and a larger average cell surface area compared with adult fibroblasts under the same culture conditions. These results are consistent with prior studies that demonstrated serum-independent adhesion by fetal sheep dermal fibroblasts (Brink *et al*., 2005) and serum-independent migration by human fetal skin fibroblasts (Kondo *et al*., 1993; Kondo and Yonezawa, 1995), in contrast to the serum-dependent behaviour reported for their adult counterparts. Although there was no significant difference in the average cell surface area of the adult dermal fibroblasts under the different culture conditions, the fetal fibroblasts grown in 0.5% serum medium had a significantly larger average cell surface area than fetal fibroblasts cultured in either the 1% or the 2% serum medium. The difference in average fetal cell surface area is likely due to the more confluent nature of the fetal dermal fibroblasts grown in the 1% and the 2% serum medium, which prevented spreading of individual cells.

Fetal and adult fibroblasts in monolayer culture also displayed differences in collagen production. After 7 days, fetal fibroblasts produced significantly more types I and III collagen compared to adult fibroblasts. These results are in agreement with previous studies that found that fetal fibroblasts elaborated more collagen than adult fibroblasts *in vitro* (Thomas *et al*., 1988; Takeda *et al*., 1992; Lorenz and Adzick, 1993; Bullard *et al*., 2003; Goodman *et al*., 2004; Brink *et al*., 2006). Normal fetal skin and tendon contain higher amounts of type III collagen compared to adult skin and tendon (Smith *et al*., 1986; Lovvorn *et al*., 1999; Woo *et al*., 2000; Bullard *et al*., 2003; Goodman *et al*., 2004), which is similar to the results reported for monolayer culture in this study.

Direct comparisons between the 2D and 3D studies are difficult, given the differences in experimental variables (i.e. surface chemistry, topography, serum content, seeding density, etc.). However, similar to the monolayer studies, when cultured in the PGA–PLLA scaffolds, fetal fibroblasts exhibited enhanced growth and ECM production compared to adult fibroblasts. Fetal rat dermal fibroblasts displayed superior adhesion to the polymer scaffolds, as evidenced by the higher DNA content per construct at day 1. This is in agreement with a previous report of improved human fetal dermal fibroblast cell attachment to collagen gels (Sandulache *et al*., 2007). Fetal fibroblasts also proliferated faster than adult fibroblasts on the polymer scaffolds, suggesting that the fetal cells acclimated more rapidly to the synthetic culture environment than the adult fibroblasts. With respect to biosynthesis, fetal fibroblasts produced significantly more total protein compared to adult fibroblasts over time. Interestingly, despite the higher protein content per scaffold, the fetal fibroblast-seeded constructs also exhibited significantly more normalized type I collagen compared to adult specimens, indicating that collagen comprises a larger proportion of the total protein in fetal samples. Conversely, adult constructs produced significantly more type III collagen compared to fetal constructs at day 7, but after day 21 there was no significant difference in type III collagen between the fetal and adult specimens. The fact that there was no significant difference in normalized type III collagen production between fetal and adult fibroblast-seeded scaffolds may be due to the *in vitro* culture environment or to the lack of specific signalling molecules present. A previous study by Rolfe *et al*. (2007) found that growth factors play an important role in regulating type III collagen expression, and that transforming growth factor-β1 induces human fetal dermal fibroblasts to produce more type III collagen, while adult fibroblasts are not affected.

In addition to improved matrix elaboration, fetal fibroblast-seeded constructs also displayed more robust mechanical properties compared to adult samples (and cell-free controls). This was not surprising, given the slower rates of cell proliferation and protein production by adult fibroblasts compared to fetal fibroblasts cultured on the polymer scaffolds. At 5 weeks, fetal specimens had an average maximum load of 0.18 ± 0.04 N, an average maximum stress of 0.10 ± 0.03 MPa and an average modulus of 0.62 ± 0.19 MPa. These values are close to those for cell-free control scaffolds after just 1 week of culture, when the materials are intact and do not exhibit signs of degradation (maximum load of 0.23 ± 0.07 N, maximum stress of 0.14 ± 0.04 MPa and modulus of 2.33 ± 0.69 MPa). This suggests restoration of functional properties due to elaboration of newly deposited matrix by the seeded cells. Nevertheless, the values are low compared to those reported for healthy rat supraspinatus tendons (1.0 ± 0.2 N for maximum load, 1.2 ± 0.6 MPa for maximum stress and 32 ± 13 MPa for modulus; Gimbel *et al*., 2004). However, the fibroblast-seeded constructs evaluated in this study are not comparable to fully developed tendons, as they were cultured for only a short time period. Furthermore, these constructs were grown in the absence of environmental stimuli that may enhance the properties of tissue-engineered constructs. For example, soluble growth factors, including platelet-derived growth factor-BB, basic fibroblast growth factor, insulin-like growth factor-1 and bone morphogenetic proteins 7, 12 and 13 have all been shown to increase proliferation and ECM elaboration by tendon fibroblasts (Fu *et al*., 2003; Wong *et al*., 2005; Costa *et al*., 2006; Yamada *et al*., 2008). Such growth

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factor-induced proliferation and matrix biosynthesis may give rise to improved mechanics of tissue-engineered analogues (Qi *et al*., 2006). Additionally, mechanical stimulation (i.e. dynamic tensile strain) may augment the functional properties of developing tissue equivalents (Juncosa-Melvin *et al*., 2006a, 2006b; Androjna *et al*., 2007; Shearn *et al*., 2007; Butler *et al*., 2008; Joshi and Webb, 2008; Wang *et al*., 2008). Therefore, it is possible that growth factor supplementation and mechanical loading, individually or in combination, may enhance matrix production and functional properties of both fetal and adult fibroblast-seeded constructs (Mauck *et al*., 2003; Augst *et al*., 2008; Chen *et al*., 2008).

This study has demonstrated that fetal fibroblasts maintain characteristics that are advantageous to wound healing in a 3D culture environment. Future studies will assess fetal and adult fibroblast-seeded constructs in an *in vivo* rat supraspinatus tendon injury model to determine whether the cell-seeded constructs can augment wound healing. In addition, mechanical stimulation or growth factor supplementation may be used to engineer more tendon-like tissue analogues prior to implantation into the wound site. The use of allogeneic fetal fibroblasts may promote improved tendon regeneration and may also present the benefit of a more immunocompatible cell source, since fetal cells have been reported to express lower levels of alloantigens and to modulate the immune response (Götherström *et al*., 2004). Characterizing the differences between fetal and adult fibroblasts may eventually lead to new methods for manipulating adult fibroblasts to display more fetal-like behaviour and alternative therapies for improving adult wound healing.

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