Bioengineering a Human Face Graft

The Matrix of Identity

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Objective: During the last decade, face allotransplantation has been shown to be a revolutionary reconstructive procedure for severe disfigurements. However, offer to patients remains limited due to lifelong immunosuppression. To move forward in the field, a new pathway in tissue engineering is proposed.
Background: Our previously reported technique of matrix production of a porcine auricular subunit graft has been translated to a human face model.
Methods: 5 partial and 1 total face grafts were procured from human fresh cadavers. After arterial cannulation, the specimens were perfused using a combined detergent/polar solvent decellularization protocol. Preservation of vascular patency was assessed by imaging, cell and antigen removal by DNA quantification and histology. The main extracellular matrix proteins and associated cytokines were evaluated. Lip scaffolds were cultivated with dermal, muscle progenitor and endothelial cells, either on discs or in a bioreactor.
Results: Decellularization was successful in all facial grafts within 12 days revealing acellular scaffolds with full preservation of innate morphology. Imaging demonstrated a preservation of the entire vascular tree patency. Removal of cells and antigens was confirmed by reduction of DNA and antigen markers negativation. Microscopic evaluation revealed preservation of tissue structures as well as of major proteins. Seeded cells were viable and well distributed within all scaffolds.
Conclusions: Complex acellular facial scaffolds were obtained, preserving simultaneously a cell-friendly extracellular matrix and a perfusable vascular tree. This step will enable further engineering of postmortem facial grafts, thereby offering new perspectives in composite tissue allotransplantation.

Keywords: bioreactors, decellularization-recellularization, extracellular matrix, face transplantation, human model, regenerative medicine, scaffold, tissue engineering

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At the dawn of the 21st century, conventional facial reconstructive techniques, based on the use of autologous tissues, have shown their limitations and inability to simultaneously restore the morphological identity, cosmetic appearance, and expressive function devoted to the human face. In 2005, the first successful facial transplantation[1,2] was applied to the highly complex three-dimensional (3D) framework of the face, relying on the vascularized composite tissue allotransplantation (VCA) principles learned from limb transplantation.[3] The success of face transplantation raised a lot of hopes for a significant improvement of the surgical treatment of severe disfigurement. A decade later, however, face transplantation remains of limited clinical applications with an overall of 35 cases reported worldwide.[4] While ethical concerns have been overcome by the outstanding superiority of the clinical results,[5] the cost-benefit

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JD conceived, designed, and wrote the study project, performed all investigations and surgeries, collected results, interpreted data, and wrote the manuscript. He was responsible for the primary undertaking, completion, and supervision of all experiments, and gave final approval to the version to be published. LM participated in the study project, assisted all investigations, collected results, interpreted data, and reviewed the manuscript, and gave final approval to the version to be published. AT, RR, and EV were responsible for the growth factor and cytokines analysis in the native and decellularized grafts, performed all related experiments, interpreted data, reviewed the whole manuscript, and gave final approval to the version to be published. GO contributed to the study design, provided input on tissue engineering aspects, edited and revised the manuscript critically, and gave final approval to the version to be published. EC performed radiogram and CT scan of the face graft, reconstructed and interpreted images, reviewed manuscript and gave final approval to the version to be published. VJ and CD were responsible for endothelial cell culture choice and production, participated to the reendothelialization methodology and results interpretation, reviewed manuscript, and gave final approval to the version to be published. JL reviewed the manuscript and provided strong insights on tissue engineering and organ transplantation aspects, edited and revised the manuscript critically, gave final approval to the version to be published. CB provided technical support, reviewed the manuscript and gave final approval to the version to be published. GC Provided support in muscular regeneration conception for the study design, reviewed and interpreted data, reviewed the manuscript, and gave final approval to the version to be published. PG contributed to the study design, provided input on tissue engineering aspects, supported analysis and review data, edited and revised the manuscript critically, and gave final approval to the version to be published. BL contributed largely to the study design, provided strong input on aspects of face anatomy, transplant surgery, tissue engineering, edited and revised the manuscript extensively, and approved its final version to be published.

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balance of the procedure remains questioned, because of need for a lifelong immunosuppression (IS).[6]

Initially, the reluctance to accept the procedure relied mostly on the risk of acute rejection, whereas nowadays the main concern regarding the future of this technique is chronic rejection. Like in other types of VCA, chronic rejection[7,8] induces distal vascular impairment, which results in a mean graft survival of 10 to 15 years. Hence, the need to open up new therapeutic pathways arose, with 2 different strategic options: the first one is aimed at reaching the ultimate goal of a long-term graft tolerance,[9,10] the second one relies on the de novo creation of new grafts, that will naturally be tolerated, through the new original pathway of regenerative medicine.[11]

In regenerative medicine, the so-called cell seeding on scaffold technology (CSST), in which cells from a patient can be grown on extracellular matrix (ECM) scaffolds to produce functional organs, has been previously investigated in solid organs[12–16] and limbs[17] with very promising results. In a previous report,[18] we demonstrated the successful application of CSST in a porcine ear graft model, designed as an emblematic initial experimental platform to start face bioengineering. The so-obtained results on the auricular facial subunit led us to draw 3 hypotheses for the present study: first, can we extend CSST to other types of facial subunits, such as the nose, lips, cheeks, or even a whole face? Then, can we transpose it to a human model? And finally, can we extend the donor criteria for face graft procurement, through the resistance of composite tissues from cadaveric donors to biological degradation?

METHODS

Our study was conducted on 5 segmental lower face grafts (SF-G) and 1 full face graft (FF-G), procured from fresh human cadavers from body donation at Université catholique de Louvain, within a median delay of 50 hours (range 12–96) from death. Median age was 82 years (range 63–96). The cadavers were stored in a cold room at 4°C until graft procurement. All the experiments were approved by the local ethical committee.

Surgical Technique

SF-G involving the nose, lips, and cheeks subunits were elevated as previously described[19] after facial artery (FA) and vein isolation, with the facial, mental, and infra-orbital nerves. Both FAs were cannulated and muscles labeled (Fig. 1A–C). In case of the FF-G, procurement was extended to the upper face, adding both superficial temporal arteries (STA) (Fig. 1K–L).

Perfusion-decellularization Technique

All grafts were immersed in a glass jar, and the arterial cannulas were connected to a Masterflex L/S peristaltic pump with 16G tubing (Cole Parmer, Vernon Hills, IL). Mean arterial pressure (MAP) was recorded with a Datex-Ohmeda S/5 monitor (GE Healthcare Life Sciences, Chicago, IL). Perfusion of both FAs, keeping the MAP below 80 mm Hg, was divided into 3 sequences, respecting volumes load indicated for SF-G. In the first sequence, adapted from Ott et al,[12] the solution consisted of heparinized saline (15 UI/mL, 1.5 L), 10 μM adenosine (A-4036, Sigma-Aldrich, St. Louis, MO); 1% SDS (70 L); deionized water (6 L); 1% Triton-X 100 (9 L); and PBS (30 L). During the second sequence, defatting was achieved using 2-propanol (ISO) (VWR, Radnor, PA). After a first overnight stirring-bath (1 L), ISO was perfused in closed circuit perfusion (2 L) for 12 hours. After a second overnight ISO stirring-bath (1 L), grafts were rehydrated, with deionized water (1 L) and PBS perfusion (26 L). During the third sequence, type I bovine DNAse (Sigma-Aldrich) (25 mg/L, 1.5 L PBS) was perfused at 37°C, then washed with PBS (10 L). During all 3 steps, MAP as well as the graft were monitored with respect to edema, blistering of facial skin and mucosae, fat and muscle bleaching. For FF-G, perfusion was done alternatively in STAs and FAs.

Produced segmental (SF-ECM) and full (FF-ECM) face ECM scaffolds were either processed for analysis or stored at 4°C in PBS.

In Vitro Vascular Assessment, Mock Transplantation, and In Vivo Reperfusion Study

SF-ECM vascular patency was assessed by fluoroscopy, acquired with a Powermobil C-Arm (Siemens, Munich, Germany). FF-ECM were injected with a barium sulfate-gel solution (40 mL), as previously described,[20] then imaged with a Senograph Essential (GE Healthcare, Chicago, IL). To mimic transplantation, the FF-ECM was replaced on the original donor head and images acquired with a computed tomography (CT) scanner (Philips Healthcare, Cleveland, OH), and analyzed using Osirix software (Pixmeo, Bernex, Switzerland).

To assess if the innate vasculature of the scaffold would support a physiological blood pressure, we heterotopically reperfused a SF-ECM graft under general anaesthesia in a porcine recipient. Both FAs were anastomosed to abdominal aorta and the inferior mesenteric artery. After systemic heparinization, reperfusion was observed for 4 hours.

Tissue Sampling

SF-G and SF-ECMs were sampled for histology, quantification of DNA and proteins, at various locations and in triplicate (Fig. 2A).

Histology, Immunohistochemistry, and Immunofluorescence

Samples were processed according to standard protocols, including 5-μm-thick sectioning and staining with H&E and Masson's trichrome. Methods followed previously described protocols.[21] For immunohistochemistry (IHC), sections were incubated with anti-MHC class I antibody (1:200, ab198376, Abcam, Cambridge, UK) and anti-CD31 (1:100, ab28364, Abcam). Nuclei were counterstained with hematoxylin. For immunofluorescence (IF), incubation was performed with anticollagen IV (1:500, ab6586, Abcam), anti-laminin (1:50, ab11575, Abcam), and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

DNA Quantification

DNA was extracted from samples with a DNeasy kit (Qiagen, Hilden, Germany) and quantified using the Quant-it Picrogreen dsDNA assay kit (ThermoFischer Scientific, Waltham, MA), according to the manufacturer's instructions. To determine the remaining DNA fragments size, samples were analyzed by gel electrophoresis, as previously described.[22]

Quantification of ECM Proteins, Growth Factors, and Cytokines

To understand how the decellularization process affected the ECM and their presence, major ECM proteins, several growth factors, and cytokines were quantified. Collagen was quantified using a Chondrex hydroxyproline assay kit (Chondrex Inc, Redmond, WA); Glycosaminoglycan (GAGs) content was evaluated using the Blyscan Sulphated-GAG assay kit (Biocolor LTD, Carrickfergus, Northern Ireland) and elastin content was quantified using the Fast Elastin Assay kit (Biocolor), according to the manufacturer's protocols. Concentration of growth factors and cytokines were quantified using the Bio-Plex Pro Human Chemokine 40-plex Panel and Bio-Plex Pro TGF-β assays (Bio-Rad, Hercules, CA) according to the manufacturer's guidelines.

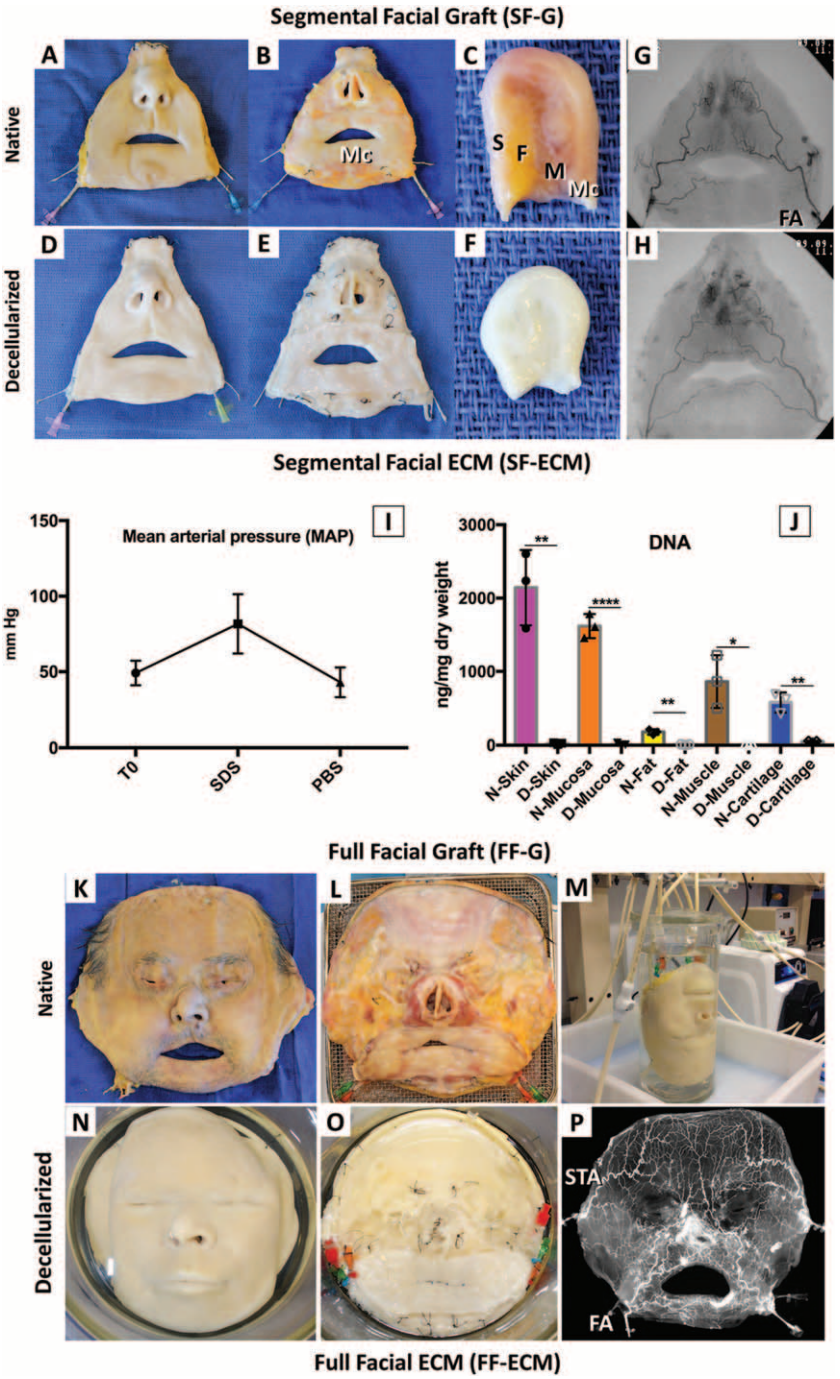

FIGURE 1. Segmental and full face grafts perfusion-decellularization. A–C, Native segmental (SF-G) nose-lip face graft with its associated vascular pedicles, in A anterior and B posterior views. C, represents the lower lip cross section with the associated tissue layers of skin (S), fat (F), orbicularis ori muscle (M), and oral mucosa (Mc). D–F, Decellularized segmental face graft (SF-ECM) demonstrating a satisfying superficial aspect of D the acellular skin, mucosa, fat and E muscle stumps labelled with a nylon suture; F, deep thickness examination of the lip, demonstrating treatment of all layers. G and H, Fluoroscopy demonstrates that patency of the facial artery (FA) and branches is preserved when comparing G native SF-G with H produced acellular SF-ECM vascular tree. I, Mean arterial pressure (MAP) during the main decellularization steps of SF-G expressed in mm Hg: baseline (T0), at the end of sodium dodecyl sulfate (SDS) perfusion, and after final rinsing (PBS). J, DNA content in native (N-) and decellularized (D-) tissues: skin, oral mucosae, fat, facial muscles, and cartilage. Results expressed in ng/mg ECM and native tissue (dry weight). Data presented as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.0001$ by 2-tailed unpaired *t* test. K and L, Native full-thickness face graft (FF-G), in K anterior and L posterior views. M, Decellularization apparatus and graft in process. N and O, Decellularized full-thickness face graft (FF-ECM), exhibiting obvious skin and annexes treatment as well as tissues on the inner side. P, Radiogram of the barium-gel injected decellularized FF-ECM; contrast injection through superficial temporal arteries (STA) and facial arteries (FA) demonstrates a very well preserved vascular tree after decellularization.

Static Disc Cell-seeding

1 cm^2 discs of acellular lips (Lip-ECM) obtained from SF-ECM were sterilized with 0.1% peracetic acid (PCA). Sixteen discs were seeded with 50×10^4 NIH-3T3 dermal fibroblast (Sigma-Aldrich) and 4×10^6 C2C12 (Sigma-Aldrich) myoblast progenitor cell lines, placed in 24-well plates, and allowed to grow in supplemented Dubelcco's Modified Eagle Medium (DMEM), changed every 2 days. For C2C12 cells, we used 1% FBS as differentiation medium. After 14 days, biopsies were either processed according to standard protocols as whole-mount for Live/Dead Cell Viability assays (ThermoFisher Scientific), prepared for analysis with a field-emission scanning electron microscope (JSM-7600F, Jeol, Akishima, Tokyo, Japan), or processed for H&E staining.

Whole Lip Bioreactor Cell-seeding

Two upper Lip-ECM were procured with their artery from SF-ECM, sterilized with 0.1% peracetic acid, setup in a 500 mL

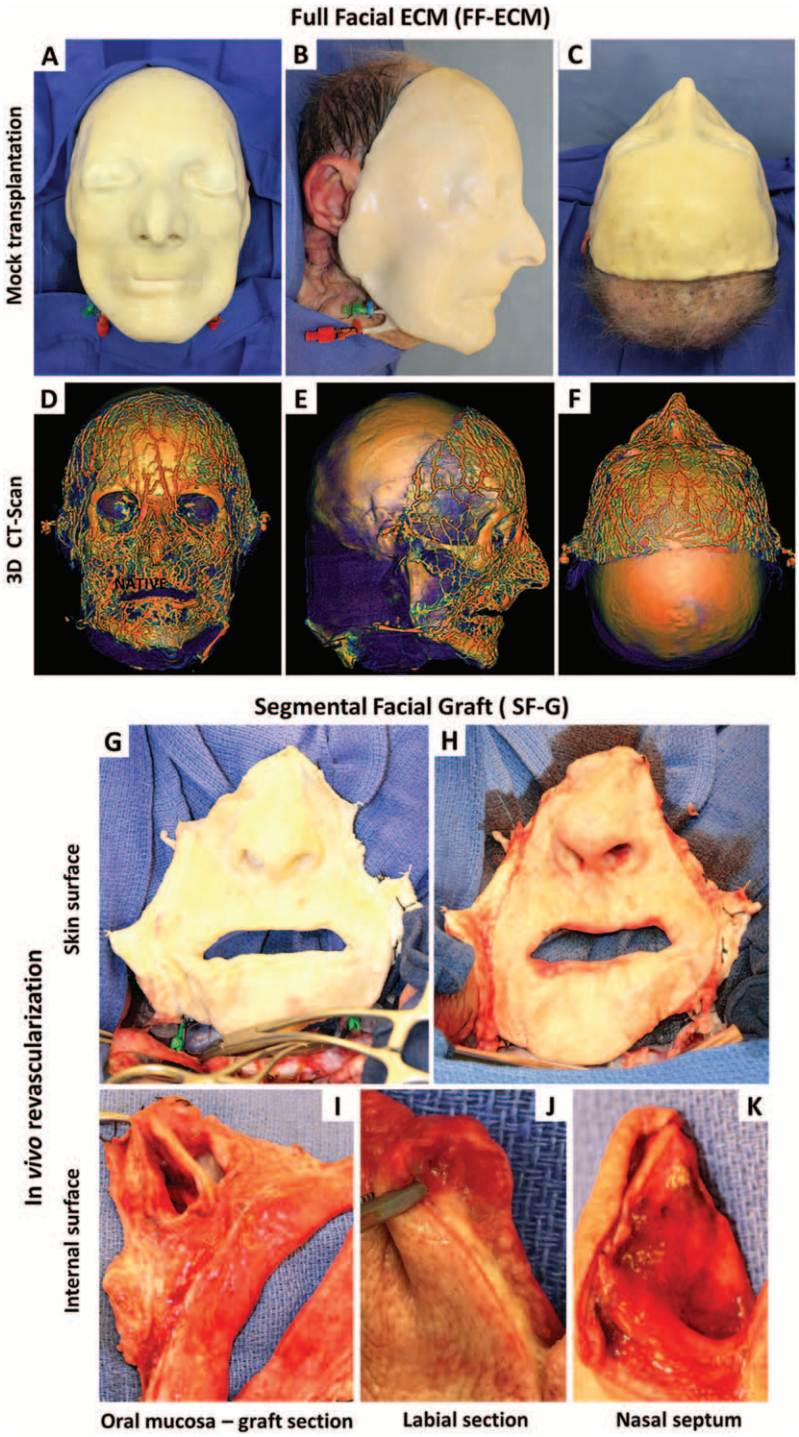

FIGURE 2. FF-ECM mock transplantation and SF-ECM in vivo reperfusion. A–F, Mock transplantation of the FF-ECM and angio-CT. A–C, Anterior, lateral, and upper views of FF-ECM replaced and sutured on the original donor head defect; preservation of facial morphology in all areas, including cartilaginous support of the nasal framework, as well as transition with original native areas. D–F, corresponding angio-CT views from previous panel, showing the perfect anatomical and plastic congruence of the acellular graft and its opacified vasculature, with the underlying bone support of the recipient. G–K, In vivo reperfusion of SF-ECM in a porcine recipient. External view G before and H after vascular clamp release; the general recoloration is obvious, especially on the lip vermillion. I, Internal aspect of the revascularized scaffold, with signs of oral mucosa reperfusion and capillary bleeding on the section lines. Similar observations on J lip section and K nasal septum.

perfusion-bioreactor and conditioned through medium perfusions for 4 hours at 37°C/5% CO_2 conditions (Fig. 5I, J).

C2C12 Cells

The bioreactor was filled with Dulbecco Modified Eagle Medium differentiation medium supplemented with 1% FBS. 70×10^6 cells were collected and delivered into the scaffold by twenty 0.1 mL injections (Fig. 5K). Perfusion flow was set at 2 mL/min for 4 hours and increased thereafter to 4 mL/min. After 2 weeks, the recellularized scaffolds were examined by Live/Dead and H&E stainings.

Human Aortic Endothelial Cells (HAEC)

After immersion in Endothelial Cell Growth Medium MV2 (Bio-Connect, Toronto, Canada), 25×10^6 HAEC (304-05A,

Sigma-Aldrich) were slowly injected through the arterial line. A static period was observed for 4 hours, to allow cell attachment. Then flow was restarted at 1 mL/min for 48 hours; thereafter, biopsies were processed for Live/Dead, H&E and CD31 IHC stainings.

Statistical Analysis

All statistical analyses were performed using Prism 7.00 (GraphPad Software, La Jolla, CA). Values were expressed as mean ± standard error of the mean, and significance was set at $P<0.05$, using 2-tailed unpaired *t* test.

RESULTS

Macroscopic Decellularization, Vascular Patency, and DNA Reduction

The used perfusion method successfully and consistently resulted in complete cell clearance of the face grafts, while preserving their ECM. Macroscopically, epidermal blistering appeared in the first 24 hours of SDS perfusion; epidermolysis was complete and muscle became whitish within a few days, with remaining lipidic content; fat became completely white after the polar solvent sequence (Fig. 1A–F). Fluoroscopy demonstrated the patency of the FA main axis and branches of the SF-ECM (Fig. 1G, H). During the decellularization process, the MAP was increased during the SDS perfusion step, associated with graft diffuse edema which resolved after the final perfusion (Fig. 1I). This phenomenon was principally located in the fat layer. The DNA content was significantly reduced in all tissues, with a mean reduction of 97% ($P < 0.001$) in decellularized biopsies compared with native ones (Fig. 1J). The remaining DNA fragments size was found heterogeneous, with some long fragments especially observed in cartilage and muscle. When extending the process to a full-face graft (Fig. 1K–M), we also observed a satisfying aspect in all graft tissues (Fig. 1N, O). The arterial radiogram revealed an extremely well-preserved vasculature (Fig. 1P).

Mock Transplantation and CT of a FF-ECM, In Vivo Reperfusion of a SF-ECM Graft

The replacement of the produced FF-ECM on the original donor harvesting site enhanced the quality of morphological preservation of the face in all anatomical areas (Fig. 2A–C) and integration to the native environment. The CT-scan examination of the mock transplantation showed an excellent integration of the graft and vessels in 3D, with a perfect plastic congruence on the underlying facial bone support (Fig. 2D–F).

During in vivo reperfusion, the SF-ECM arteries could be easily anastomosed to the recipient vessels. A restored flow was immediately observed in the lips, then on the nasal septum and graft margins and was patent for the 4-hours observation period (Fig. 2G–K).

Cell and Antigen Clearance

H&E staining performed in all anatomical subunits did not identify any basophilic staining indicative of cell nuclei (Fig. 3B–E). This was confirmed by negative MHC-I antigen staining (Fig. 3F–H). These findings indirectly indicate the cellular clearance. However, the cartilage did retain some cells, as we reported in the porcine ear engineering[18] and in the literature[23] with a negligible impact on DNA content.

Preservation of ECM Proteins and Growth Factors

Masson's trichrome showed a structural preservation of whole collagen (Fig. 3J, K), and especially type IV collagen (Fig. 3L), involved in basal membrane preservation, for dermal junction, vessels, and glands. For the muscle, laminin was very well preserved, as confirmed by laminin staining (Fig. 3M), a positive factor for subsequent muscular regeneration.[24] Regarding the major ECM protein content (Fig. 4A), collagen was found important in all tissues, with a high relative increase. For elastin, the reduction was significant in all tissues with exception of fat. For the GAGs, a significant decrease was observed in skin, mucosa, and muscle; for fat and cartilage they were relatively well preserved.

Regarding decellularization effect on cytokines and growth factors (Fig. 4B), we observed in skin a significant decrease of IL-8 and GM-CSF; SDF-1alpha, IL-10, TNF-alpha and IL6 did not decrease significantly due to their high variation in the native tissues. In mucosa, a significant decrease of all analyzed cytokines was observed with the exception of IL-8 and IL-6, which nevertheless showed a clear tendency to decrease. The fat presented the highest grade of preservation, with a significant decrease of GM-CSF, IL-10, and TNF-alpha. In facial muscles, a significant decrease of GM-CSF and IL-10, a tendency to decrease of IL-8, TNF-alpha, and IL-6 with partial preservation of SDF-1alpha were observed. Finally, cartilage presented a significant decrease of all analyzed cytokines but SDF-1alpha.

Cell Compatibility and Distribution on Discs

For both NIH-3T3 and C2C12 cell-seeded constructs showed viability, attachment, and proliferation of the cells on the scaffold surface in all the samples. Cells remained at the surface of the scaffolds with a limited deep migration, relying on the passive aspect of cell seeding and on the high density of the ECM (Fig. 5B–D). For C2C12, cells adopted a different muscular phenotype (Fig. 5E–H).

Whole-lip Bioreactor Vascular Seeding (Fig. 5I–P)

C2C12-injected lip demonstrated cell groups at different superficial and deep levels, mainly located in the parenchyma, forming clusters, and infiltrating the matrix with some migration. Cell viability was confirmed by Live/Dead staining (Fig. 5K–M).

HAEC, observed homogenously in vessels, throughout the scaffold whole-thickness, were viable (Fig. 5N) and exhibited vascular attachment. Cells with a typical endothelial morphology were located on the vessel wall, in both large and small vessels, rather than remaining in the lumen (Fig. 5O). CD31 staining was observed on the vascular wall similar to normal endothelium phenotype (Fig. 5P). Some cell clusters could be found at the border of the graft, as vascular leakage.

DISCUSSION

This report describes matrix production from human cadaveric face grafts. Our protocol successfully decellularized segmental and total faces, while keeping their morphology, sustaining vascular perfusion, and allowing cell engraftment.

Previous work by our group on the porcine ear model has allowed scaling up to the entire face in the human model, with the main difference of adding a polar solvent step, as described for isolated adipose tissue decellularization.[25,26] Indeed, contrary to solid organs, adipose tissue is a true anatomical and functional compartment in the face.[27] Finally, we confirmed that deceased donors from even several days can be used: this resistance to degradation of composite tissues could be explained by their low metabolic cell activity, and a high collagen content.

Growth factor preservation in matrix is a key factor, as investigated in organ CSST.[28] Our decellularization method allowed for a partial preservation of growth factors, which were still detectable in skin, mucosa, fat, muscle, and cartilage. Interestingly, in

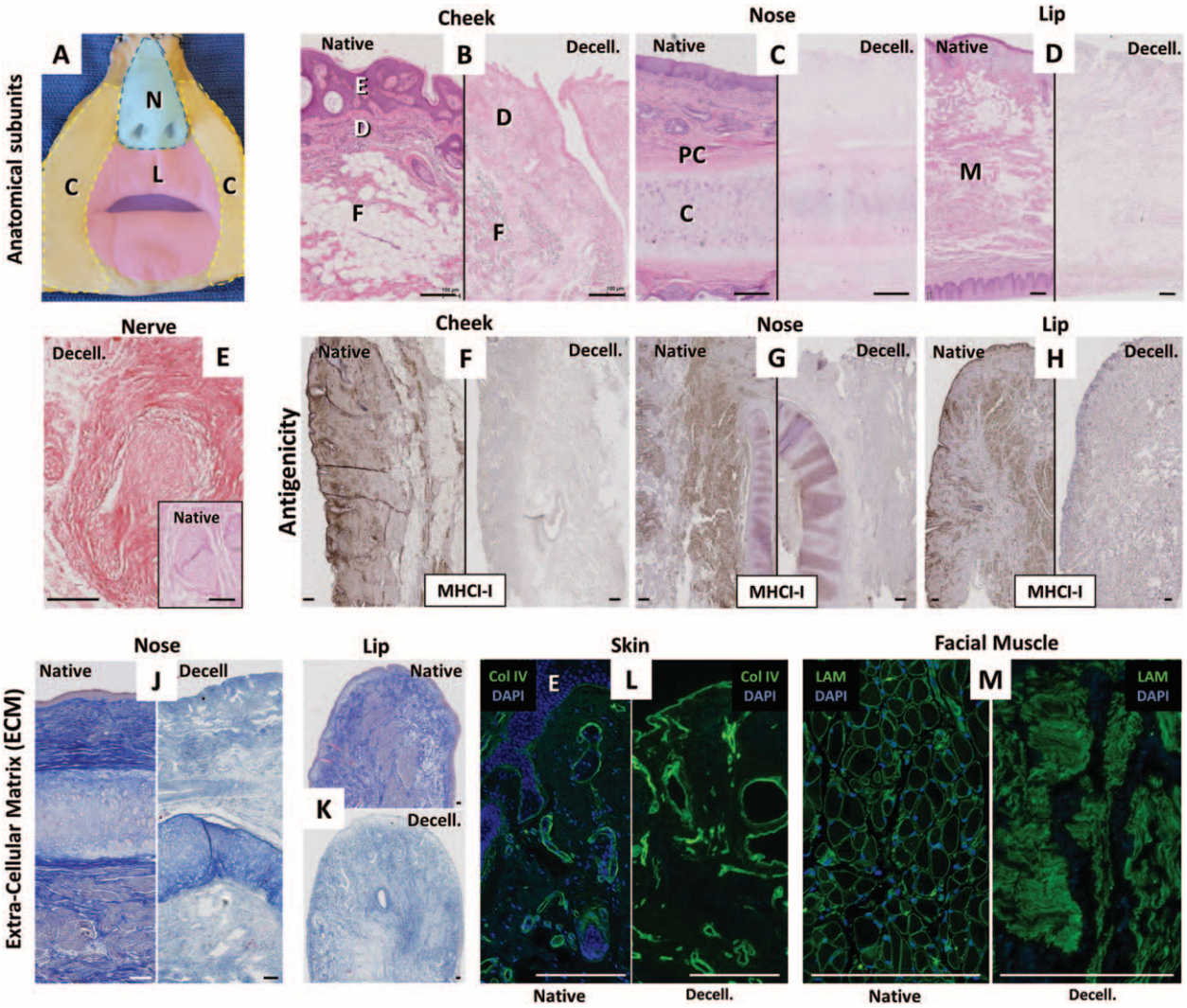

FIGURE 3. Microscopic assessment of SF-ECM. A, The mapping for SF-ECM biopsies is defined on the subunits division of this segmental face graft: nose (N), cheek (C), lips (L). B–D, H&E staining of the 3 different subunits, from native and decellularized grafts, with cell removal at all levels, confirming the DNA content analysis (see Fig. 1J), with preservation of the ECM structure. B, In the cheek, epidermis (E) and skin appendages exhibit clear cell clearance, leaving an acellular dermis (D); this anatomical location allows a more important observation of the adipose tissue (fat, F). C, The nose allows a more specific study of the elastic cartilage (C) tissue layer, demonstrating structural preservation after decellularization. Remaining cell nuclei can be observed in the deep layer, but not correlated to high remaining DNA. D, In the lip, facial muscle (M) can be specifically studied and exhibits complete cell clearance with structural preservation. E, Decellularized nerve compared with native (insert). F–H, Anti-MHC class I IHC staining in the cheek, nose, and lip biopsies, comparing native (left) and decellularized (right) tissues, with absence of staining in the latest. J–M, Examination of specific ECM structural proteins. J and K, Masson's trichrome stain demonstrates collagen preservation in the nose and lip areas. L, Anti-type IV collagen IF staining in native (left) and decellularized skin (right): the staining is positive for both vessels, glands, and dermal basal membranes. M, Anti-laminin IF staining in native (left) and decellularized (right) facial muscles, with evidence of its preservation in the scaffold. Scale bars represent 200 μm.

all the analyzed tissues, TGF-beta1 and TGF-beta3 were only minimally decreased with a significant decrease only in mucosa. Other detectable cytokines have a role in stem cell chemotaxis and survival (SDF-1alpha), in differentiation and migration of monocytes and neutrophils (GM-CSF and IL-8), and in the modulation of the inflammatory process (TNF-alpha, IL-6, and IL-10). Although we found some remaining long DNA fragments, discussed as a possible negative predictive factor in the host,[22,29,30] this observation was balanced by a very small DNA matrix content, by the modulated content of inflammatory cytokines, and finally the negative HLA markers in our scaffolds.

Face is a very unique anatomical and functional organ, consisting in a very complex association of different tissues, which must be uniformly treated. Further studies in facial CSST should concentrate on a subunit approach, as we did with the lip in the present study, to allow an anatomical selectivity of tissue associations and a discrimination of specific tissues: nose and ear for cartilage; lips and eyelids, for muscle; cheeks for adipose tissue and muscle.

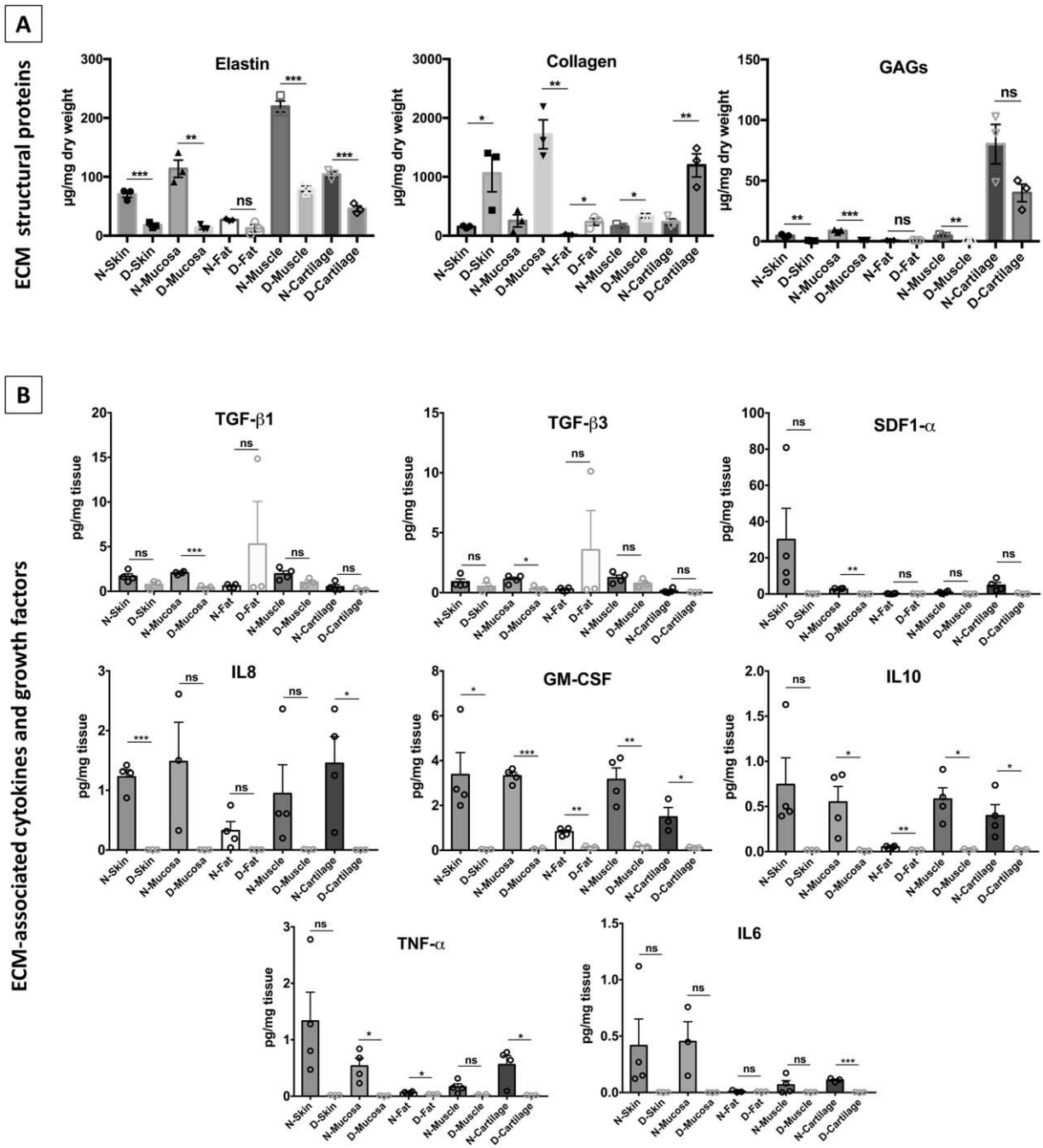

FIGURE 4. Evaluation of the decellularized process on the preservation of collagen, elastin, glycosaminoglycans, growth factors, and cytokines. A, Quantification of elastin, collagen, and GAGs in native (N-) and decellularized (D-) skin, oral mucosa, fat, muscle, and cartilage tissue biopsies from native segmental face grafts and decellularized segmental face ECM. Elastin, even with a certain degree of preservation, is significantly reduced in all tissues, with exception of the adipose tissue. The collagen is highly preserved, and even demonstrates an important increase after decellularization, due to its relative gain with decrease of the other ECM proteins. GAGs are significantly reduced in skin, mucosa, and muscle, whilst relatively preserved in the fat and cartilage tissues. B, Cytokine concentrations of TGF-beta1, TGF-beta3, SDF1-alpha, IL-8, GM-CSF, IL-10, TNF-alpha, and IL-6 in biopsies collected from native and decellularized tissues, analyzed by Luminex-like multiplex assay. Preservation of the analyzed cytokines ranged from 57.4 ± 31.1 for TGF-beta3 to 0.4 ± 0.1 for IL-8 in skin, from 71.5 ± 13.9 for TGF-beta3 to 0.1 ± 0.03 for IL-8 in mucosa; from 178.3 ± 15.6 for SDF-1α to 4.8 ± 0.4 for IL-8 in fat; from 62.7 ± 16.9 for TGF-beta3 to 2.0 ± 0.4 for IL-8 in muscle and from 38.6 ± 17.3 for TGF-beta1 to 0.2 ± 0.05 for IL-8 in cartilage. Data presented as mean $\pm$ SEM, ns = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by 2-tailed unpaired *t* test. The content is expressed in µg/mg dry weight for ECM proteins and in pg/mg for cytokines.

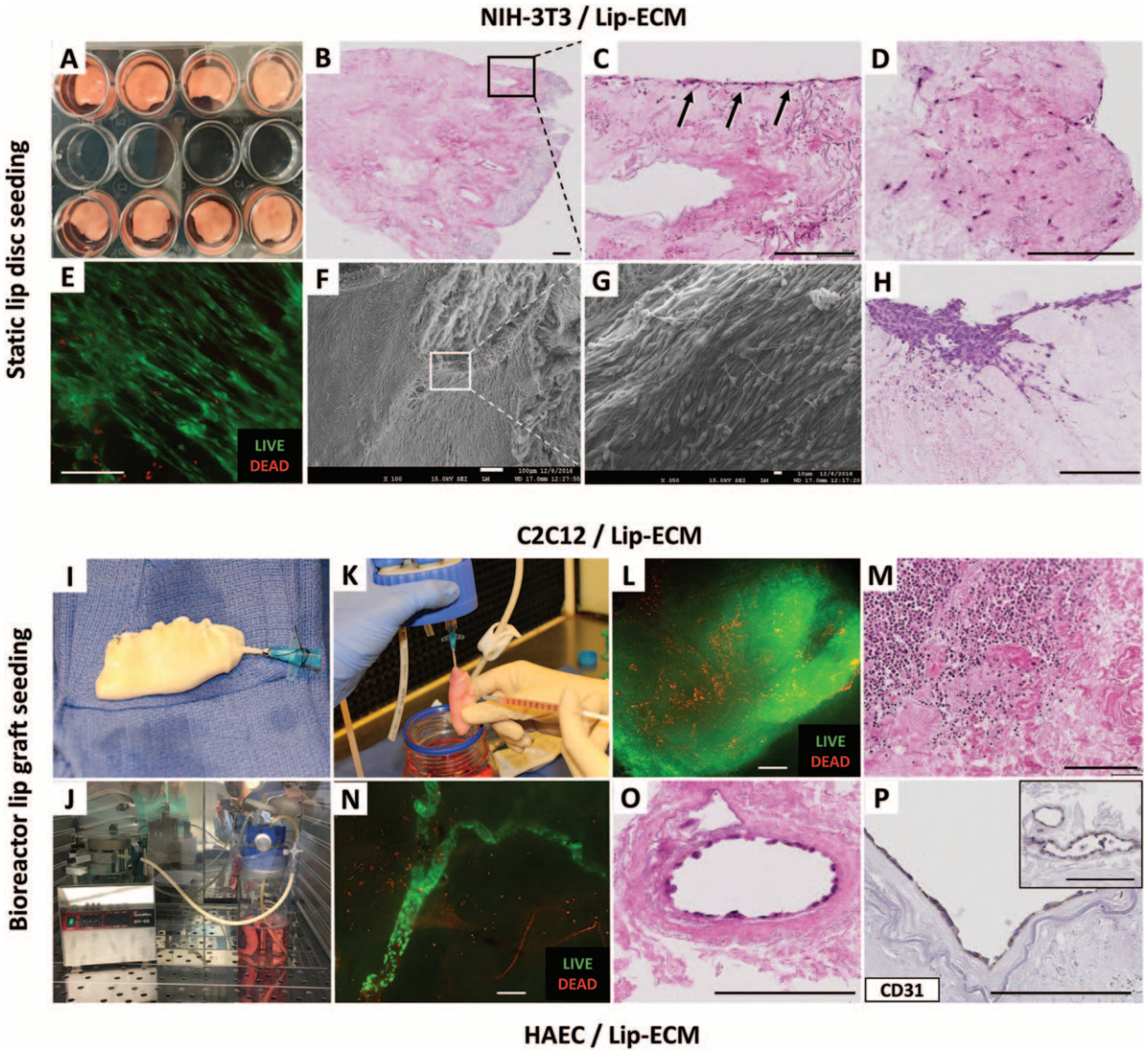

FIGURE 5. Acellular lip (Lip-ECM) disc culture and bioreactor recellularization after 14 days of culture. A–H, Static NIH-3T3 and C2C12 cells seeding. A, Acellular lip discs in the 24-well plate and conditioned with medium prior to seeding. B–D, NIH-3T3 dermal fibroblast cell line seeding result on the acellular lip side section. B, Cells are found on the whole section, as seen on C, enlarged view from B, with a remaining mainly at the scaffold surface (arrows). D, In some locations, a certain degree of cell migration in the scaffold can be seen. E–H, C2C12 myoblast progenitor cell seeding results. E, Live/Dead whole-mount staining revealing a majority of living cells; C2C12 cells have taken an elongated morphology, typical of ongoing muscular differentiation. F and G, SEM observation of the cultured discs, showing an important contact of the cells with the scaffold ECM. H, H&E observation of cells forming proliferative conglomerates at the surface, with a limited degree of migration into the scaffold. I–P, Perfusion-recellularization of a whole upper-lip acellular graft in a perfusion-bioreactor. I, Lip-ECM with the cannulated labial artery, extracted from a segmental face scaffold. J, Perfusion-bioreactor apparatus in a CO_2 incubator, with preconditioning of the lip with medium. K–M, C2C12 cells injection seeding. K, Seeding through 20 0.1 mL injections of cells and cultured for 2 weeks. L, Live/Dead of recellularized lip, with observation of a large proportion of living cells, organized in clusters. M, H&E examination of the cell clusters showing signs of cell contact with the ECM. N–P, Human aortic endothelial cells (HAEC) vascular seeding and culture for 48 hours. N, Live/Dead observation shows positivity of living cells, located in the vessels. O, H&E examination demonstrates attachment of endothelial cells on the intimal vessel wall, with excellent endothelial morphological aspect. P, Anti-CD31 IHC staining demonstrates positivity of the seeded cells, at all vessels calibers, from arterial pedicle to distal vessels (insert). Scale bars represent 200 μm.

Moreover, given the importance of the neuromuscular component in the facial function, research on face bioengineering should also focus on this aspect, to ultimately warrant the motility of the construct. Restoration of face discriminative expressivity is indeed the ultimate goal of any reconstructive procedure, especially in the lower and midface areas. Applied to VCA in general, this technology could result in functional developments, critical for bioengineering of other highly complex body parts, like hands.

Contrary to solid organs,[31] xenotransplantation cannot be applied to face which is species-specific, and CSST techniques must apply to human models. Ideally, facial ECM should reproduce the exact missing morphology of the patient, and serve as a template for the ex novo bioengineering of the new face to implant. As current state-of-the-art synthetic technologies[32] have not yet reached this step, due to the unfeasibility of replicating the complexity of the matrisome and 3D framework of the innate human ECM, human organs are being proposed as source of ECM. Since the first report on discarded human kidneys as a platform for organ bioengineering and regeneration,[11] numerous studies have corroborated the idea that ECM scaffolds obtained from the decellularization of human organs may represent a valuable template for organ biofabrication[15,28,33–39] Importantly, these studies have shown that partial regeneration of the cellular compartment of the 3D innate ECM can be obtained, but to an extent that it is still not compatible with physiological functional needs. Our experience confirms these findings, as our cells were not able to diffusely migrate within the 3D framework of the ECM. As stigmatized by the experiences with kidney bioengineering, major physical barriers remain in CSST that limit in vitro recellularization of acellular scaffolds, and numerous are the obstacles that must be investigated to effectively advance this strategy for regenerative medicine.[40] While the regeneration of the endothelium should be considered the *sine qua non* condition for bedside application, research should aim at identifying alternative regenerative strategies to solve the high complexity of recellularizing acellular ECM scaffolds of composite grafts, possibly using the recipient as its own bioreactor, as intelligently suggested by others.[41]

In the history of surgery, solid organ transplantation preceded VCA. This sequence also occurred for tissue engineering. The two domains, however, are now merging in an unprecedented synergetic effort in the whole field of transplantation surgery.[42]

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DISCUSSANTS

Michael Olausson (Gothenburg, Sweden):

First, I would like to thank for the opportunity to discuss this interesting paper on tissue engineering of face transplants. Ever since the landmark paper of Doris Taylor in Nature Medicine in 2008, a growing number of laboratories have tried to use decellularizartion techniques, followed by recellularization with stemcells, to regrow the organ with autologous recipient cells. So far, with limited success, ending in a clinical transplantation, has been seen. It is my belief that is wise to use nature's own matrix in recreating life. The alternative, using an artificial matrix, and build something that took 5 billion years to create, seems even more difficult.

First, regarding the decellularization process. You used SDS and Triton-X 100. Could you elaborate how you can be sure that all detergents are removed after its use as well as explaining why DNAse was not used?

Second, regarding the ECM proteins. How large proportion of the original matrix proteins was preserved and how do you think this could affect the recreation of the organ? Did you use proteomics using MS-LC techniques to see if any proteins disappeared?

Third, regarding the migration of the cells. You have suggested that a dynamic seeding through the vasculature would ensure the migration. Could you please enlighten us on the density of cells in the bioreactor vascular recellularized whole-lip in comparison to the same tissue before decellularization?

Finally, I want to congratulate you on an interesting paper.

Response from Jerome Duisit (Brussels, Belgium):

Thank you for the comments. For the decellularization, actually we know that SDS is efficient to treat the matrix but is difficult to remove: That's why we used additionally used Triton-X for this purpose. We could use for example mass spectrometry, to have an estimation of the residual SDS inside the matrix. But performing an important PBS solution rinsing, with an equivalent volume of perfused SDS, as we did, has been proven efficient in the literature. Our best proof was the survival of the cells. When we will go further into refining our method, we will certainly have to perform mass spectrometry. I think SDS is the best experimental solution for composite tissues. About the DNAse, we actually used it. For the remaining DNA, we assessed it by quantification and the length of fragments. We used the enzyme in this study to obtain the lowest DNA value that could be obtained. However, it shouldn't be mandatory when dealing only with in vitro studies. It will be more important to consider for in vivo studies, because of the residual DNA possible adverse events on immune response.

For the study of structural ECM proteins, we first used histological qualitative techniques, as well as quantification with colometric assays for collagen, elastin, and GAGs. For the ECM-associated proteins, growth factors, and cytokines, we used quantitative multiplex assays. The structural proteins were very well preserved, especially collagen above 100%. This relative increase is explained by the removal of cells, and the decrease of GAGs and elastin. So, the relative collagen content in a same biopsy volume became predominant. We observed for example a reduction of about 2/3rd of the GAGs and a preservation of half of elastin in the cartilage. For the cytokines, what was more important was to see that it didn't disappear totally and the types of proteins remaining: If you want to orientate stem cells differentiation, you will prefer SDF-1alpha or TGF-beta content to be high in your scaffold. If you target proteins that are involved into in vivo immune reactions, you want more TNF-alpha or IL-10. For the moment, the most important was the preservation of the structural proteins, associated to the mechanical properties.

About the migration of cells: we first tried to obtain a uniform distribution of cells through the scaffold, as assessed by different observers, for the same biopsies at different locations. For the endothelium, we looked for the diameters the seeded vessels, and the continuity of the cells layer. We didn't try for the moment to regenerate the media with smooth muscle cells, or with another type, like pericytes, which are going to be critical in vivo to limit the problem of edema formation. This is a big matter in vasculature of composite tissues, because they are less robust compared with the solid organs, with a very delicate and fragile vascular bed, and so very sensitive to an increase of perfusion pressure. We also observed an early edema associated to the adipose tissue, which retains a lot the fluid extravasation. It's more during a next step that we will try to repopulate the thickness of the vessels, and especially the artery, and try to see how we can regenerate a functional distal micro circulation.

Henri Bismuth (Villejuif, France):

First of all, I compliment you for this impressive presentation. I think that you are modest when you say that the face is not a vital organ. It is a vital organ and you and another group showed that the patients that are not well repaired, are socially excluded, have a poor professional life and sometimes in despair commit suicide. So, it is a vital organ and I think that you are right to work on the ways to increase the number of grafts. My question is about the other modality to obtain the scaffold. You spoke about human graft, using decellularization process in order to obtain scaffold. What about bioprinting, which is used as you know for plain organs such as the liver. I imagine there is special difficulty for the nerves, which are of course very important for the face, or for the vessels. So, I would like to know if there is some attempt of bioprinting of the face in order to have the scaffold which could be easier than human graft decellularized?

Response from Jerome Duisit (Brussels, Belgium):

Thank you for the question, Professor Bismuth. Actually, it's very interesting because our presentation's title was extended to ''the matrix of identity.'' We meant the restoration of a biological identity, while replacing donor cells by the recipient's ones. But for our disfigured patients, we will also have to restore their lost morphological identity and provide a customized reconstruction. It is going to be achieved through bioprinting for sure. Decellularization/recellularization technology is a step, and we will make it available for patients; but we have already to consider bioprinting. For the moment, this technology is available for simple and small tissues, due to a lack of vasculature and an access to it: thus, current scaffolds are limited to a few cm^3, because relying on a passive penetration of the nutrients. What we are trying to do for the moment is to extract the 3D structure information from the decellularized scaffolds we produce. We have to understand first the Nature's genius before trying to reproduce it. Indeed, the strategy in bioprinting is more about materials, but the fine structure of the tissues is missing. The efforts will also need the unify microscopic features with gross anatomy. It's a very emerging field we have here for the future,

but for the moment what does exist in bioprinting remains quite rudimentary. In the examples of the ear and nose, printed versions remain homogeneous, with only 1 tissue layer, and lacking a vascular tree: the face or even a subunit are by definition composite: the only nose contains multiple tissues like mucosa, skin, cartilage, vessels, nerves: this is impossible to reproduce by bioprinting for the moment. What we try to learn is how to deal with complex scaffolds, identify the patient's cells reservoir, and how to bring the cells into the matrix: from decellularized origin now, and later on from synthesis.

Inne Borel-Rinkes (Utrecht, The Netherlands):

Very nice paper. Compliments and congratulations. I would like to elaborate a little bit more on the different types of cells, you told us that you see cells as I understand correctly with different methods and techniques to get the cells where you want them to be. What is, for instance, your experience, what is the source of the cells? How do you isolate them, how do you keep them alive and particularly if you've seeded them? What's the longevity and what's the vitality of the cells? What percentage of cells actually do take and are capable of staying there?

Response from Jerome Duisit (Brussels, Belgium):

Thank you for the question. Well, actually we've been trying different types of cells, with different seeding routes, in a sterile perfusion-bioreactor. You can perform graft injections, seed through the vasculature, or topically for the skin. This is the difficulty with composite tissues: We have different compartments to treat at once, with different accesses. We have been working mainly with cell lines, because we targeted a critical number of cells, as well as mesenchymal stem cells. The main difficulty was to obtain an important density of cells within the scaffold: Even a human half-lip represents much more volume than a heart of a rat (in reference to the seminal tissue engineering work from Ott et al). In this study for example, we have been seeding up to 16 millions of cells, which finally was low. But, what we screened was more the quality of the spreading, than the density obtained. Badylak in his last Nature's editorial, supported the strategy of a combined approach: seeding in vitro the minimum cells and rely on the body to complete the scaffold regeneration, which is actually regenerative medicine. Bringing both together is very likely to be a long-term strategy, relying first on a vascular regeneration, with an access allowing replantation. In a previous paper in Annals of Surgery, our idea was to see if the stem cells were able to differentiate on the matrix, driven by the ECM: if you remember the cross-section of our lip scaffold, you could see that all tissue layers are joined together. With the vascular route, you can't select where in the different tissues the cells will be going. So, we will have to rely on the stem cells, like IPS, matrix-orientated differentiation. We know that's not particularly limiting for the moment, but will be needed to validate this aspect, critical for composite tissues. For the cells spreading, we are currently adapting seeding parameters: If the cells should remain inside the vascular tree, then you have to perform a slow perfusion, to stick to the vessels only. If you want the cells to spread into the tissue, seeding will be done with a lot of pressure, to force the cells to go in the extravascular space. Our main initial mistakes were a too weak or too strong perfusion. ''Fine tuning'' is a next step, but we have already all the elements to start new researches. We hope that other groups will join the effort in this new field: active collaborations will be critical in composite tissue engineering.