SPECIAL FOCUS: ANIMAL MODELS IN TISSUE ENGINEERING. PART II*

Tissue Engineering Strategies to Improve Osteogenesis in the Juvenile Swine Alveolar Cleft Model

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Alveolar (gumline) clefts are the most common congenital bone defect in humans, affecting 1 in 700 live births. Treatment to repair these bony defects relies on autologous, cancellous bone transfer from the iliac crest. This harvest requires a second surgical site with increased surgical time associated with potential complications, while providing only limited cancellous bone. Improvements in treatment protocols that avoid these limitations would be beneficial to patients with clefts and other craniofacial bone defects. There have been steady advances in tissue-engineered (TE) solutions for long-bone defects and adult patients, but advances for the pediatric craniofacial skeleton have been slower to emerge. This study utilizes a previously established juvenile swine model with a surgically created, critical size alveolar defect to test the efficacy of umbilical cord (UC) mesenchymal stem cells (MSCs) treatments on nano-microfiber scaffolds. At 1 month after implanting our TE construct, mineralized tissue in the surgical gap was quantified through computed tomography (CT), and histology, and excised tissue was subjected to mechanical testing. Both undifferentiated and predifferentiated (toward an osteogenic lineage) UC MSCs generated bone within the cleft on a scale comparable to iliac crest cancellous bone, as evidenced by histology and CT scans. All of the pigs treated with scaffold/stem cell combinations had mineralized tissue within the defect, although without filling the entire defect. Several of the experimental animals exhibited poor and/or asymmetric maxillary growth 1 month after the initial surgery, especially if the surgical defect was located on the smaller side of an already asymmetric pig. Our results demonstrate that tissue engineering approaches using UC MSCs are a promising alternative for repair of the alveolar cleft. Data in the pig model demonstrate that implanted scaffolds are at least as good as the current gold standard treatment based on harvesting cancellous bone from the iliac crest, regardless of whether the cells seeded on the scaffold are precommitted to an osteogenic fate.

Keywords: cell differentiation, mesenchymal stem cells, alveolar cleft surgery, animal model, nanocomposite scaffolds

Introduction

FOR CRITICAL SIZE bone defects of the craniofacial skeleton in pediatric patients, tissue-engineered (TE) solutions have been slow to develop for a complex set of reasons, including a higher standard for safety and efficacy in children and the need to accommodate growth. To accelerate progress in this area, it is necessary to have a large animal model and an established, efficacious treatment protocol for the target disease. Craniofacial anomalies in children occur in 1 in 250 live births. Cleft lip (including a defect of the alveolus/gumline) and palate (defect in the midline of the maxilla) are the most common congenital bone defects in humans (1/700 live births).^{1,2}

Historically, these defects were corrected surgically at 1 year of age with bone harvested from multiple locations including rib, tibia, and calvarium³; however, as these patients grew, it became increasingly apparent that they developed growth restriction of the treated maxilla, which did not keep pace with growth of the remaining craniofacial skeleton. This growth restriction resulted in smaller bones, often associated with asymmetric craniofacial shape, resulting in functional impairments, such as breathing, feeding, and malocclusion.^{4–8}

Patients affected with restricted growth after early surgery require multiple additional surgeries to correct the maxillary growth restriction. In response, practitioners now delay treatment of the alveolar cleft until 7–12 years of age, allowing further skeletal growth before treatment.^{1,4,9} As a

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*This article is part of a special focus issue on Animal Models in Tissue Engineering. Part II.

result of this delay, the uncorrected anomaly persists, leading to on-going sinusitis, potential speech anomalies, and dental problems. Because the defect is maintained beyond the age of self-awareness there is potential for a negative psychological impact on these patients.^{10–13}

The current clinical gold standard for treatment of the alveolar cleft is cancellous bone harvested from the anterior iliac crest.^{14,15} Cancellous bone offers an immunocompatible source of bone progenitor cells, allows for rapid generation of vascularized, alveolar bone that can fully integrate with the maxilla leading to improved tooth eruption.^{12,16} Importantly, there is sufficient bone volume for safe harvest by the time a child is 7–12 years of age.^{3,4,7,9,17} Disadvantages of this protocol include a second surgical site for donor tissue,^{16,18} with possible lack of integration and resorption of the transplanted graft. For secondary bone grafting, done before eruption of the permanent lateral incisor (7–10 years-of-age), or permanent canine (10–12 years-of-age), resorption occurs in many patients, with 10% of patients experiencing sufficient bone loss that necessitates repeat bone grafting.^{13,19}

TE strategies using scaffolds (of which there are a variety) and cells (usually mesenchymal stem cells (MSCs) for skeletal defects) are potential treatments for filling bone defects in the growing craniofacial skeleton, including alveolar clefts.^{20–23} Wharton's Jelly, a constituent of the umbilical cord (UC), is an abundant source of MSCs,²⁴ with no ethical concerns for harvest,^{25,26} high proliferative capability, low tumorigenic potential, and privileged immune status^{27,28} preserved after differentiation.²⁹

The clinical application of MSCs must address whether these cells can be utilized without any *in vitro* manipulation or if they require prior differentiation for full efficacy. Although not previously reported^{30,31} it is possible that undifferentiated (UD) cells may proliferate into tumors, as their terminal differentiation is not controlled. On the other hand, differentiated (DI) cells require expansion time *in vitro* with added growth factors, experience replicative senescence^{32,33} and other stress factors that can limit their use for clinical trials.³⁴

In this study, we present the results of experiments to determine the efficacy of UD and DI cells to generate new bone in a swine alveolar cleft model, with a surgically created weight-based critical size defect.³⁵ In this study, we generated TE grafts using Poly(Lactide-co-Glycolide) copolymer (PLGA) electrospun scaffolds,³⁶ seeded with green fluorescence protein (GFP)-expressing autologous MSCs derived from the UC. Efficiency of bone formation was evaluated histologically, with CT scans, and mechanical testing. Results were compared among UD, DI, and iliac crest cancellous bone (CB) to determine bone formation differences among treatments. Asymmetry of the craniofacial skeleton was also tracked to verify whether growth restriction similar to clinical results in young children were replicated. These results will be useful in establishing whether MSCs require differentiation before application in TE alveolar cleft repair, or whether UD cells can be used, avoiding the need for *in vitro* exposure to culture conditions and growth factors.

Materials and Methods

Animals

Three to 4-week-old Yorkshire pigs obtained from Michael Fanning Farms (Howe, IN) were transferred to the Cincinnati

Children's Hospital Medical Center (CCHMC) animal facility following weaning, and allowed to acclimate for 2 weeks before surgery. A total of 22 piglets (17 females, 5 neutered males), with a weight-range of 7–18 kg (mean of 11 kg) at the time of surgery were used for this study. Sixteen animals received TE treatments, 8 UD and 8 with DI UC MSCs; 3 had the surgical defect filled with iliac crest CB; and 3 surgically created defects were left unrepaired (nontreated; NT) to serve as controls. The defect side was chosen randomly (left 13, right 8).

Piglets were housed in a temperature and light-controlled environment and fed hard pellets, with twice-a-day diet enrichment using a combination of apples, bananas, carrots, potatoes, sugar cane, peanut butter, dried fruit, and vegetables. During the first week after surgery, piglets were fed with medicated 22% protein starter feed, with water added to the pellets during the first 24 h. All animal care adhered to NIH guidelines and was performed under an IACUC approved protocol (IACUC2014-0062) at CCHMC.

Surgical procedure

A unilateral alveolar defect was surgically created following a previously reported protocol.³⁵ Following appropriate general anesthesia, a hemi-maxilla was prepared for creation of the surgical defect from the central incisor anteriorly to a posterior position near the canine; the defect size was normalized for weight (0.17 cm/kg). Following creation of the defect, the site was filled with one of three conditions: nanofiber scaffolds (described in *Nanofiber scaffold generation*) with UD UC MSCs, scaffolds with DI UC MSCs or CB. CB was harvested from the anterior superior iliac crest using a 2 cm incision. All surgical incisions were closed with absorbable sutures.

Nanofiber scaffold generation

Mixed fiber diameter nanofiber scaffolds were prepared by electrospinning Delta System™ polylactic and polyglycolic acid (PLGA) implants from Stryker®, following a previously described protocol.³⁶ PLGA was chosen because it is both biocompatible and biodegradable, encourages cell attachment, proliferation, and differentiation,^{36–41} and is FDA approved for use in pediatric bone repair.^{23,42} Scaffolds were sterilized in 70% ethanol under UV light for at least 4 h, washed with phosphate-buffered saline (PBS), and primed with growth medium overnight for cell seeding.

MSC isolation and growth

Swine UC were harvested at the time of birth, as previously reported,³⁵ and MSCs were isolated and expanded from Wharton's Jelly by explant technique, as previously described.⁴³

Cell labeling

To track transplanted cells, swine UC MSCs were labeled with the TMEW-GFP-lentivirus (Viral Vector Core, CCHMC). Transduction was performed using a multiplicity of infection of 10 IU/cell in growth medium containing 8 g/mL protamine sulfate (Sigma). Media were changed 24 h later. Before seeding, transduction efficiency higher than 95% was confirmed by fluorescent microscopy on a Nikon Eclipse Ti-S inverted microscope.

Cell seeding and differentiation

Sterilized electrospun PLGA mats with a diameter of 1 cm were seeded with passage one MSCs at a density of 5×10^5 cells per scaffold. Cells were either maintained at subconfluence in growth media (DMEM-10% FBS, 2 mM glutamine, 100 U/mL/100 μ g/mL Penicillin/Streptomycin; Gibco) or DI 48 h postseeding in osteogenic media (growth media with 10 mM beta-glycerophosphate, 0.1 μ M dexamethasone, and 50 μ M ascorbic acid; Sigma) for 7 days before implantation.⁴³ Cells were monitored microscopically for attachment and GFP expression. Immediately before surgical implantation, the constructs were washed with PBS and implanted into the surgically created defect.

Histological analysis

Thirty days postimplantation, piglets were sacrificed and both maxillae were harvested. The boundary between the implant and the remaining intact bone was marked with a histological pen, for later identification in paraffin sections. The anterior halves of the alveolar cleft sites were fixed for 72 h and decalcified in 10% HCL/EDTA pH 7.4 (Fisher Scientific) for 24 h and processed for staining as previously described.³⁵ Briefly, 5 μ m slides of paraffin sections were stained with hematoxylin and eosin (H&E; Fisher Scientific) to analyze matrix architecture, inflammatory response, bone ingrowth, and cellular organization. Immunohistochemical analysis was performed in contiguous paraffin slides using anti-GFP antibody (A-6455, Invitrogen) and developed with Impact NovaRED peroxidase substrate (Vector Laboratories). Hematoxylin was used for counterstaining. Samples were analyzed using a Nikon Eclipse Ti-S inverted microscope.³⁵

Volume calculations of bone formation

To estimate the volume of new bone grown into the defect for each of the treatments, *in silico* calculations based on three-dimensional (3D) reconstructions were completed. Each pig skull was subjected to a CT scan immediately after surgical creation of the defect (D0) and again at sacrifice, 30 days later (D30), with exposure doses determined by the size of the animal (monoplane flat-panel detector C-arm angiographic system; FD20, version 8.1.2; Philips Healthcare, IL). A 3D reconstruction of each skull was generated, based upon the total number of voxels meeting or exceeding the mean attenuation coefficient for bone, excluding teeth (Amira 6.1). Segmentation of the bones was accomplished by a single individual blinded to treatment conditions at the time of data collection (D.C.J.).

In the initial scan, the location of the defect was noted in reference to anatomic landmarks related to the position of the teeth and the curvature of the dorsal/lateral margin of the maxilla. These were visualized on the pig's second scan (D30) to position the clipping planes used to delineate the region of the defect: maxillary midline, surgically defined anterior and posterior osteotomies, and the limit of sufficiently mineralized tissue in the dorsal and lateral directions (Fig. 3). This was done because the length of the surgical defect increased in size along with the balance of the maxilla during the experiments due to pig growth. Once the clipping planes were placed, all of the voxels meeting or exceeding the attenuation coefficient for bone inside the region of the defect were converted into

volume estimates (mm^3), based upon individual scan parameters. For the purpose of comparisons and to account for variability in the growth of individual pigs, all volumes were standardized to weight at the time of sacrifice (mm^3/kg).

Qualitative asymmetry determinations

Because early surgical treatment of alveolar clefts in children can lead to growth restriction and facial asymmetry, the 3D reconstructions of all pigs were examined to qualitatively determine baseline asymmetry in each pig at the time the defect was created (D0) and at sacrifice (D30). Asymmetry was defined as one hemi-maxilla being smaller than the other, and obvious differences between bilateral aspects of the facial skeleton (e.g., size and position of the zygomatic arches, orbits, canals in the maxilla or mandible). If asymmetry was found, the discrepancy was documented, the two time points were compared, and the side of defect was noted.

Quantitative asymmetry determinations

A collection of seven landmarks were placed on 3D skull reconstructions for each pig (surgical and sacrifice days). These included three pairs of bilateral landmarks and one at the midline. Landmarks were positioned away from the defect to avoid missing a landmark in some specimens and to test for asymmetry in craniofacial bones outside of the immediate surgical defect (Fig. 5A). To ensure precise placement, landmark locations were determined by toggling between the surface reconstruction (based on x-ray attenuation as detailed above) and the volume rendering setting of Amira 6.1.

A validation study on a sample of 10 scans was conducted, with landmarking sessions occurring three times, with at least 48 h between sessions. Variation among sessions (Procrustes ANOVA, MorphoJ⁴⁴) determined that variance in landmark placement was insignificant. The Cartesian coordinates for each landmark were collected and a mirror-image set of landmarks for each individual was generated across the sagittal plane by interchanging the left and right landmarks, and taking the negative value of X for each coordinate (Excel 2013). Data were converted from the CVS file format utilized in Excel into NTS format in a custom-written program.

A Procrustes superimposition was performed and the distance in shape space (i.e., Procrustes distance, D^{45}) between the cloud of landmarks representing each pig at each age and its mirror image was determined (TpsSmall v1.33, Rohlf 2015). A Procrustes superimposition was selected because it calculates the differences between landmarks in a collective manner, ignoring differences of scale, rotation, and position. By calculating the distance between the two mirrored clouds of landmarks, this metric provides an estimate of how different the left and right sides are from each other: if at D0, the specimen has perfect symmetry, increasing asymmetry is represented by a linear increase in this value. For comparisons among treatments, calculated values of D were recorded for each scan and the data were fit with a general linear model to determine the contribution of treatment to the variance (SYSTAT 13), with age of pig included as an independent variable.

Mechanical testing

The mechanical behavior of the different treatments at 30 days postsurgery was evaluated by conventional indentation

measurements, using an Instron 4465 universal testing instrument (Instron). The posterior halves of the alveolar cleft sites were frozen immediately after sacrifice and defrosted before measurement. To normalize measurements between samples, a grid was created with three positions that were 4 mm apart on a line drawn at 3/4 distance from piriform aperture to the palate. The entire bone sample was fixed on a stainless steel plate with Body Filler (MSC, Industrial Supply).

Tensile testing was performed at a rate of 3.6 mm/min with a 1.62 mm probe at room temperature. The force and corresponding deformation were recorded after the load exceeded 0.2 N and the Young's module was automatically calculated by the InstronSoftware (TESTWORK 4). The software calculates the slope of the force-deformation curve in its elastic deformation region to obtain the Young's modulus. A total of 16 pigs were used for testing (UD=6, DI=5, CB=2, NT=3). Normalization was performed using maxillary bone contralateral to the defect ($n=14$).

Statistical analysis

All data are reported as mean $\pm$ standard deviation. Statistical analysis for volume filling was performed through an analysis of variance (ANOVA). For quantitative asymmetry a Procrustes ANOVA was conducted in MorphoJ,⁴⁴ and the ANOVA for mechanical testing was performed with a Tukey's honestly significant difference test as a *post hoc* test. Statistical analyses were performed with SYSTAT v13, unless otherwise stated.

Results

A total of 22 piglets were included in this study. The alveolar cleft was surgically created (D0) and the piglets received one of the following treatments: UD, DI, CB, NT. Subjects were sacrificed 1-month postsurgery (D30), except for one each of the UD and DI treatment pigs that were sacrificed at 3 months postsurgery (D90). All animals survived surgery, with piglets recovering normal activity within a day after surgery. No infections or healing problems were noted for any study animal. All animals demonstrated pro-

gressive weight gain through the 30- and 90-day studies. Upon sacrifice, gross examination of the defect demonstrated new tissue within all of the defects. The contour of the bone defect was easily distinguishable on NT samples but not for any of the other treatment groups.

FIG. 1. Histology of new bone formation in the surgically created alveolar cleft defect. Representative pictures span the area from intact bone to the area of the defect (*left to right*) for each treatment type, including nontreated and intact bone. The *dotted line* represents the intersection of intact bone and the defect area; *arrows* point to representative areas within each defect. UD treatment (**A**) shows higher cellularity with less organized bone in the defect, while DI (differentiated) treatment (**B**) demonstrates more organized bone and greater integration with the surrounding, intact bone. The intersection of intact bone with the area of the defect cannot be visualized. CB treatment (**C**) also demonstrates new bone formation integrated with surrounding normal bone. The NT samples (**D**), demonstrate no evidence of new bone formation with fibrotic tissue filling the defect. Histology of normal bone is shown (**E**). Samples were stained with hematoxylin/eosin; slides are presented at 400 $\times$ magnification. Adobe Photoshop was used to generate composite images. CB, cancellous bone; DI, differentiated; NT, no treatment; UD, undifferentiated. Color images available online at www.liebertpub.com/tec

Histological assessment of new bone formation

Bone formation in all treated and untreated animals was evaluated by H&E staining, with similar results for D30 and D90 samples. Although, UD samples demonstrated greater cellularity, with less organized trabeculae than DI samples, both had similar morphological appearance with new bone formation in the defect. New bone had the typical appearance of maxillary bone, with osteoid, connective tissue, trabeculations, and characteristics of more mature bone (Fig. 1). In some samples, particularly DI samples, integration with surrounding, intact bone obliterated any visual evidence of a cut bone surface. CB samples demonstrated new bone formation and mineralization, however, the new bone appeared more immature, demonstrating greater hematoxylin staining with high cell density, randomly arranged cells, and fewer incipient or organized trabeculae than UD and DI samples. Integration with the surrounding intact bone was not as defined as with the DI treatment, where a visible line at the osteotomy site was noticeable. Modeling of the intact bone was detected at the borders of the defect (Fig. 1).

For all three treatments, histologic analysis revealed areas with fibrotic, collagenous tissue within the defect, indicating the surgically created defect was not completely filled by bone with any of the treatments. Distinct from the other three treatments, histology for the NT samples demonstrated ubiquitous fibrous connective tissue in the defect, with no bone formation, and higher rates of modeling in intact areas. In these samples, there was also a clear boundary between the defect and the intact bone.

There was no histological evidence of an inflammatory response in any of the treatment groups or in the NT defects. However, an inflammatory reaction was detected when scaffold alone was implanted (data not shown). No histological evidence of bone resorption was noted. There was no evidence of multinucleated, darkly stained cells in the H&E stains (a defining characteristic of osteoclasts), or of enlarged lacunae (Howship lacunae) inside or along the edges of the defect area.

Localization of transplanted cells within the defect

Cells labeled with lentivirus expressing GFP were tracked to evaluate the ability of implanted UC MSCs to generate new bone and integrate with the surrounding intact bone.

Before implantation, fluorescent expression demonstrated that more than 95% of transfected cells were GFP+. Cells expressing GFP were demonstrated within the surgically created defect using immunohistochemistry (Fig. 2) for both TE treatments (UD and DI). Although areas with more organized trabeculae were detected in UD treatment samples, the majority of the assessed areas demonstrated immature bone, with higher numbers of GFP+ cells continuing to display a mesenchymal-like morphology, randomly distributed, and not actively lining the bone matrix for mineralization.

FIG. 2. GFP immunochemistry to determine the presence of implanted cells in newly created bone. Implanted cells were at least 95% GFP+ by microscopic visualization. Histologic samples from TE conditions were treated with anti-GFP antibody and visualized with ImPACT NovaRED peroxidase substrate and hematoxylin counterstaining. Representative pictures from the defect are shown (A, B). A stained sample of normal bone is also shown as a negative control (C). Both TE treatments demonstrated GFP+ cells within the defect (red staining); UD conditions (A) demonstrated disorganized cells in the more central parts of the defect; DI treatments (B) resulted in GFP+ cells that are more flattened, lining the new trabecular bone. Arrows point to representative areas. In both treatments, the implanted cells contributed to bone formation. Magnification is 400 $\times$. Adobe Photoshop was used to generate composite images. GFP, green fluorescence protein; TE, tissue engineered. Color images available online at www.liebertpub.com/tec

FIG. 3. Comparison of day 0 (D0) and day 30 (D30) 3D CT scans for different treatments. Treatments at D0 are in the upper panel (A–C) and D30 are in the lower panel (D–F). The maxilla is shown in green; the anatomic location of the defect is determined for each animal at D0 (boxed); this region of interest is located in the corresponding D30 scan (also boxed). The volume of mineralized tissue deposited in the region of interest at D30 is shown in yellow (D–F). Volume filling for UD; (D) DI; (E), and CB; (F) were equivalent. 3D, three dimensional; CT, computed tomography. Color images available online at www.liebertpub.com/tec

More GFP+ cells were present in the osteoblast lining on the surface of the mineralizing areas in the majority of the DI treatment samples. In both cases, the transplanted cells could be seen contributing to new bone formation. Most of the positive cells remained within the defect area, with a few GFP+ cells detected in the intact surrounding bone for some samples, although migration to areas other than the defect was not specifically tested.

Volume filling analysis

To determine volume filling with new bone for the different treatments, D0 CT scans were compared to those taken at D30 and D90. After 3D reconstruction, the anatomic location of the initial defects was noted and the new bone volume was measured (Fig. 3). All implants, independent of treatment (UD, DI, or CB) showed a significant increase in the amount of new bone content within the defect when compared to the NT controls (Fig. 4). No defect was completely filled with bone, but all UD, DI, and CB pigs had new bone within the defect. This is an expected finding given the limited, 1-month healing time. All treatments provided similar amounts of new bone growth (UD = $7.23 \pm 4.99 \text{ mm}^3/\text{kg}$, DI = $5.82 \pm 4.48 \text{ mm}^3/\text{kg}$; $p=0.04$, CB = $2.58 \pm 2.83 \text{ mm}^3/\text{kg}$, $p=0.400$), with high variance among the individual pigs (Fig. 4).

Asymmetry

Both quantitative and qualitative approaches were used to determine whether the juvenile pigs (age-matched for 1-year-old children) would develop growth restriction and

asymmetry similar to that demonstrated in children with early surgical repair of the alveolar cleft. Morphometric analysis of seven landmarks (Fig. 5) demonstrated no statistically significant asymmetry among the treatments at the time of defect creation or at 1 month postsurgery ($p=0.4148$). However, a qualitative, individualized review of all the 3D reconstructions demonstrated noticeable

FIG. 4. Day 30 3D CT scan estimates of volume filling. Volumes are reported for scaffolds seeded with either UD or DI cells, compared to CB as a positive control. All treatments demonstrated a significant increase in the amount of new bone content within the defect when compared to controls with NT; no statistical difference was found between the different treatments, due to the high variability between individual subjects ($p=0.400$). Two additional animals (a single UD and one DI) were followed for 3 months postsurgery (bone volume formed was 11.7 and 11.9 mm³/kg). Color images available online at www.liebertpub.com/tec

FIG. 5. Assessment of asymmetry in experimental pigs at day 0 (D0) and day 30 (D30). Placement of landmarks (A) used both the segmentation surface and volume rendering options in Amira 6.1 to optimize precision. The average Procrustes distance (B) between an individual and its mirror-image among treatment groups is reported: UD, DI, CB, and defects with NT. No difference in asymmetry was noted when comparing any of the treatment groups ($p=0.564$). Color images available online at www.liebertpub.com/tec

asymmetry in seven of the piglets at the time of surgery (Table 1). One month later, this asymmetry did not appear to have qualitatively changed when the smaller side was coincident with the location of the surgical defect; however, there was qualitative evidence of asymmetry affecting the side of the surgically created defect when it was on the opposite side of the smaller hemi-maxilla.

Three out of the eight pigs that were qualitatively symmetric at the time of surgery became qualitatively asymmetric 1 month later (two out of these three were also quantitatively asymmetric by morphometric analysis), with the side of the defect shorter than the unoperated hemi-maxilla. In total, 9 out of the 22 pigs demonstrated some degree of increased asymmetry, either quantitatively, qualitatively, or both, at 1-month postsurgery (Fig. 5 and Table 1). Both piglets sacrificed at 3 months postsurgery demonstrated asymmetry anteriorly, with the maxillary bone from the normal side curving toward the side with the surgically created. The frontal bone also demonstrated asymmetry (Fig. 6), indicating a propagation of the asymmetry to other parts of the craniofacial skeleton. It should be noted that a similar asymmetry, but to a lesser extent, was already present at the time of surgery for both D90 piglets.

Mechanical properties of newly formed bone

Indentation studies were performed to report the mechanical properties of new bone formation (Fig. 7). The average elastic modulus of new bone within the defect was

TABLE 1. QUALITATIVE ANALYSIS OF ASYMMETRY IN TREATED PIGLETS

	D30		
	Symmetric	Smaller side Left	Smaller side Right
D0			
Symmetric	2 ^a (R); 3 (L) ^b	1 (L)	3 (R)
Smaller side left	1 (R)	6 (L); 2(L)	
Smaller side Right	1 (L)	1 (L)	1 (R)

Samples were analyzed independently by two different individuals, using unidentified 3D CT scans (Amira 6.1). Asymmetry was defined as both individuals agreeing there was visible difference between the two hemi-maxillae. Though no correlations were found between treatment and degree of asymmetry, a total of 9 out of 22 pigs were found to be asymmetric following treatment. These included those that were originally symmetric at D0, and then were either smaller on the left (1) or on the right (3); those that were smaller on the left at D0 and then symmetric at D30 (1); smaller on the left at D0 and still smaller at D30 (2 out of 8); and lastly, those that were smaller on the right at D0 and either symmetric (1) or smaller on the left (1) at D30. These data suggest that nearly half of the pigs (9/22) demonstrated preliminary evidence of growth restriction.

Bold: Samples with increased asymmetry D30.

^aNumber of pigs fitting this description at day 0 (D0) and the new designation at day 30 (D30).

^bParentheses designate the side of the surgically created defect. Left (L); Right (R).

3D, three dimensional; CT, computed tomography.

255 ± 376 MPa (UD), 513 ± 618 MPa (DI), and 95.7 ± 41 MPa (CB). Samples from untreated animals, which did not lead to any bone formation, had tissue samples with moduli too low to permit accurate measurements. Differences among treatments were not statistically significant and all treatments demonstrated significantly lower elastic moduli than normal bone from the contralateral maxilla.

Discussion

The current gold standard for treatment of the alveolar cleft associated with cleft lip and palate, involves harvest of cancellous bone from the iliac crest, and is generally performed between 7 and 10 years of age. Delaying the surgical procedure until a child is older has reduced growth restriction (loss of size in the maxilla) and implant resorption^{3,4,7,14,17} that was noted after early surgical procedures. However, there continue to be problems associated with the procedure: harvest of cancellous bone still requires a secondary surgical site^{3,9,11,12,14,16,18} and is associated with increased surgical time, pain, and risk of infection.^{18,46}

TE grafts have been identified as an alternative to autologous bone grafting. Benefits include avoidance of a donor site with its associated morbidity, and unlimited supply that is uniformly available, independent of patient age.⁴⁷ Several polymers can be used to generate scaffolds.⁴⁸ In this study we used PLGA, an FDA-approved biodegradable polymer, which is currently used for resorbable plates and screws in pediatric craniofacial reconstruction.^{49,50} Our preferred stem cell source is UC-derived MSCs. UC MSCs have several advantages with respect to other sources of cells: they can be harvested at the time of birth from discarded tissue, have a high cell yield, excellent osteogenic potential, and anti-

FIG. 6. 3D CT scans of pigs 90 days following repair of a surgically created defect (A–C) and an age-matched control pig (D–F). Note the growth restriction in the maxilla (B, C) relative to normal growth (E, F) and that the asymmetry was propagated into portions of the skull at a distance from the cleft (A, D). Increased bone growth on the nondefect side (B), along with collapse of the defect side (C), may have contributed to the marked asymmetry. Statistical analysis of asymmetry based on mirror reflection of landmark locations in the maxilla indicated no difference among treatment types ($p=0.464$). Color images available online at www.liebertpub.com/tec

immunogenic properties, making them a favorable source of MSCs for clinical trials.⁵¹

In this study, we seeded autologous UC MSCs onto PLGA scaffolds for TE treatment of the alveolar cleft. Results were compared to iliac crest CB grafts, in a swine

FIG. 7. Elastic modulus evaluation by indentation of new bone generated with the different treatments. Mean elastic modulus values are shown for UD, DI cells, and CB treatments. A single NT sample was testable; the remainder were too soft for testing. Indentation values are compared with the contralateral NL of all the subjects tested. Although TE treatments appear to have a slightly higher elastic modulus, no statistically significant difference was noted between the treatments. NL, normal side. Color images available online at www.liebertpub.com/tec

model of the alveolar cleft.³⁵ TE treated animals were divided into two groups: the first received scaffolds seeded with UD UC MSCs and the second group with DI UC MSCs. One-month postsurgery, samples were analyzed by histology and 3D reconstructions of CT scans to analyze bone formation, volume fill of the defect, and craniofacial asymmetry.

Histological studies demonstrated the presence of immature woven bone with osteoblastic activity within the alveolar gap indicating active osteogenesis for all three treatments. Integration of this newly formed bone with the intact bone boundaries was also noted. In untreated samples there was minimal osteoblastic activity at the intact bone boundaries, with no osteogenic activity detected within the defect. Both UD and DI treatments appeared to have similar bone-forming potential comparable to the iliac crest CB grafts; defects treated with DI constructs demonstrated increased mature bone within the defect compared to the other treatments.

Although intense osteogenic activity was detected within the defects from all treatments groups, there were also regions with collagen fibers and fibroblast-like cells, indicating the presence of fibrotic tissue within the alveolar gap, demonstrating that none of the treatments were sufficient to completely fill the defect with bone after 1 month. No remodeling seemed to be present at 30 days postsurgery, indicated by a lack of histological evidence of osteoclast

activity. This is to be expected, because mineralization was not yet complete 30 days postsurgery. The majority of cells contributing to bone formation within the defect were GFP+, indicative of implanted cells as the major source of osteogenic progenitors. In some samples, isolated GFP+ cells were noted beyond the surgical boundary in normal bone; this however, was a rare event, indicating that implanted cells generally remained in the implanted area.

There are controversial reports in the literature regarding the need for the presence of cells on scaffolds to effectively regenerate tissue.^{52,53} Our experience with implantation of scaffolds alone within the surgical defect induced an inflammatory response with no bone formation. In the treatment conditions where cells were implanted with the scaffold, there was no histological evidence of an inflammatory response. This may support the idea of the immunomodulatory properties of MSCs, preserved even after *in vitro* differentiation.^{29,54,55}

3D CT scans indicate that TE grafts promote new bone formation in a periphery-to-center pattern, indicating good integration with surrounding bone and a reduction of the defect volume, but without complete filling of the defect. There were no statistically significant differences between either of the TE treatments (UD, DI). Both treatments showed similar volume fill when compared to autologous CB treatment. The incomplete volume fill may be, at least in part, due to the structural limitations of the scaffolds used to fill the defect. The nanoscaffolds provided the architecture to maintain implanted cells at the surgical site, allowing osteogenesis, but not the adequate structural integrity to maintain the space of the defect and avoid soft tissue collapse.

Previous studies have demonstrated that surgical alteration of craniofacial bone (removal, implants, or onlays) induces craniofacial asymmetries.^{56–58} Although there are no statistically significant differences in symmetry using morphometric examination, we did detect asymmetries associated with all three treatments, including the untreated controls in individual pigs. No variations were detected between treatment groups, suggesting that differences in these individual pigs were as likely to be from any one of the treatment groups, and may be a finding intrinsic to the pigs themselves. Excessive bone growth on the nondefect hemi-maxilla, along with collapse of the affected side, appeared to contribute to the noted asymmetry.

Growth restriction was not measured directly in this study due to the short-term follow-up of the animals. We do, however, see evidence of qualitative growth restriction in one of the 3-month CT scans; the operated maxilla was shorter in total length than the contralateral normal bone. As a whole, both maxillae and the nasal bones were shorter when compared to normal age-matched pigs. Factors that may contribute to growth restriction include the loss of blood supply due to surgical bone removal,⁵⁹ unilateral removal of teeth,⁶⁰ and subsequent alteration of the dental midline. The presence of soft tissue in nonbone filled areas alters the stress and strain distribution pattern associated with normal function of the maxilla,⁶¹ potentially contributing to both the asymmetry and the growth restriction.

Although, 1 month is insufficient time to allow complete mineralization of new bone, which subsequently defines its mechanical properties, mechanical testing demonstrated that TE engrafted material increased the strength (a higher

Young's modulus) of the tissue within the defect when compared to untreated controls. This may indicate improved bone mineralization for TE-based treatment, in particular the DI treatment. CB graft also had little effect on providing mechanical stiffness in this short time period. All treatments had a significantly lower elastic modulus than contralateral, unoperated bone. Longer-term studies (6 months to a year) are required to make a final determination of peak modulus values capable for TE bone.

A previous report in a rat alveolar cleft model demonstrated differences in reduction of defect volume between UD and DI MSCs seeded on hydroxyapatite scaffolds, with UD cells producing significantly more bone within the defect volume.⁶² In our own work, though UD treatment histologically demonstrated areas of more immature bone than DI treatment at 1 month, the differences appeared insignificant. In addition, we did not see differences between the two TE treatments with respect to volume fill and asymmetry. Inducing *in vitro* differentiation of UC MSCs before implantation does not appear to be justified or necessary in our swine model.

TE constructs based on a combination of PLGA-UC MSCs appear to be appropriate candidates for repair of alveolar cleft defects. Our findings demonstrate that MSCs can contribute to bone regeneration, independent of previous *in vitro* priming toward an osteogenic phenotype. This study also shows that the swine model can be used in preclinical studies to test novel TE-based repair strategies. Most importantly, the model can be used to study the mechanisms that cause asymmetry and growth restriction after early surgical correction of craniofacial bone defects. This requires longer-term studies (6 months or greater) that utilize scaffolds with greater structural integrity and have mechanical properties that prevent soft tissue collapse.

Acknowledgments

Cincinnati Children's Hospital Veterinary Services and the Imaging Research Center; Stryker Craniofacial for donation of the PLGA plates to create the mixed fiber scaffolds, and to GR Liu, PhD and Zimo Zhu, PhD for their assistance with changes to software used to convert files from CVS to NTS. This project was supported by NIH (NIDCR K08 DE023124-01; KL2TR000084), and internal funding from Cincinnati Children's Hospital Medical Center.

Disclosure Statement

No competing financial interests exist.

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Received: March 30, 2017

Accepted: July 24, 2017

Online Publication Date: August 31, 2017