EPA Public Access

Author manuscript

Birth Defects Res. Author manuscript; available in PMC 2019 September 06.

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Published in final edited form as:

Birth Defects Res. 2018 October 16; 110(17): 1322–1334. doi:10.1002/bdr2.1394.

Development of an organotypic stem cell model for the study of human embryonic palatal fusion

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Abstract

Background.—Cleft palate (CP) is a common birth defect, occurring in an estimated 1 in 1000 births worldwide. The secondary palate is formed by paired palatal shelves, consisting of a mesenchymal core with an outer layer of epithelial cells, that grow toward each other, attach and fuse. One of the mechanisms that can cause CP is failure of fusion, *i.e.*, failure to remove the epithelial seam between the palatal shelves to allow mesenchyme confluence. Epidermal growth factor (EGF) plays an important role in palate growth and differentiation, while it may impede fusion.

Methods.—We developed a 3D organotypic model using human mesenchymal and epithelial stem cells to mimic human embryonic palatal shelves, and tested the effects of human EGF (hEGF) on proliferation and fusion. Spheroids were generated from human umbilical-derived mesenchymal stem cells (hMSCs) directed down an osteogenic lineage. Heterotypic spheroids, or organoids, were constructed by coating hMSC spheroids with extracellular matrix solution followed by a layer of human progenitor epithelial keratinocytes (hPEKs). Organoids were incubated in co-culture medium with or without hEGF and assessed for cell proliferation and time to fusion.

Results.—Osteogenic differentiation in hMSC spheroids was highest by day 13. hEGF delayed fusion of organoids after 12 and 18 hours of contact. hEGF increased proliferation in organoids at 4 ng/ml, and proliferation was detected in hPEKs alone.

Conclusion.—Our results show that this model of human palatal fusion appropriately mimics the morphology of the developing human palate and responds to hEGF as expected.

Keywords

Cleft palate; Organotypic models; Spheroid; Human stem cells; Fusion

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Conflict of Interest

The authors declare that they have no conflict of interest

1 Introduction

Cleft palate (CP) is a common congenital malformation. Incidence varies geographically but is high worldwide, reported at about 1.55 in 1000 births for orofacial clefting of any kind (Panamonta, Pradubwong, Panamonta, & Chowchuen, 2015) and as high as 1 in 2500 births for isolated CP (Burg, Chai, Yao, Magee, & Figueiredo, 2016). During embryological development, several fusion events occur in the body in which two apposing structures come together and fuse to form one unified structure, such as in the eye, heart, neural tube, and palate. In the case of the palate, the palatal shelves must grow toward each other, meet, and fuse to form a continuous palate. This is one of the later morphogenetic processes to be completed in the fetus. While CP can result from an aberration of any of the processes that form the palate, it can also result from failure of the fusion event alone. The etiology of this malformation involves multiple genetic and environmental factors (Abbott, 2010; Cobourne, 2004; Murray & Schutte, 2004) although all of the etiological pathways have not yet been elucidated. Study of CP has traditionally been performed with animal models in vivo or with non-human organ culture in vitro. However, these options are time consuming, expensive, and generally restrict the study of CP to species other than human. With recent advances in stem cell and 3D culture technology, CP could be studied in humans using human stem cells in an organotypic culture system that closely mimics the cell type, morphology, nutrient requirements and signaling milieu of human palatal shelves and incorporates a simulated fusion event.

Palatogenesis is a complex process involving cell-cell crosstalk and the morphogenesis events are sensitive to chemical or mechanical perturbations. The human palate formed during embryonic development consists of an anterior or primary palate which encompasses the upper lip and anterior portion of the palate, and the secondary palate which is the bony palate at the roof of the mouth. These portions are formed from five separate protuberances that initiate from tissue surrounding the oral cavity. The frontonasal prominence and the two maxillary prominences fuse to form the upper jaw, lip and anterior palate. The secondary palate is formed later by two outgrowths from the maxillary prominences which protrude on either side of the oral cavity to flank the sides of the tongue, elevate to position themselves horizontally over the tongue to form palatal shelves, grow toward each other, come into contact, adhere to each other, and fuse (Bush & Jiang, 2012; Ferguson, 1988; Ray & Niswander, 2012). Disruption of any of the developmental processes forming the secondary palate can lead to CP. The palatal shelves consist of a neural crest-derived mesenchyme core with an ectoderm-derived epithelial outer layer (Bush & Jiang, 2012; Lane & Kaartinen, 2014). The epithelial outer layer is separated from the mesenchyme by a basement membrane. The epithelial cells in the epithelial layer are rich in adhesion molecules and proteins derived from the extracellular matrix (ECM) including collagen, laminin, fibronectin, integrins and hyaluronic acid (d'Amato et al., 2012; Martinez-Sanz et al., 2008). During growth and reorientation, both cell types of the palatal shelves undergo proliferation. By the time the palatal shelves are about to fuse, the mesenchyme has differentiated down the osteogenic lineage and the cells become osteoblasts, resulting in a bony structure separating the roof of the mouth from the nasal cavity (Baek et al., 2011). The epithelial outer layer on the medial surfaces of the palatal shelves, referred to as the medial edge

epithelium (MEE), has thinned to one layer of basal cells (Brinkley, 1984; Farbman, 1968; Fitchett & Hay, 1989) after the outermost layer, or periderm, has sloughed off and proliferation in the MEE has ceased (Brinkley, 1984). The human palatal shelf, measured from the nasal to oral side at the medial edge, is about 150–200 microns thick immediately prior to fusion (Abbott, Diliberto, & Birnbaum, 1989; Taya, O’Kane, & Ferguson, 1999). As the two palatal shelves meet during fusion, the MEEs of each shelf come into contact and adhere to each other to form an epithelial seam, or the midline epithelial seam (MES). During the process of fusion, the MES is removed. Failure to remove the epithelial seam between the palatal shelves and allow the mesenchyme to merge and form a continuous palate results in clefting of the secondary palate, and is the focus of this study.

The process of palatal fusion, as well as all of palatogenesis, requires proper synchronization of many signaling events that are mediated by epithelial-mesenchymal interactions. These events include signaling by bone morphogenic proteins (BMPs), sonic hedgehog (Shh) and various growth factors including FGF and TGF families that result in growth, proliferation, migration, cell death, and differentiation of cells. While Shh and FGF10 are implicated in shelf outgrowth (Rice et al., 2004), FGF10 and its downstream Jag2 and transcription factor Irf6 are largely responsible for differentiation of the MEE in preparation of the fusion event, which includes loss of periderm epithelial cells and thinning of the epithelium to one layer (Lane & Kaartinen, 2014). Adhesion of the MEE of the two palatal shelves involve ephrins, cell surface ligands that bind their receptor on the opposing shelf. EphrinB2 is expressed at high levels in the MEE just prior to adhesion and fusion (Ray & Niswander, 2012). Subsequent to contact and adhesion of the palatal shelves, removal of the MES is accomplished by one or more of three proposed mechanisms: epithelial migration, epithelial to mesenchymal transition, and apoptosis (reviewed by Gritli-Linde, 2007). The definitive mechanism or combination of these mechanisms has yet to be elucidated. Nevertheless, it is agreed that the main driving force in fusion is TGF β signaling, predominantly expression of TGF β 3 (Ahmed, Liu, & Nawshad, 2007; Bush & Jiang, 2012; Lane & Kaartinen, 2014; Murray & Schutte, 2004).

The initiation of palatal fusion can be perturbed by an imbalance of regulatory factors in the signaling pathways. Epidermal growth factor (EGF) is known for its induction of proliferation in general, and regulates proliferation in the embryonic palatal shelves. EGF and the EGF receptor (EGFR) are expressed in the mesenchyme (Brunet, Sharpe, & Ferguson, 1993) and the MEE prior to contact of both rodent and human palatal shelves (Abbott & Pratt, 1988; Brunet et al., 1993; Citterio & Gaillard, 1994) and are responsible for proliferation and growth of both the epithelium and mesenchymal compartments, and ECM generation (Dixon & Ferguson, 1992). Immediately prior to fusion, EGF receptor levels decrease (Abbott, Adamson, & Pratt, 1988) and proliferation ceases (Brinkley, 1984; Hudson & Shapiro, 1973) in the MEE. Elevated levels of EGF have been shown to prevent or delay fusion. EGF added to palatal organ culture prevented the natural reduction in proliferation of the MEE, interfering with fusion of palatal shelves (Greene & Pratt, 1976; Tyler & Pratt, 1980). The success of palatal fusion may lie in a balance of the actions of the growth factors TGF β 3 and EGF. A recent study using in silico modelling of signaling pathways of the palate suggested that an increase in TGF β 3 levels relative to EGF levels

drives fusion, while an increase in EGF levels relative to TGF β 3 levels would delay fusion of the palatal shelves (Hutson, Leung, Baker, Spencer, & Knudsen, 2017).

Many studies on CP have been performed in animal models or organ culture models, mostly in the mouse. Although the mouse is generally considered to be an appropriate model for the human because palatogenesis between the two species is similar, differences in molecular signaling and other processes may be great enough to impact the regulation of palatal fusion or the response to external insult. CP is difficult to study in the human and relies mainly on epidemiological studies, but we can recapitulate the human palate by using human cells in a three dimensional organotypic culture model. We can use spheroids to study human palatal fusion, specifically the removal of the MES and confluence of the mesenchyme core, as cultured spheroids have been shown to fuse and form a continuous structure (Jakab, Damon, Neagu, Kachurin, & Forgacs, 2006; Perez-Pomares & Foty, 2006; Rago, Dean, & Morgan, 2009). Here we designed a novel 3D organotypic model to closely mimic the morphology and cellular components of human palatal shelves at the time of fusion. We created heterotypic spheroids, or organoids, consisting of an umbilical Wharton's Jelly-derived mesenchymal stem cells differentiated along the osteogenic lineage, covered with ECM and an outer layer of progenitor epithelial keratinocytes. We conducted experiments to characterize factors affecting spheroid and organoid size, growth, differentiation, and cellular survival and proliferation, and tested the effects of EGF on proliferation and fusion of the organoids.

2 Materials and methods

2.1 2D Cell culture

Cells were cultured and passaged according to manufacturer's guidelines. Human mesenchymal stem cells (hMSCs) derived from umbilical Wharton's Jelly (PCS-500-010; ATCC, Manassas, VA) were seeded into CellBIND® cell culture flasks (Corning) at 5000 cells/cm², cultured in mesenchymal growth medium (hMSC-G) consisting of basal medium (PCS-500-030, ATCC) with added growth supplements as instructed by the kit (#PCS-500-040, ATCC), and dissociated for passaging using trypsin (ATCC). Human progenitor epithelial keratinocytes (hPEKs) derived from pooled juvenile skin (HPEKp; CellnTec, Switzerland) were seeded into CellBIND® culture flasks at 4000 cells/cm², cultured in epithelial keratinocyte culture medium (hPEK-G) (CnT-PR, CellnTec) and dissociated during passaging using Accutase (CellnTec). All cells were passaged at 70 – 90% confluency and cultured at 37° C and 5% CO₂ in a humidified incubator.

2.2 Generation of Spheroids, Organoids, and hPEK-coated Microcarrier Beads

Definitions: 3D spherical cultures consisting of one cell type (hMSCs) or an unorganized mixture of two cell types (as discussed in the Supporting Information section) will be referred to as spheroids. 3D cultures consisting of multiple cell types (hMSC+hPEK) in an organized, layered architecture that depicts the architecture of the palate will be referred to as organoids.

2.2.1 Dish preparation.—Spheroids were generated in microwells of agarose dishes. Dishes were made by pouring molten 2% solution of UltraPure™ Agarose (Invitrogen, Carlsbad, CA) into a MicroTissues® 3D Petri Dish ® (Sigma-Aldrich, St. Louis, MO) containing either 5×7 (#Z764051, Sigma-Aldrich) or 16×16 (#Z764000) grid microwells and cooling for 15 min. Agarose dishes were transferred to a 24 or 12 well culture plate in 500 µl hMSC-G and equilibrated at 37° C and 5% CO₂ for 30 min.

2.2.2 Spheroid/Organoid generation and culture.—hMSCs at passage 3 – 10 were counted and either left unstained or stained with CellTracker™ (CT) orange CMTMR (Invitrogen) at 10 µM for 25 min, pelleted, rinsed in sterile Dulbecco's phosphate buffered saline (DPBS) and resuspended in hMSC-G with 0.2% Penicillin/Streptomycin/Amphotericin B (P/S/A; ATCC). Agarose dishes were drained of media and 75 µl hMSCs in hMSC-G were seeded into dishes at various cell densities. Cells were allowed to settle into microwells for ~15 min, medium was restored to each well to cover the agarose dish, and the plate was placed in incubator overnight. hMSC spheroids formed overnight and the next day, day 1 (d1), medium was changed to various ratios of O medium and keratinocyte differentiation (K) medium (CellnTec) for experimentation, or to StemPro® Osteogenic Differentiation (O) medium (Gibco, Gaithersburg, MD) with 0.5% P/S/A and 10µg/ml Kanamycin, and cultured up to 20 days. For the final spheroid model, hMSCs were formed in hMSC-G medium overnight, and cultured in O medium from d1-d13.

To generate heterotypic spheroids and organoids, hMSC spheroids were coated with 50% Maxgel™ (MG) extracellular matrix (Sigma-Aldrich) by immersing spheroids in cold 1:1 MG:hMSC-G medium mixture for 2 h, rinsing with DPBS, and transferring spheroids by pipette to a fresh agarose dish in hMSC-G medium. hPEKs were harvested from passage 3–10, suspended in either hPEK-G, K, or serum-free epithelial/stromal co-culture medium (CC; #CnT-PR-CC, CellnTec), and either left unstained or stained with CellTracker™ green CMFDA (Invitrogen). Dishes of hMSC spheroids were drained of media and 75µl of hPEK cell suspension at various densities was seeded onto hMSC spheroids in the agarose dish on d2, d8, or d13 of hMSC culture. After settling of hPEKs, appropriate media was restored to cover dishes and spheroids with media. Spheroids and cells were incubated at 37° C and 5% CO₂ in a humid environment on a rotator at a gentle rotation (~60 rpm) for 6 –24 h (d0). Heterotypic spheroids were completely formed as organoids, with hPEKs attached to the outer surface of the hMSC core, by the next day (d1). See Supporting Information for spheroids generated from co-suspension of hMSCs and hPEKs.

2.2.3 3D culture of hPEKs on microcarrier beads.—CT green-stained hPEKs suspended in hPEK-G were seeded onto Corning® enhanced-attachment microcarrier polystyrene beads (Corning #3779; Sigma-Aldrich) at 320,000 cells to 350 beads in a microcentrifuge tube, transferred to a well of a 96-well culture plate, rotated (swirled) at ~150 rpm for 4–5 h, and 100 µl hPEK-G was added to the well. hPEK-seeded beads were cultured in conditions described for spheroids to produce hPEK-coated microcarrier beads.

2.3 Assessment of Spheroid Diameter and Gross Epithelial Coverage

Spheroids and organoids were imaged in the dish at 4X magnification in bright field on an Olympus inverted microscope fitted with a RS Photometrics CoolSnap camera (Roper Scientific, Sarasota, FL) with the RS Imaging system (v 1.9.2, Roper Scientific) and diameter was measured using ImagePro Premier 9 image analysis program (Media Cybernetics, Rockville, MD). Spheroids were flushed out of the agarose dish at the end of culture and transferred to a well of a 96 well optical plate for fluorescence analysis. Epithelial coverage of organoids was determined by subjective visual assessment. Spheroids and organoids were visualized by fluorescence imaging on either a Leica DMIL inverted microscope (Leica Microsystems, Wetzlar, Germany) fitted with GFP (green; 492nm ex/ 517nm em) and Y3 (orange; 541nm ex/565nm em) fluorescence filter cubes, equipped with a camera (Leica DM7000-T) and images captured with Leica Application Suite X (LASX) program (Leica Microsystems), or on a Nikon A1 confocal laser scanning microscope (Nikon Instruments, Melville, NY) at green (495ex/519em) and orange (541ex/565em) wavelengths, and images analyzed with NIS Elements Advanced Research program, v4.51 (Nikon Instruments). Organoids that appeared to have roughly greater than 40% surface coverage with epithelial cells were used in the fusion experiments.

2.4 Alkaline Phosphatase Assay for assessment of Osteogenic Differentiation

Samples of mesenchymal spheroids ($n = 5-10$) were transferred to a well of a 96-well plate daily from d1 to d21. hMSCs spheroids on d1 were cultured in hMSC growth medium, and hMSC spheroids on d2 through d21 were cultured in osteogenesis differentiation medium. Alkaline phosphatase substrate solution was prepared daily immediately prior to exposing spheroids, following vendor's instruction for the Vector Red Alkaline Phosphatase substrate kit (Vector Labs, Burlingame, CA). Sets of spheroids at various days of culture in separate wells were exposed to the same solution each day. Spheroids were exposed to five drops of substrate and incubated at room temperature in the dark for 6 h and rinsed in DPBS. Fluorescence in the spheroid was visualized on a Leica imaging system with a Tx2 fluorescence filter cube (577nm ex/602nm em). Images were captured with LASX program and fluorescence intensity was measured with ImagePro Premier 9 program keeping exposure and RGB settings constant.

2.5 Live/Dead Assay

Mesenchymal spheroids of various diameters were tested for viability through day 26 using the Live/Dead® Viability/Cytotoxicity kit (Molecular Probes, Invitrogen) following vendor's instructions. Briefly, 1 μ l calcein AM was added to a solution of 4 μ l of Ethidium D-1 in 1 ml of DPBS, and the solution was kept away from light. Spheroids in a multiwall plate were rinsed in DPBS and immersed in the ethidium-calcein solution or the ethidium-calcein solution plus 50% ethanol as a positive control for dead cells, and incubated at room temperature for 45 minutes in dark conditions. Spheroids were rinsed in DPBS twice, and imaged with LASX or confocal fluorescence microscopy for fluorescence for green (492nm ex/517nm em) and red (577nm ex/602nm em).

2.6 Proliferation Assay in Organoids and hPEK-coated Microcarrier Beads

The proliferation assay was performed using the Click-iT™ EdU Alexa Fluor 647 imaging kit (Invitrogen) with an adaptation of the manufacturer's instructions to suit 3D culture. Briefly, organoids on d0 or hPEK-coated beads were cultured overnight in CC or K medium with 0, 2, or 4 ng/ml hEGF in separate wells per treatment group. The next day, d1, media was removed and fresh media with hEGF treatment and Click-iT™ EdU (5-ethynyl-2'-deoxyuridine) at 10 μ M was added to the organoids or hPEK coated beads. Organoids or hPEK-coated beads were cultured for 48 hours, rinsed in DPBS and fixed in cold 4% paraformaldehyde / 1% Triton X-100 (Sigma-Aldrich) solution for 3 h at 4° C, rinsed in DPBS and incubated in 3% BSA (Sigma-Aldrich) overnight in the dark at 4° C. Organoids or beads were rinsed in DPBS, transferred to respective treatment wells in a 96 well plate and incubated in Click-iT™ EdU reaction cocktail for 3 h at room temperature in the dark. Organoids or beads were rinsed and counterstained with Hoechst dye at 10 μ g/ml for 1 hour, rinsed in DPBS, and fluorescence was imaged on the confocal microscope (Nikon A1) at wavelengths for green (495ex/519em), orange (541ex/565em), blue (352ex/461em), and far red (577ex/602em). Cell counts of different cell types and of EdU-incorporating cells were analyzed using NIS Elements Advanced Research program and recorded in Microsoft Excel. The ratio of proliferating cells to all cells was measured and calculated. At least 5 separate experiments were performed from separate passages of cells with one dish of 35 organoids or microcarrier beads, in each treatment group and 5–12 organoids or hPEK-coated beads per image.

2.7 Fusion Experiments

Organoids of approximately 200 μ m were cultured in CC medium with 0, 4, or 8 ng/ml hEGF (BioLegend, San Diego, CA) beginning on day 0 (day of epithelial coating). The next day (16 hours later), two pairs of organoids from each treatment group were randomly selected every 6 h, removed from the original dish and transferred to trough recesses of a fusion dish, an agarose dish made from a MicroTissues® 3D petri dish® rod mold (#Z764078; Sigma-Aldrich) in respective hEGF treatment groups where they were placed in contact (Figure 1a), and cultured for up to 5 days. Each pair was monitored every 6 h and new pairs were introduced to a new trough in the same dish every 6 h (Figure 1b). This experimental design allowed for pre-contact hEGF exposure for various lengths of time in the original dish as well as a post-contact hEGF exposure period for various lengths of time up to 5 days in the fusion dish (data recorded up to 4 days; Figure 1c). Treatment media was refreshed daily in original dishes and in fusion dishes. Bright field images of fusing organoids were taken at these same 6-hour time points at 4X magnification on an Olympus microscope fitted with a Photometrics CoolSnap camera and imaged with the RS Imaging program. Images of each pair at each time point were scored independently by two scorers blind to time and treatment using a scale of 0 (not touching) to 5 (completely fused; oval or round shape) (Figure 1d).

2.8 Statistical Analysis

All experiments were performed with an n of 3 – 7 trials, with the exception of an n of 2 in the 200K cell group in the spheroid diameter experiment. All data were normalized to d1 or

to control groups where appropriate. Fluorescence intensity data for the alkaline phosphatase assay were analyzed in GraphPad Prism v.6 (San Diego, CA) with linear regression models and ANOVA. Proliferation data for two media types were analyzed in GraphPad Prism by two-way ANOVA. When an overall effect was found, differences between test groups and the control group were analyzed using Dunnett's multiple comparisons test. Fusion scoring data were analyzed with repeated measures analyses within mixed effects linear models using SAS (Cary, NC) PROC Mixed. The repeated measures structure of the data was modeled using an autoregressive covariance matrix, and each run of the experiment was included as a random effect. The fixed effects portion of the model began with the factors dose, pre-contact exposure time (hours) and post-contact hours with all 2-way and the 3-way interaction terms. Nonsignificant interaction terms were removed in a backwards stepwise fashion. The final statistical model included significant fixed effects for dose, pre-contact hours, post-contact hours and the interaction of pre- and post-contact hours. F-tests for the effect of pre-contact hours were calculated within the model at each post-contact time point. Since pre-contact exposure had limited effect, a similar model was run after removing the pre-contact hours (and its interaction terms) from the fixed effects to better visualize the effect of hEGF on fusion across time. The effect of dose was tested at each post-contact time point within this second model. Statistical significance was set at a P value less than 0.05 for all analyses.

3 Results

3.1 Spheroid and Organoid Diameter, Growth, and Viability

One of the goals of this study was to define conditions that would generate spheroids of a size comparable to that of the human embryonic palate, approximately 200 μm , and of a size that would be optimal for confocal imaging, approximately 100 μm . This was accomplished by varying cell seeding number and microwell number of the dish. Spheroid diameter was a function of the number of hMSCs seeded into a dish and distributed to a given number of microwells, or a function of the number of cells per microwell. In general, higher numbers of hMSCs seeded into a dish produced larger spheroids ($p < 0.01$ by linear regression; Figure 2a). A seeding number of 40,000 hMSCs in the 5 $\times$ 7 grid agarose dish satisfied our goal of producing spheroids of approximately 200 μm in diameter on d1. These spheroids were significantly smaller in diameter compared to spheroids produced from seeding the highest seeding number used in the same 5 $\times$ 7 grid dish; 280,000 hMSCs ($p < 0.05$). In the 16 $\times$ 16 grid agarose dish, a seeding number of 80,000 and 60,000 hMSCs produced spheroids of approximately 137 μm and 133 μm in diameter, respectively, on d1, considered close to optimal for confocal assessment (Figure 2a). Growth of spheroids was positive under most conditions and for those groups with positive growth, spheroids grew in diameter roughly 16–20% throughout culture from d1 to d20 (Figure 2b). Spheroids generated from any seeding density in the 5 $\times$ 7 grid dish grew to the same extent (slopes not significantly different; Figure 2b). The spheroids generated from 40,000 hMSCs had significant growth across time ($p < 0.01$). Spheroids generated in the 16 $\times$ 16 grid dish from 80,000 hMSCs did not grow, while those from seeding 60,000 hMSCs had significant growth ($p < 0.001$; Figure 2c).

Diameter and growth of heterotypic (hMSC+hPEK) spheroids, or organoids (see Definitions in section 2.2), was minimally influenced by media type (Figure 3). Large (~200 μm) hMSC spheroids were coated with hPEKs on d1 or d2 (Figure 3a), and small (~100 μm) spheroids were coated with hPEKs on d13 (Figure 3b) to produce organoids. In an effort to formulate a suitable co-culture media to support the growth of both cell types, we combined O medium and K medium in different ratios (see Supporting Information) and measured diameter and growth of organoids. Growth increased in large organoids in medium formulated with O:K at 1:3 (slope increase $p < 0.05$; Figure 3a). We then compared organoid growth in K medium to growth in CC medium. Organoids that were coated with hPEK on d13 had significant growth over time in either K medium alone or CC medium (slope increase $p < 0.0001$ for both media types; Figure 3b), and the rate of growth of spheroids cultured in K medium alone or CC medium did not differ between media type (slopes not different $p = 0.156$). CC medium was used for culture of the final organoid model (see section 3.3) and for the fusion assay, to ensure support of both cell types. More of this work on media formulations was performed on heterotypic spheroids generated from co-suspensions of hMSCs and hPEKs (See Supporting Information).

Viability of hMSC spheroids of 200 – 400 μm remained high up to 26 days. This is evidenced by green fluorescence in the spheroids with the Live/Dead assay (Figure 4). Viable cells are detected by a green fluorescence of calcein-AM esterase activity of living cells, as seen in the control spheroids on d13 and d26 (Figure 4b and e, respectively). Dead cells are detected by red fluorescence of ethidium homodimer penetrating a disrupted plasma membrane, as seen in the ethanol treated spheroids on d13 (Figure 4i), but not seen in any of the controls. Further evidence of viability of the spheroids is supported by positive growth through time (Figure 2) and by proliferation of both cell types in the spheroids (see Proliferation in Results section).

3.2 Osteogenic Differentiation

Fluorescence imaging of the alkaline phosphatase (AP) reaction revealed that hMSC spheroids cultured in O medium differentiated along the osteogenic pathway/lineage (Figure 5). AP fluorescence intensity increased gradually from d0 to d13 and was significant relative to controls on d6, then reached a plateau where it was significant on d13, d15 and d20 (Figure 5b). Days 13 and 15 of hMSC spheroid culture were the highest points of the curve of alkaline phosphatase activity, indicating maximum osteogenic differentiation. Based on the data we obtained for osteogenic differentiation and diameter, day 13 of hMSC spheroid culture was selected for the day of addition of hPEK cells to the hMSC spheroid to generate a model that includes mesenchymal cells exhibiting evidence of osteogenic differentiation.

3.3 Final Organotypic Model

The final 3D organotypic model of human embryonic palatal fusion used for fusion experiments in this study is a 2-cell type heterotypic spheroid composed of an osteogenically differentiated mesenchymal core coated with ECM and an outer layer of epithelial cells. This model was derived from the aforementioned findings, namely, mesenchymal spheroid size of 100 – 200 μm , good viability of hMSC spheroids, osteogenic differentiation, and greater than 40% coverage with seeding of hPEKs onto a MG ECM-coated d13 spheroid,

and continued growth and viability. Representative samples of this model can be seen in Figure 6 in confocal maximum intensity projection (MaxIP) images of a whole spheroid (Figure 6a) and a Z plane section (Figure 6b). Our heterotypic spheroids meet the minimum criteria for being referred to as organoids, or a collection of stem or progenitor cells that have differentiated and sorted into a morphology and cell type similar to the tissue it is modelling (Lancaster & Knoblich, 2014).

3.4 Proliferation in Organoids and hPEKs on Microcarrier Beads

Organoids were tested for proliferation with and without hEGF in order to determine whether hEGF could increase the number of hPEKs covering the hMSC core of the organoid. Proliferating cells were detected in organoids cultured for two days in either CC or K medium, as evidenced by fluorescence of Alexa Fluor-tagged EdU (Figure 7a). Treatment with hEGF at 4 ng/ml significantly increased proliferation in the spheroids in K medium (Figure 7b). Although treatment of spheroids with hEGF at 2 or 4 ng/ml in CC medium resulted in a slight increase in proliferation, this increase was not statistically significant (Figure 7b). Since the quantification of proliferating cells in the heterotypic spheroids did not differentiate between hMSCs and hPEKs, hPEKs were isolated for assessment. We first attempted to generate hPEK spheroids using the agarose dishes, but these did not produce discrete spheroids after 24 h or 48 h in hPEK growth medium, or those that did form a spheroid disassociated after a day (observation). We then seeded hPEKs onto microcarrier beads to isolate hPEKs in a context similar to hPEKs surrounding the surface of hMSC spheroids in 3D culture. hPEKs on microcarrier beads were cultured in CC medium to be consistent with the medium chosen for fusion experiments, to better compare with fusion results. Proliferation was detected in hPEKs alone after 2-day culture (Figure 7c). Although the number of proliferating cells increased with hEGF treatment at 4 and 8 ng/ml, the increase was not significant (Figure 7d). Proliferation in hPEKs attached to microcarrier beads is an approximation of proliferation in organoids due to differences in cell to cell interaction.

3.5 Fusion

Organoids in non-adherent culture in CC medium fused over the course of four days as quantified by microscopy and scoring at each time point, and fusion was delayed by hEGF. The experimental design for the study of fusion allowed for pre-contact culture time of individual organoids in hEGF-treated CC media in the original agarose dish as well as a post-contact culture time for organoid pairs in hEGF-treated CC media in the trough dish (Figure 1c). Pre-contact exposure of organoids to hEGF addressed the concern that pre-exposure of the embryonic palatal shelves to chemicals *in vivo* may disrupt fusion occurring later. For example of the study design, organoids incubating in a holding dish in hEGF-treated medium for 16 h (pre-contact time) may be transferred as a pair to the fusion dish with the same concentration of hEGF and placed in contact. This organoid pair would then be monitored 6 h later (post-contact time) and each successive 6 h and an image captured, at which time a new organoid pair would be added to the fusion dish and an image captured, so that the image captures the fusion status of the current pair and each previous pair (Figure 1b, c). In this way, each organoid pair has a pre-contact and a post-contact exposure time and

is monitored every 6 h post-contact for the progression of fusion. These images were then scored blind to treatment or time point to generate data to assess the progression of fusion.

Dose, pre-contact exposure time, post-contact exposure time, and the interaction between pre- and post-contact exposure all significantly affected fusion at some time points in the final mixed effects model ($p < 0.05$). Within that model, pre-contact exposure time had an effect on post-contact fusion at random time points, 12 and 18 hours ($p < 0.001$, $p = 0.005$, respectively), where longer pre-contact exposure of organoids to hEGF advanced fusion. As this was not generalized effect across all fusion time, pre-contact exposure time was disregarded. In general, fusion of organoid pairs progressed at a steady rate for 36 h and then slowed. Exposure to 4 or 8 ng/ml hEGF significantly delayed the rate of fusion in the first 24 h, and fusion progressed to nearly the stage of the control group after that time (Figure 8). Fusion was delayed at 12 and 18 h by 4 ng/ml hEGF and at 12, 18, and 24 h by 8 ng/ml hEGF. After 48 h, fusion in the 8 ng/ml hEGF group appeared not as progressed as that in the control stage. Complete fusion to a spherical shape (stage 5) was reached in all groups by day 4 or 5 (not shown in figure).

4 Discussion

The ability to study cleft palate in humans has been limited to epidemiological studies, culture of human palatal cells, and human palatal organ culture. Studies are often performed using non-human species and include in vivo studies, culture of palatal cells, and palatal organ cultures. With the advances of 3D organotypic culture, we can address this topic using human cells in a microtissue model that represents the morphology and cellular components of the human palate. Described herein is a 3D organotypic model of human palatal shelves for studying the fusion process in palatal formation, response to external growth factor exposure, and aberrations that may lead to cleft palate. This study outlines the processes that were employed to develop the model which was subsequently modified and described in (Belair et al., 2017). Here, we created heterotypic organoids composed of human stem cells to model the human palatal shelves and evaluated the effects of human epidermal growth factor (hEGF) on cell function and organoid fusion. This organotypic model is composed of osteogenically-differentiated human mesenchymal stem cells (hMSCs) derived from umbilical Wharton's Jelly aggregated into a spheroid, covered by an extracellular matrix (MaxGel™), and further covered with human progenitor epidermal keratinocytes (hPEKs). Developing the model involved determining the number of hMSC cells seeded in an agarose spheroid dish that could generate spheroids of optimal diameter for studying the spheroid characteristics, defining a method that would result in appropriate hPEK coverage of the hMSC spheroid, and identifying the culture media formulations that best support the health of both cell types including growth and fusion and responsiveness to fusion challenges. After successfully establishing the physical characteristics of the organoid model, we found that the model also simulates the functional characteristics of human palatal shelves in that they respond to hEGF with an increase in proliferation and a delay in fusion between spheroid pairs.

Our work confirms the work of others (Napolitano et al., 2007; Rago et al., 2009) showing that viable spheroids can be generated by seeding adherent cells into microwells of agarose

dishes (MicroTissues® 3D Petri Dish) formed in a polydimethylsiloxane (PDMS) mold. This spheroid-generating culture system is a fast and reliable method for the production of spheroids that are easily removed from the non-adherent agarose dish for processing. In our study, human mesenchymal stem cells aggregated into uniform, spherical microtissues. Fibroblast cells work well in this system to form spheroids, as opposed to other cells, such as epithelial cells (Yen, Chan, & Lin, 2010). Our attempt to generate spheroids with hPEKs in this agarose dish system was unsuccessful. hMSC spheroids produced by this method were uniform in size and shape due to the uniformity of the microwells of the agarose dish, unlike spheroids or microtissues produced by other methods (Huang et al., 2015; Yen et al., 2010). The size of the spheroid generated by this system is dependent on the number of cells seeded into the agarose dishes, and we identified the cell seeding density required to generate spheroids of the sizes desired for our study, 100 μm and 200 μm . In our system, the smaller size allows penetration of laser light and luminescent or fluorescent emissions from within the sample so that organoids can be studied with confocal and fluorescence microscopy while maintaining viability.

The culture environment influenced spheroid health, growth, epithelial cell attachment, differentiation and function. Generally, in all media tested, spheroids in our study had positive growth trajectories through time for up to three weeks regardless of starting diameter, and in all media tested, except for larger spheroids formed using 80,000 cells/sphere. The microwells of the dishes are narrow and deep, and it is possible that 80,000 cells seeded into 256 microwells in the 16 $\times$ 16 grid dish filled the spaces of the microwells, and the volume and dimension restrictions of the wells did not allow enough nutrient exchange for the spheroids to grow, a theory postulated previously by Napolitano et al. (2007). Mesenchymal spheroids and organoids consisting of mesenchymal and progenitor epithelial cells grew and were viable for weeks in culture. The media types tested had little influence on growth (i.e. increased diameter). Organoids were expected to grow as long as components to support both mesenchymal and epithelial cells were present, however, organoids grew well even in keratinocyte growth medium alone.

In addition to supporting growth, culture medium was used to manipulate the differentiation status of the cells to reflect the embryonic palate at the time of fusion. In the embryonic palate, the mesenchyme undergoes intramembranous ossification to form the hard, secondary palate. Early stages of this differentiation occur at the time the palatal shelves are ready to fuse (Baek et al., 2011; Bush & Jiang, 2012; Levi et al., 2011), although ossification is not completed until after the shelves are fused. In murine palatal shelf cultures, Runx2 gene, an initiator of osteogenesis, was expressed as early as embryonic day (E)11 and alkaline phosphatase (AP) expression, an indicator of osteogenesis, was significantly increased by E13 (Levi et al., 2011). In our study, we successfully differentiated the hMSC spheroids along the osteogenic lineage within 6 days, and more robustly by d13 and d15, using osteogenic differentiation medium. This stage of osteogenesis corresponds to the increase in AP expression in murine explants in the study by Levi et al. (2011) and occurs at gestational week 9 in the human palate (Sadler et al., 2000). The progenitor epidermal keratinocytes we used for the outer epithelial layer of our model were not stimulated to differentiate further, i.e. toward a columnar oral mucosal phenotype, as they adequately resemble the differentiation state of the epithelial cells of the palatal shelf at the time of

fusion, a single layer of relatively simple epithelium (Bush & Jiang, 2012; Tyler & Pratt, 1980).

In an effort to reconstruct the layered cellular architecture of the embryonic palate, we experimented with two methods for generating organoids. In the first attempt, we relied on the phenomenon of autonomous cell sorting reported in various studies previously (Kelm & Fussenegger, 2004; Lin, Ki, & Shih, 2015; Napolitano et al., 2007; Rago et al., 2009). Cell sorting is based on the differential adhesion hypothesis, that presumes that cells rearrange in three-dimensional cultures based on their different cell-cell adhesion properties, and is based on tenets that they are mobile, discrete units, and have different adhesion and cohesion properties (Foty & Steinberg, 2005). This cell sorting or co-suspension method in our laboratory did not produce organoids that recapitulate the architecture of the palate, with epithelial cells attached to the outer surface only and not within the mesenchymal center. Many epithelial cells were observed inside the spheroid 24 hours later and remained there when viewed several days later. This failure to self-sort may be influenced by the media, or the different adhesion properties of the cells. It was shown by Yen et al. (2010) that two cell types, mesenchyme-derived dermal papilla cells and keratinocytes, would not self-sort when co-seeded into hanging drops, but that they did sort into a mesenchymal fibroblast core with a keratinocyte outer layer when mesenchyme cells were preferentially adhered to a specially treated surface first such that the keratinocytes adhered later to the aggregating mesenchyme cells. It is possible that the cell types we used in the current study, progenitor epidermal keratinocytes and Wharton's jelly mesenchymal cells, were not optimal in conjunction for cell sorting activity. Although it is also possible that the proportions of epithelial cells in relation to hMSCs may have been too low to show robustly populated localization of hPEKs on the outer surface, epithelial cells were retained in the center of the spheroids with the co-suspension method. For the purposes of analyzing fusion, wherein the epithelial cells on the surface are removed at contact zones between adherent organoids, having epithelial cells co-localized with the mesenchyme cells in the core of the spheroid was not desirable. For this reason, we abandoned the co-suspension method. In the other method, mesenchyme cells were formed into a spheroid core, and then coated with ECM material to enhance attachment of hPEK cells, which were seeded over the hMSC spheroids in the dish. This method produced heterotypic organoids that ensured that the epithelial cells would be located on the outer surface of the spheroid and not in the interior, important for modelling the *in vivo* palatal morphology.

Our final model included a layer of extracellular matrix (ECM) between the hMSC and hPEK layers to mimic the basement membrane (BM) separating the layers of mesenchyme and epithelial cells in the embryonic palate. We added the commercially available MaxGel™, a human ECM formulation, to the surface of the spheroids before adding the hPEKs. The ECM is found surrounding mesenchyme cells within the palate and plays a role in cell-to-cell communication between mesenchyme cells and between mesenchyme and epithelial cells. Removal of the BM is an important process during palatal fusion. The BM and ECM contain proteins and enzymes that may be important factors in palatal fusion as well as palatal shelf elevation (d'Amaro et al., 2012; Kerrigan, Mansell, Sengupta, Brown, & Sandy, 2000; Martinez-Sanz et al., 2008).

Our ultimate goal in this study was to model fusion of human embryonic palatal shelves using human cells in an organoid model. Spheroids of a single cell type or multiple cell types will fuse in culture (Napolitano et al., 2007; Perez-Pomares & Foty, 2006; Rago et al., 2009) as our multicellular organoids fused in culture. Our method of scoring bright field images of stages of fusion was very effective in rating the progression of fusion between organoid pairs. The scoring method that we applied to the images, rating the effects based on a defined progression, is one that is commonly used in developmental studies (Abbott, Buckalew, & Leffler, 2005; Hotchkiss, Ostby, Vandenberg, & Gray, 2003; Tucker et al., 2015). The use of agarose trough dishes allowed fusion to proceed as unimpeded and naturally as possible. In the troughs, the organoids are free to move within the fluid environment and are placed but not pushed into contact. The organoids are not subjected to forces such as gravitational force pushing them into contact as would be the case were organoids placed in a round-bottomed well.

Our model also responded to the growth factor EGF as would the fusing palate *in vivo*. EGF is well known for its ability to induce proliferation and for promoting growth of the palatal shelves, and exposure to excess or exogenous EGF can alter the morphology and persistence of the epithelial layer in explants in palatal explants *in vitro*. The peridermal epithelial cells at the medial edges of the palatal shelves are desquamified and reduced to a one or two cell basal layer prior to contact and fusion of the opposing shelves. At this initial stage, the EGF receptor is down-regulated in the remaining basal MEE cells (Abbott et al., 1988). Exogenous EGF, or treatment with agents that interfere with down regulation of the EGF receptor, result in persistence of EGF pathway signaling, resulting in excess proliferation of the MEE cells, preventing fusion (Abbott & Birnbaum, 1990; Abbott, Probst, Perdew, & Buckalew, 1998). In our model, hEGF induced an increase in proliferation of both mesenchymal and epithelial cells, determined by EdU incorporation in organoids and in hPEKs attached to microcarrier beads. Treatment of fusing organoids with hEGF elicited a delay in fusion which may be related to the increase in proliferation of hPEKs stimulated by hEGF. Organoids were also pretreated for various times (up to 48 h) with hEGF before placing into contact to address a concern that exposure of embryonic palates to teratogens prior to the time of fusion may alter regulation of fusion. Pre-contact exposure time in hEGF had a sporadic effect on fusion at some time points, so the general contribution of pre-contact exposure on the regulation of fusion could not be determined. These effects of hEGF in our organoid model, stimulating epithelial proliferation and delaying fusion, are similar to the functional response of the MEE in palatal shelf organ culture after exposure to EGF.

In this study we successfully developed a 3D organotypic model that mimics human embryonic palatal shelves in morphology and function. We developed a method to create a complex heterotypic spheroid, or organoid, composed of two human cell types with appropriate differentiation and morphology to depict the architecture of the human embryonic palatal shelves. We determined the culture conditions for optimal size, growth and performance and devised methods to analyze proliferation and fusion. We determined that fusion of these organoids is responsive to EGF in ways predicted by *in vivo* studies. We can investigate this model in future studies to further characterize the cellular components of the model, and to identify key morphological and signaling events that are critical to epithelial seam disappearance. This foundational study has supported additional work in our

lab refining the model and testing the effects of potential palate teratogens on fusion using this human palate model. This model provides a basic approach which could be modified for studying other embryonic tissue fusion events. With modifications of parameters such as cell types, differentiation status, culture platform, and media, the model has the potential to be applied to studies of embryonic fusion events such as those forming the eye, heart, spinal cord, and genitalia.

Supporting Information

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Description of Fusion study. Samples of organoids were cultured in original dish of treated CC medium (0, 4, or 8 ng/ml hEGF), removed every 6 h and placed in pairs into a trough dish of matching treated medium, and imaged at 0 h and every 6 h thereafter. A) organoid pair placed in contact in trough, in BF. B) two control organoid pairs in trough dish at 0 h and 6 h, BF. C) Design of study showing pre- and post-contact exposure time. Column on left (Pre-contact exposure time in original dish): Hours samples of organoids were in original medium with 0, 4, or 8 ng.ml hEGF before removing and placing in contact in fusion dish of corresponding hEGF concentration. Each row represents each new set of organoid pairs. Columns to the right (Post-contact exposure time in fusion dish): For each row, length of time the organoids were in contact in hEGF-treated medium (increasing numbers along the row) and imaged. D) Extent of fusion of each pair was scored using the illustrated scale of 0 – 5. BF, bright field. CC, co-culture medium. hEGF, human recombinant epidermal growth factor.

A

	5x7 dish				16x16 dish	
Seeding #	280,000	200,000	100,000	40,000	80,000	60,000
Mean Diameter (μm)	341	304	273	206	138	133
SEM	27	49	13	11	2.4	1.8
n	3	2	3	3	5	3

Figure 2.

Diameter of hMSC spheroids is correlated with seeding density. A) Diameter of spheroids on day 1 or 2 after seeding into dish. Lower seeding density is correlated with smaller spheroids in the 5×7 grid agarose dish by linear regression ($p < 0.01$), and diameter of spheroids seeded at 40K cells is significantly lower than those seeded at 280K ($p < 0.05$). Diameter of spheroids generated in the 16×16 grid dish were not different for 60K and 80K seeded dishes. Spheroids grown in hMSC-G medium. B, C) Growth of hMSC spheroids cultured in a 5×7 grid agarose dish (B), or 16×16 grid agarose dish (C). Spheroids grew at approximately the same rate, except for those seeded at 80,000 cells. Spheroids made from 40,000 cells show a significant growth trend across the culture period ($p < 0.01$). All sets of spheroids were cultured in CC medium except for two sets of spheroids seeded at 200K that were cultured in 3:1 K:O medium. hMSC, human mesenchymal stem cells; hMSC-G, hMSC growth medium; CC, co-culture medium. * $p < 0.05$.

Figure 3.

Diameter and growth of organoids is minimally affected by media type. A) Growth of organoids generated from 200,000 hMSCs and seeded with hPEKs on d1 in a mixture of O and K media showed a significant increase in size across time ($p < 0.05$). B) Growth of organoids generated from 40,000 hMSCs and seeded with hPEKs on d13 cultured in K medium versus CC medium in the 5×7 grid agarose dishes increased significantly in both media from d1 to d21 ($p < 0.001$). Growth trajectories of these organoids are not significantly different. hPEK, human progenitor epithelial keratinocytes; O, osteogenic differentiation medium; K, keratinocyte differentiation medium; CC, co-culture medium.

Figure 4.

hMSC spheroids cultured in hMSC-G are viable to 26 days. Spheroids were untreated or treated with 20% ethanol for 45 minutes and tested with Live/Dead™ viability assay. A) untreated hMSC spheroid at day 13, BF; B) untreated hMSC spheroid at day 13, live test; C) untreated hMSC spheroid at day 13, dead test; D) untreated hMSC spheroid at day 26, BF; E) untreated hMSC spheroid at day 26, live test; F) untreated hMSC spheroid at day 26, dead test; G) ethanol-treated spheroid at day 13, BF; H) ethanol-treated spheroid at day 13, live test; I) ethanol-treated hMSC spheroid at day 13, dead test. All images at same exposure. Green, live by calcein-AM; Red, dead by ethidium homodimer-1. hMSC, human mesenchymal stem cells; BF, bright field; hMSC-G, hMSC growth medium.

Figure 5.

Alkaline Phosphatase (AP) activity indicative of osteogenic differentiation in hMSC spheroids cultured in O medium starting on day 1 of culture. A) fluorescence in hMSC spheroids on consecutive days of culture. B) AP intensity relative to intensity on day 1. The curve shows an increase through d7 and a plateau at later days. AP intensity is significantly higher on d6, d13, d15 and d20. $n = 6$ independent experiments. * $p < 0.05$. hMSC, human mesenchymal stem cells; O, osteocyte differentiation medium.

Figure 6.

Final heterotypic stem cell spheroid, or organoid, model of human embryonic palate for fusion study. Human mesenchymal stem cell (hMSC) spheroids cultured 13 days in osteogenic differentiation medium, covered with MaxGel extracellular matrix in hMSC growth medium, seeded with human progenitor epithelial keratinocytes (hPEKs), and imaged the next day (hMSC d14, hPEK d1) on a confocal microscope. (A) Maximum Projection Image showing epithelial coverage on the surface. (B) Optical Z section through center of spheroid showing hPEKs on the outside and hMSCs throughout the center. Green, hPEK cells. Red, hMSC cells. hMSC, human mesenchymal stem cells; hPEK, human progenitor epidermal keratinocytes.

Figure 7. Proliferation in organoids or hPEK cells on microcarrier beads treated with hEGF. Confocal image of (A) hMSC+hPEK organoids, control group, and (C) hPEK-seeded microcarrier beads, control group, expressing fluorescence-tagged EdU incorporation (pink), denoting proliferating cells. Proliferation in organoids (A) relative to control is visible in both cell types, and was increased in the organoid as a whole by hEGF when cultured in K medium, but not in CC medium (B). Proliferation in hPEKs alone (C), was increased by hEGF in CC medium, although the increase was not significant (D). Pink, EdU-incorporating cells; Blue, Hoechst DNA staining; Red, hMSCs; Green, hPEKs. hMSC, human mesenchymal stem cells; hPEK, human progenitor epidermal keratinocytes; K, keratinocyte differentiation medium; CC, co-culture medium. * $p < 0.05$.

Figure 8.

Progression of organoid fusion through time with or without hEGF treatment. Organoids were cultured in CC medium alone or with 4 or 8 ng/ml hEGF for up to 2 days and imaged every 6 hrs, generally. Images of fusing organoids were given a score on a scale of 0 (not touching) to 5 (completely fused). Treated groups were compared to control group at same time point. * $p < 0.05$ ** 0.01 ***0.0001. CC, co-culture medium. hEGF, human epidermal growth factor.