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Towards Microsurgical Correction of Cleft Lip *Ex Utero* via Restoration of Craniofacial Developmental Programs

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Abstract

Background—Cleft Lip with or without Palate (CL/P) is present in approximately 1 in 500–700 live births, representing the most common congenital craniofacial anomaly. Previously, we developed a unique murine model with compound Pbx deficiency that exhibits fully penetrant CL/P. To investigate the possibility of tissue repair at an early gestational stage, we designed a minimally invasive surgical approach suitable for intrauterine repair using Wnt9b-soaked collagen microspheres to restore craniofacial developmental programs for cleft correction.

Methods—Collagen microspheres with diameters ranging from 20–50 microns were fabricated to serve as a delivery vehicle for Wnt9b. At gestational day 11.5, wild type and Pbx-deficient murine embryos were isolated. Microspheres soaked in murine purified Wnt9b protein were microsurgically implanted at the midface λ junction. Embryos were cultured in a 37°C modified Whole Embryo Culture (WEC) system.

Results—Targeted release of Wnt9b resulted in augmented Wnt expression at the λ junction. Microsurgical implantation of Wnt9b-soaked microspheres resulted in cleft correction in 27.1% of

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the Pbx-deficient embryos. The difference in the ratio of the areas of clefting between implanted and non-implanted embryos was significant ($p<0.05$).

Conclusion—Ex utero correction of CL/P in our murine model via microsurgical intervention and targeted delivery of Wnt proteins is an innovative and promising strategy. Although further refinement and optimization of this technique will be required to improve efficacy, we believe that this approach will open new avenues towards unconventional prenatal interventions for patients with CL/P, as well as provide future approaches for prenatal repair of other congenital head and neck disorders.

Introduction

Cleft Lip with or without Palate (CL/P) occurs in approximately 1 in 500–700 live births worldwide, representing the most common congenital craniofacial anomaly in humans [1]. These malformations result in facial disfigurement, impaired feeding and defective speech and create psychological, emotional and economic hardships for patients and their families [2]. Proper treatment of this deformity requires a multidisciplinary team consisting of pediatricians, plastic surgeons, geneticists, neurosurgeons, otolaryngologists, dentists, audiologists, speech-language pathologists, and psychologists [1,2].

Mice share similar craniofacial morphological development with humans and thus are commonly used to study embryonic processes [3,4]. At gestational day 10.5 (E10.5) in mice (equivalent to late 4th week in humans), the morphology of midface, including the upper lip and primary palate, is gradually formed by fusion of the maxillary processes (mxp), medial nasal process (mnp), and lateral nasal process (lnp) at the lambdoidal junction (λ) [3,4,5] (Figure 1). CL/P most often occurs without an identifiable cause (70%) and less frequently presents in association with other defects in syndromic forms caused by teratogenic exposure, chromosomal abnormalities, or single gene mutations [6,7].

Earlier studies identified a complex, genetically controlled, and tightly regulated molecular network that mediates craniofacial morphogenesis [4]. Previous reports show that the fusion at the midface λ junction is characterized by high expression of *Wnt* and *Bmp* genes, as well as *Irf6* and *p63* in both mice and humans, aberrations of which can lead to CL/P [4,8,9]. Furthermore, among the genetic mechanisms that come into play, it has been demonstrated that the canonical Wnt signaling pathway controls a cascade of morphogenetic events [9,10,11] (Figure 1)(See Supplemental Digital Content 1, Figure shows Wnt/β-catenin signaling pathway. In the absence of Wnt, cytoplasmic β-catenin is constantly degraded by the Axin complex, which is composed of the scaffolding protein Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). CK1 and GSK3 sequentially phosphorylate the amino terminal region of β-catenin, resulting in β-catenin ubiquitination and degradation. By preventing β-catenin from reaching the nucleus, the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) protein family represses Wnt target genes. However, the binding of WNT ligand to the Frizzled (FZD) receptor and the low-density lipoprotein-related receptor protein (LRP) 5 or 6 co-receptor, and interaction of FZD with the cytoplasmic protein Disheveled (DSH) lead to inactivation of the Axin/APC/GSK3β/CKI complex and stabilization of cytoplasmic β-catenin. β-catenin then

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accumulates and travels to the nucleus to form complexes with TCF/LEF and activates Wnt target gene expression, INSERT LINK). Notably, we previously reported craniofacial defects in embryos with loss of the Pbx1 homeodomain protein [12]. We also established that genes involved in midfacial development, *Pbx1*, *Pbx2* and *Pbx3,* co-localize in the developing frontonasal processes, maxillary process, and olfactory placode, wherein they are expressed at high levels in both in the ectoderm and mesenchyme resulting in apoptosis of the epithelial lining at λ and subsequent fusion of the underlying mesodermal tissue. Compound loss of function of Pbx genes results in unilateral or bilateral CL/P [4] (Figure 2).

Disruption of the Pbx-directed *Wnt9b/Wnt3a* signaling pathway in the midfacial epithelium results in suppression of apoptosis at the midface λ junction, which in turn causes epithelial persistence and abnormal lip and palate fusion [4]. We developed a unique murine model with compound Pbx deficiency that exhibits fully penetrant CL/P. Furthermore, we demonstrated partial rescue of clefting using genetic strategies to reconstitute Wnt canonical signaling in the cephalic ectoderm [4].

To this date, no effective tissue repair of clefting has been conducted during fetal development in engineered animal models of CL/P. Alternative options to correct clefting such as retroviral gene therapy to alter Wnt signaling are potentially dangerous, given the possibility of retroviral induction of oncogene activation [13]. In order to investigate the possibility of obtaining tissue repair of orofacial clefting at an early gestational age, we utilized our Pbx-deficient embryonic murine model and sought to develop a minimally invasive correction method via microsurgical implantation of Wnt9b-soaked collagen microspheres suitable for intrauterine repair.

Methods

Animals

Pbx1$^{-/-}$, *Pbx2*$^{+/-}$ mutant mice on a BALB/c genetic background (Department of Cell and Developmental Biology, Weill Cornell Medical College, NY), *BAT-gal* mice and wildtype mice of C57BL/6J and CD-1 (Charles River Laboratories, Wilmington, MA) genetic background strains were used in these experiments. Transgenic BAT-gal mouse lines and embryos were produced from B6D2F1 females mated with B6D2F1 males (Charles River Breeding Laboratories, Wilmington, MA) using standard protocols. Mouse lines with compound Pbx deficiency exhibited CL/P with full penetrance. Unilateral clefting (either right or left) was reported in 4.8% of the embryos analyzed, while bilateral clefting was present in 95% of the cases [4].

Serum-free media preparation

A serum-free culture medium prepared from commercially available stem cell media supplements was utilized in this study [14]. Knockout serum replacement (Invitrogen^{TM}), Knock DMEM (Invitrogen^{TM}), and Dulbecco's modified Eagle's medium (DMEM, Invitrogen^{TM}) were used to maintain a consistent formulation for all embryos, as described previously [14,15]. Culture medium was filter sterilized through a 0.22mm filter into a 50mL conical tube and allowed to equilibrate for at least 5min at room temperature.

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Preparation of collagen microspheres

Type I collagen was extracted from rat-tails following an established method (Pel-Freez® Biologicals, Rogers, AK) [16]. Collagen microspheres were fabricated in sterile conditions using a method based on a modified emulsification process and a self-assembled collagen fiber reconstitution process [17]. One hundred collagen microspheres (diameter 20–50 micron) were soaked in 50μl 100μg/mL Wnt9b protein (R&D System, Minneapolis, MN) at 37°C overnight in the Wnt9b solution and stored in 4°C.

Preparation of whole embryo culture (WEC) system

Glass culture bottles and rubber bottle corks were sterilized by autoclaving. The roller culture chamber of WEC system (Ikemoto Rika, Tokyo, Japan) was sprayed with 70% alcohol and warmed to 37°C before starting the culture [18]. A gas cylinder containing a gas mixture of 95% O_2 / 5% CO_2 was connected to the rotating culture chamber and the gas flow was adjusted to flow through the embryo culture setup at a rate of 50–75 cc/min and ensure adequate oxygen supply to the embryos. The rotation speed of the drum was set up at 20 rpm/min [14].

Embryo dissection and culture

Wild type and mutant female mice were assessed for vaginal plugs every morning and with vaginal plugs found, female mice were considered as E0.5. At gestational day 11.5 (E11.5), pregnant mice were euthanized as specified by IACUC guidelines. After the uterus was removed from the abdomen, embryos were dissected from the deciduum and freed from maternal membranes. The embryonic head was then gently exteriorized from the yolk sac while maintaining the integrity of the embryonic vasculature with that of the yolk sac. Each embryo was morphologically assessed for stage-dependent characteristics such as headfold development, somite development, and heart rate. Embryos were then placed in 2mL of equilibrated culture medium in a sterile glass vial and placed in the rotator drum, at 37°C [19]. The embryos were cultured for 24 hours.

Microsurgical Implantation of Collagen Microspheres

Under a stereomicroscope, embryos at E11.5 were placed with their face up in order to expose the midface lambdoidal junction. Collagen microspheres either with or without Wnt9b were implanted within the midface λ junction on the left side of wildtype and Pbx-deficient embryos; the contralateral (right) side of each respective embryo remained unperturbed. After implantation, embryos were then placed in labeled rotator bottles by using a plastic Pasteur pipette and cultured at 37°C using the mixture of 95% O_2 / 5% CO_2 supplied at a constant rate. After 24 hours, each embryo was gently transferred to a Petri dish. Viability was assessed by observing the heartbeat, morphological features, and transparency (dead embryos are usually opaque and can show hemorrhage or edema).

Assessment of Wnt9b release from collagen microspheres

Thirty collagen microspheres soaked with Wnt9b were suspended in 100ul of 1X PBS in each sealed Eppendorf tube and incubated in a 37°C water bath. 100ul of supernatant in each

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Eppendorf was withdrawn precisely at 30 minutes, 1 hour, 3 hours, 6 hours, 12 hours and 24 hours, respectively. Western blot was performed as described previously [20]

Histology

Samples were fixed with 4% paraformaldehyde and then were dehydrated with sequential ethanol washes in a Vacuum Infiltration Processor (SAKURA VIP Tek Tissue Processor, the Netherlands), embedded in paraffin, and cut into 10 μm thick sections. Hematoxylin and eosin (H&E) staining was performed. Stained sections were imaged with an inverted microscope using bright field microscopy (Olympus 1X71, Olympus America).

Scanning Electron Microscopy

Embryos were fixed with 4% paraformaldehyde in 1X PBS followed by OsO_4 and gradually dehydrated in ethanol. The embryos were critical point dried under CO_2 and sputter coated with 30nm gold particles. Images were obtained using a Scanning Electron Microscope with field-emission electron gun (ZEISS LEO 1550, Germany). The observed rate of rescue was calculated dividing the area of the cleft from the treated side by the untreated side. Area sizes were measured using ImageJ [21].

B-galactosidase assay/X-gal staining

We evaluated Wnt9b canonical activity in the midface of Pbx compound mutants by using Wnt reporter (*BAT-gal*) mice [22]. Wnt9b-impregnated collagen microspheres were implanted in the foreheads of BAT-gal mice (a region of baseline low expression) and cultured for 24 hours. X-gal staining was performed as previously described [23].

Results

Upon initial embryo dissection, wildtype mouse embryos at E11.5 exhibited 100% survival when transferred into culture as evidenced by visible normal heartbeat and circulation. Additionally, after 24 hours of culture in the whole embryo culture system embryos at E12.5 showed normal growth and morphological development of facial structures (Figure 3).

The type I collagen microspheres utilized in this study were shown to have a stable structure and a diameter between 20 to 50 microns (Figure 4). To demonstrate the feasibility of an *ex vivo* microsurgical approach utilizing collagen microspheres as drug delivery vehicles, trypan-blue soaked microspheres were implanted at the midface λ junction in wildtype mice to assess the reliability of the microsphere to remain in the target area throughout the culture period (Figure 5). Approximately 95% of microspheres implanted in the E11.5 embryos remained within the λ junction after 24 hours in culture.

Microsurgical implantation of collagen microspheres at the λ junction in cultured Pbx mutant embryos did not impair overall midface morphogenesis and growth compared to those without microspheres implantation (($p>0.05$). Furthermore, Wnt9b-soaked collagen microshpere implantation resulted in a significant rescue of the clefting defect on the implanted side ($p<0.05$) (Figure 6, 7). A total of 59, $Pbx1^{-/-}$, $Pbx2^{+/-}$ embryos with fully penetrant cleft lip phenotype were analysed in our study. Notably, there was comparable

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growth and morphological development (aside from clefting) in Pbx mutant mouse embryos cultured in this whole embryo culture system for 24 hours when compared to wildtype control embryos.

Rescue was defined as a ratio of less than 50% in the area of clefting (complete or incomplete closure) comparing the treated side to the untreated/control side (Table 1). Sixteen out of the 59 Pbx mutant embryos with orofacial clefting analyzed exhibited significant rescue, totaling an observed 27.1% rate of rescue. It has to be underscored that the rate of rescue after microsurgical implantation of Wnt9b-soaked collagen microspheres might be overestimated by 1.35% (a factor that equals the percentage of unilateral clefting [4] over the total percentage of observed rescue). This takes into consideration that compound Pbx mutant embryos display a fully penetrant CL/P phenotype with an overall incidence of bilateral clefting of 95% and unilateral clefting of 2.4% for either right or left side [4]. Using the implantation protocol described above, *in vivo* experiments to study the spatial extent of action of Wnt expression in implanted Bat-gal embryos were also conducted. Given endogenous Wnt signaling at the lambdoidal junction of Wnt-reporter Bat-gal embryos, it was difficult to estimate the expression of exogenously delivered Wnt in this region. Thus, targeted delivery of Wnt9b was demonstrated by implantation of Wnt9b-soaked microspheres at the forehead (an area of low endogenous Wnt), resulting in augmented canonical Wnt signaling in the area surrounding the collagen microsphere when compared to the contralateral side (Figure 8). In order to gauge the range of action and optimize the dose of Wnt protein released by the collagen microspheres, the release kinetics from Wnt9b-soaked microspheres was quantified using Western blot. By three hours nearly half of the of the loaded Wnt (approx 50ng/microsphere) was released into the solution; by 6 hours almost 90% had been released (**See** Supplemental Digital Content 2, Figure shows the levels of Wnt9b released from Wnt9b-soaked collagen microspheres by using Western blot. Similar to methods used in the in vivo experiments, an aliquot of 50μl (100μg/mL) of Wnt9b protein was used to soak 100 collagen microspheres. Thirty Wnt9b-soaked microspheres were suspended in 1X PBS in each sealed Eppendorf tube and incubated in a 37°C for 30 minute, 1 hour, 3 hours, 6 hours, 12 hours and 24 hours, respectively. Analyses of band intensity were presented as the relative ratio of Wnt9b protein released from 30 microspheres to an appropriate Wnt9b solution at known concentration. Three hours after soaking of the collagen microspheres in the solution nearly half of the loaded Wnt (approx 50ng/microsphere) was released into the solution; by 12 hours almost two-thirds had been released. Con. 10μL 100μg/mL Wnt solution. *P<0.05 versus Con. #P>0.05 versus Con, INSERT LINK).

Discussion

CL/P has dramatic consequences for the affected child and their family, requiring many cycles of surgery, speech therapy, specialized orthodontics, and dentistry [2,4,6,7]. Despite multiple interventions, patients with CL/P are often left with significant sequelae including visible scars, abnormal dentition, nasal deformity, lack of mid-facial growth as well as hyper-nasal speech, all of which can significantly impact patients' future life and associated health care costs.

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Presently, no single genetic lesion has been identified as a universal culprit for CL/P but it is understood that both genetic and environmental factors contribute to its etiology [2,3,5]. Murine models of clefting have been widely used based upon the fact that mice and humans share similar craniofacial developmental process [3,4] Genes encoding secreted growth factors and their receptors (such as the *Tgf-βeta* [24,25] */Bmp* superfamily [12,26], *Fgf* [27], *Shh* [4,28] and their effectors, *Wnt* and *Lef* [29]) have been shown to be essential for the normal development of head and face structures in mice. Likewise, transcription factors (such as homeobox genes [29], bHLH-related genes [29], Paired-box genes [29], Winged Helix/Forkhead [29] and Pbx [11,12]) have been found to be crucial in initiating patterns of gene expression that result in morphological changes in the developing face and head. In support of this evidence, previous findings have shown that mutations in Wnt-p63-Irf6 regulatory network play a key role in the pathogenesis of CL/P in humans and mice [4]. In a previous study, we successfully established a strong genetic interaction between Pbx and Wnt9b and demonstrated that Pbx homeoproteins directly control Wnt signaling [4,12]. In our murine model, with simultaneous loss of multiple Pbx genes and consequent loss of function of Wnt/β–catenin pathway components, failure of apoptosis along the lambdoidal junction results in CL/P [4].

In the present study, our aim was to develop a new microsurgical technique to deliver intrinsic growth factors that may be absent or reduced in cases of CL/P. Whole embryos provide the tissue integrity and support appropriate for the tissue interactions that are crucial for timely occurrence of signaling mechanisms essential for different cellular processes during organogenesis [6,18]. The serum-free culture medium used to culture mid-gestation stage wildtype embryos allowed for progressive growth and morphological development for 24 hours, which permitted normal cellular behaviors (proliferation, migration, differentiation and tissue interactions) comparable to those observed in embryos developing in utero [14]. In addition, mutant mouse embryos cultured for 24 hours under these defined conditions exhibited normal progression of frontonasal morphologenesis and development of the upper lip, which provided the possibility for evaluating and measuring the area of clefting.

Wnt, a potent stem cell-inducing growth factor, has been labeled as a therapeutic protein in regenerative medicine, even though it can be extremely deleterious if delivered in high dose [30,31]. However, targeted localization of Wnt-impregnated collagen microspheres to the midface lambdoidal junction as shown in this study does not raise serious concerns for global Wnt-activation. Further, the collagen microsphere carrier utilized did not alter (nor improve) normal development at the lambdoidal junction. Therefore, our microsurgical technique offers the proof of concept for a minimally invasive approach at very early stages of development and defines a new potential method for fetal surgery to correct CL/P.

The possibility for intrauterine correction of CL/P would take advantage of the fact that fetal wound healing is phenotypically superior than adult or neonatal wound healing [2,3,4] and thus fetal CL/P surgery is an attractive surgical option [32,33]. In support of this approach, several groups have used intrauterine repair as a way to prevent scarless healing [2,34]. Even though fetal surgical interventions for CLP have been performed and relevant surgical techniques have improved over the past 30 years, there are still limitations and feto-maternal risks during fetal surgical procedure [34]. Because this particular model requires

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intervention at day 10.5 of gestation in the mouse, which is equivalent to 4 weeks in the human fetus, there remain significant barriers prior to translation of this approach to cleft repair. However, with further investigation, fetal surgery may yet allow for scarless repair of CL/P.

Our overall rate of rescue of 27.1.% can be attributed to multiple factors. Undetectable injuries caused by the microsurgical procedure were inevitable, and even with 100% survival rate, growth status of each embryo could not be the same as in normal in utero development, given that minor but significant experimental variations occur when using the whole embryo culture system. Further, although our microsphere delivery system did result in a localized elevation in Wnt activity, optimization of the release kinetics and the dosage used are necessary to improve the efficacy of this technique.

Taking into account the benefits and risks mentioned above, microsurgical implantation of Wnt protein for extrauterine and even intrauterine CL/P repair cannot be implemented as yet for clinical applications. We will need to overcome significant technical limitations, as well as improving tissue engineering methods, in order to advance this approach from the laboratory to the clinical field.

Conclusion

In summary, we have demonstrated a proof of concept approach to rescue CL/P using exogenously delivered Wnt protein so as to restore critical developmental programs. Furthermore, our novel *ex vivo* whole embryo culture system can be utilized to open new avenues towards unconventional and innovative prenatal interventions for patients with CL/P, as well as providing new solutions towards future pre-natal repair approaches to correct other congenital head and neck disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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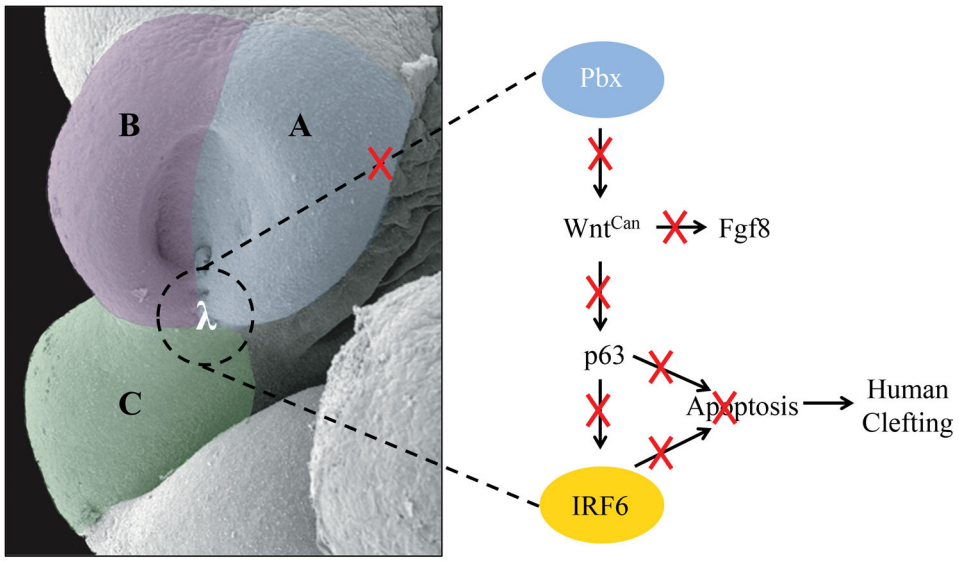

Figure 1.

Model of essential role of Pbx in midfacial morphogenesis. At the lambdoidal junction (dashed circle), a Pbx-dependent Wnt-p63, Irf6 regulatory module mediates apoptotic programs. Dysregulation of the module, which is fully conserved only within mammals, results in localized suppression of apoptosis and orofacial clefting[4]. (**(A)** Medial Nasal Process (MNP), **(B)** Lateral Nasal Process (LNP), **(C)** Maxillary Process (Mx)) (Adapted from 'A Conserved *Pbx*-Wnt-p63-Irf6 regulatory module controls face morphogenesis by promoting epithelial apoptosis' by Ferretti E. et al, Dev Cell 2011)

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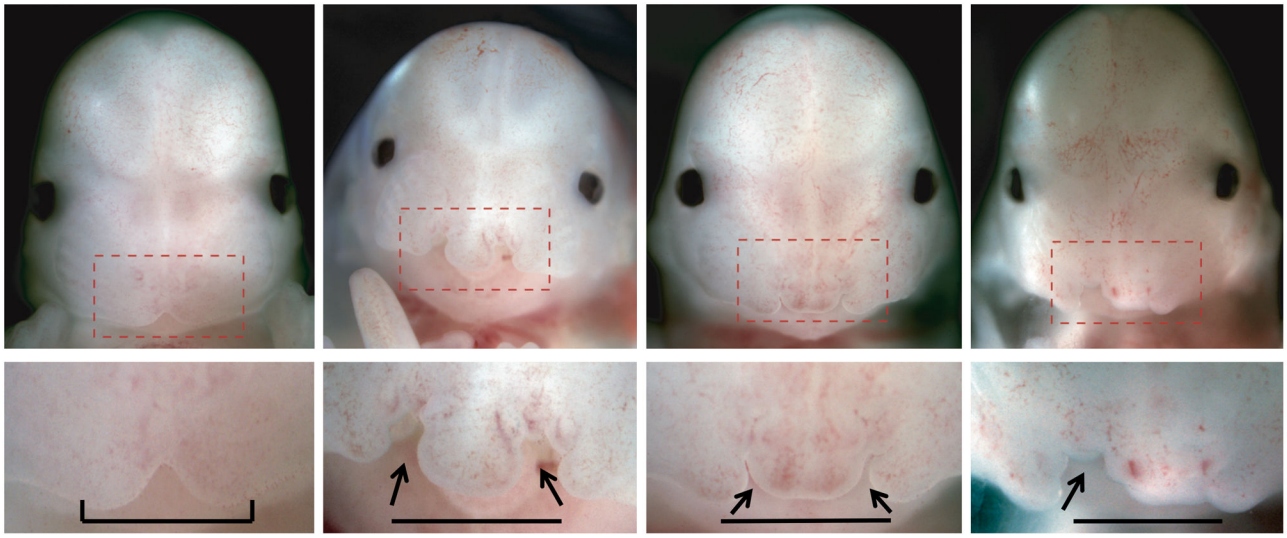

Figure 2.

Gross Morphology of *Pbx1*$^{-/-}$, *Pbx2*$^{+/-}$ **(B)** and *Pbx1*$^{-/-}$; *Pbx3*$^{+/-}$ **(C,D)** compound mutant embryos at E13.5 reveals bilateral **(B,C)** or unilateral **(D)** cleft lip (black arrows) *versus* wild-type control **(A).** (Adapted from 'A Conserved *Pbx*-Wnt-p63-Irf6 regulatory module controls face morphogenesis by promoting epithelial apoptosis' by Ferretti E. et al, Dev Cell 2011)

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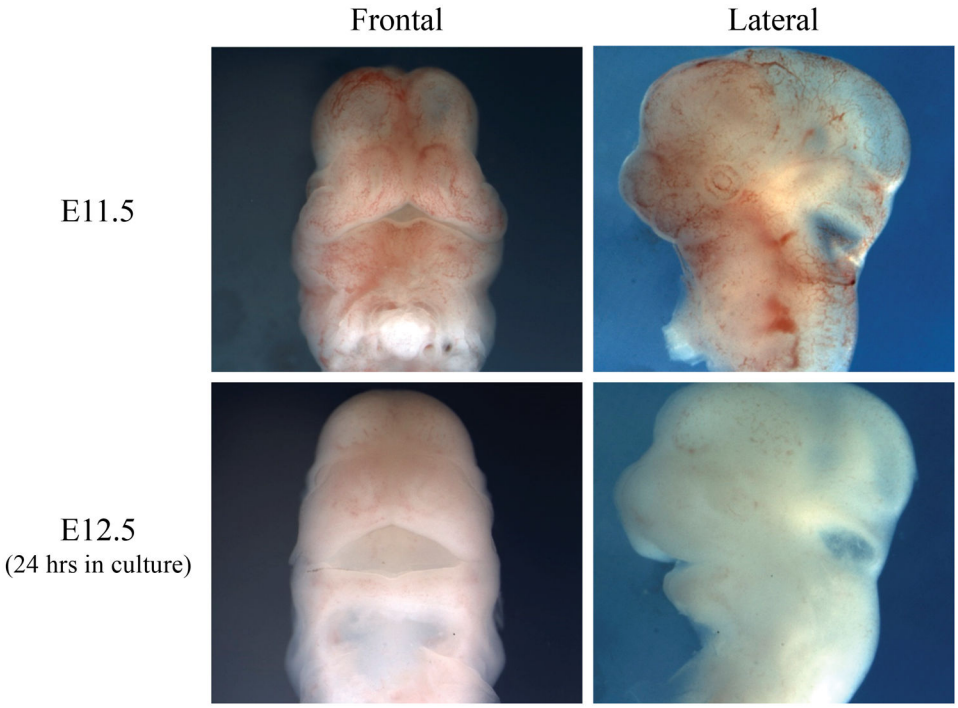

Figure 3.

Morphological development of Wildtype embryos in our whole embryo cultured system upon removal at E11.5 when compared to Wildtype embryos immediately removed from the uterus at E12.5. Normal upper lip development was noted in embryos culture in our whole embryo culture system.

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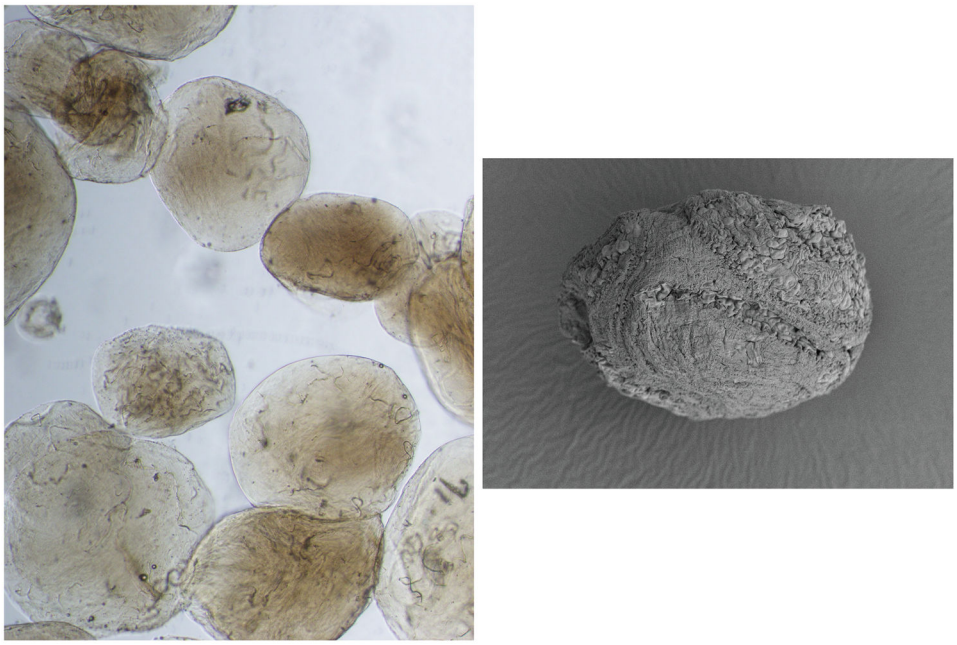

Figure 4.

Photomicrographs showing the morphology of microspheres after fabrication via double emulsion technique. Diameters of microspheres range from 20–50 microns. (**A**) Microspheres seen under light microscopy and (**B**) Scanning electron microscopy (SEM).

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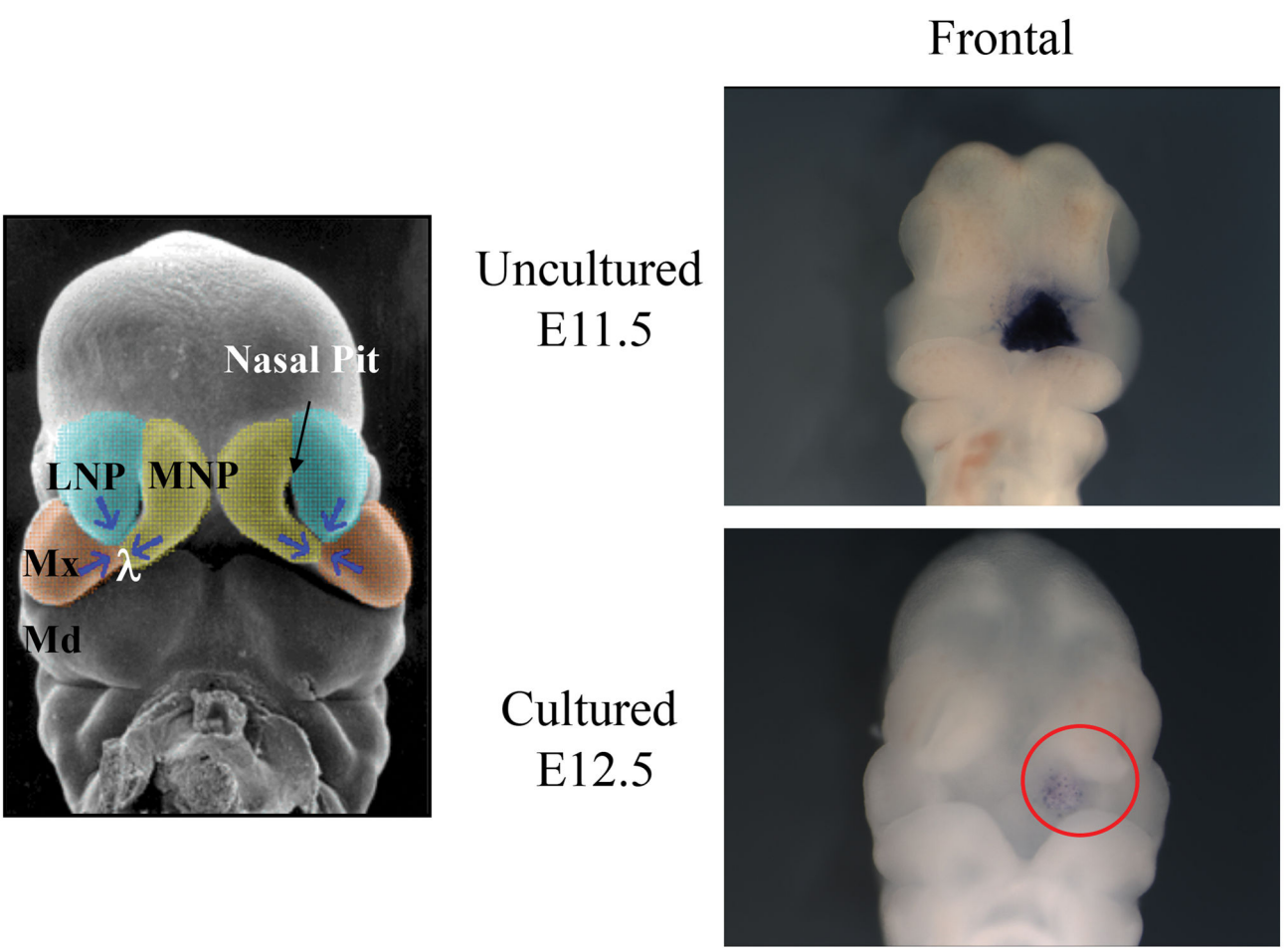

Figure 5.
Implantation of Trypan-blue soaked microspheres to study the orientation and the ability of the microsphere to stay implanted throughout culture. Note that the Trypan-blue has been largely released from the microsphere after 24 hours in culture (Collagen microsphere labeled with the red circle).

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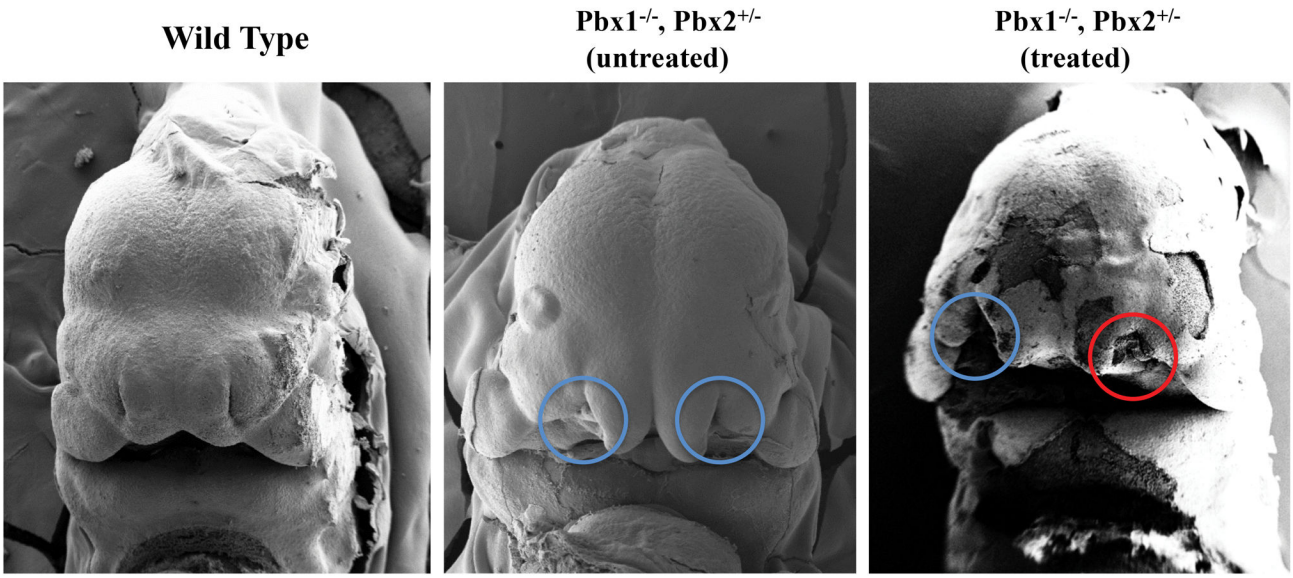

Figure 6.

Rescue of Cleft Lip in $Pbx1^{-/-}$;$Pbx2^{+/-}$ mutants at E12.5 after Wnt-soaked microspheres Implantation at E11.5. Scanning Electron Microscopy (SEM) of E12.5 mouse embryos (frontal views): wildtype (A), *$Pbx1^{-/-}$;$Pbx2^{+/-}$* mutants (B, C). *Pbx1/2* mutants after Wnt9b-soaked microsphere implantation at E11.5 exhibited a decrease in the extent of clefting (>50%) on the left implanted side (labeled with a red circle) when compared to the right untreated side (labeled with the blue circle).

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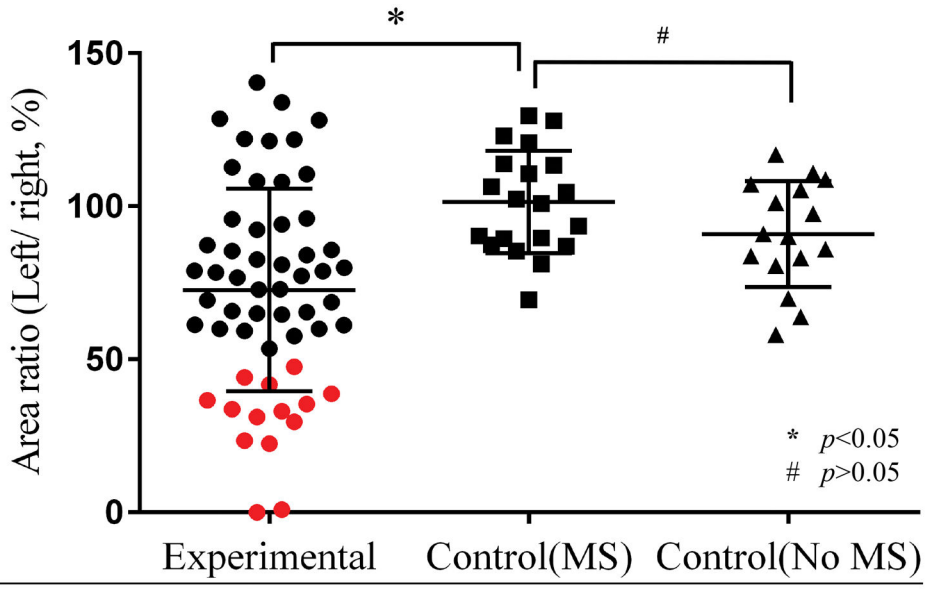

	Experimental (Wnt9b-soaked Microsphere)	Control (Microsphere)	Control (No Microsphere)
Mean	72.20	101.4	90.9
Std. Deviation	32.96	16.75	17.34

Figure 7.

Area ratio of clefting in $Pbx1^{-/-};Pbx2^{+/-}$ mutants with or without implantation of microspheres. Implantation of Wnt soaked microspheres resulted in a significant reduction in area ratio of clefting, compared to blank microspheres and the unimplanted contralateral side, $p<0.05$. Blank microsphere implantation did not affect the area ratio of clefting, $p>0.05$ vs unimplanted contralateral side. An area ratio of clefting (treated side/untreated side of bilateral cleft lip) < 50% is considered a rescued cleft lip. Red box represents the rescued mouse embryos.

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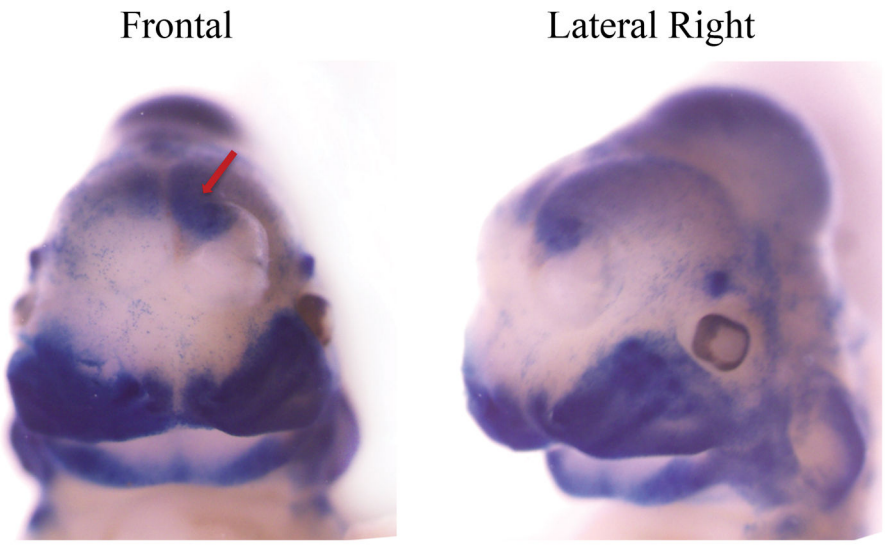

Figure 8.

Wnt soaked microspheres increases Wnt expression only locally. X-gal staining of E11.5 BAT-gal embryos implanted with Wnt9b-soaked microspheres on the left forehead and cultured for 24 hours demonstrate an amplified intensity of Wnt expression (red arrow) when compared to the normal endogenous Wnt signaling pattern present on the contralateral side.

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Table 1

Post-Wnt9b Microsphere Implantation and incidence of Cleft Lip in $Pbx1^{-/-};Pbx2^{+/-}$ mutant embryos. Implantation of Wnt9b-soaked microspheres can partially repair cleft lip in $Pbx1^{-/-};Pbx2^{+/-}$ mutant embryos.

Implantation site	Total cleft lip (n)	Bilateral cleft lip after treatment (n)	The area ratio of clefting less than 50% of bilateral cleft lip after treatment* (n)	Unilateral cleft lip after treatment (left, n)	Unilateral cleft lip after treatment (right, n)	Incidence of rescued cleft lip with implantation * (%)
Left	59	55	15	3	1	27.1

*An area ratio of clefting (treated side/untreated side of bilateral cleft lip) < 50% is considered a rescued cleft lip.

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