Pediatric RESEARCH

BASIC SCIENCE ARTICLE

Maternal folic acid supplementation reduces the severity of cleft palate in *Tgf-β_3* null mutant mice

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BACKGROUND: Cleft palate (CP) constitutes the most frequently seen orofacial cleft and is often associated with low folate status. Folate plays an essential role in the human body as a major coenzyme in one-carbon metabolism, including DNA synthesis, repair, and methylation. Whether the administration of isolated folic acid (FA) supplements prevents the CP caused by genetic mutations is unknown, as is its effect on the mechanisms leading to palate fusion.

METHODS: FA was administered to females from two different strains of transforming growth factor β_3 heterozygous mice. Null mutant progeny of these mice exhibit CP in 100% of cases of varying severity. We measured cleft length, height of palatal shelf adhesion, and the number of proliferating mesenchymal cells. Immunohistochemistry was also carried for collagen IV, laminin, fibronectin, cytokeratin-17, and EGF.

RESULTS: FA supplementation significantly reduced CP severity and improved palatal shelf adhesion in both strains both in vivo and in vitro. Medial edge epithelium proliferation increased, and its differentiation was normalized as indicated by the presence and disposition of collagen IV, laminin, fibronectin, and cytokeratin-17.

CONCLUSIONS: A maternal FA supplementation reduces the CP appearance by improving the mechanisms leading to palatal shelf adhesion.

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INTRODUCTION

The palate forms from two anlages. The primary palate develops from the frontonasal process of the head and gives rise to that part of the palate bearing the four incisors. The secondary palate appears in the form of two palatal shelves from the medial aspect of the maxillary processes of the first branchial arch. The secondary palate is the origin of the rest of the hard and soft palates. In mice, palatal shelves appear in the oropharynx on embryonic day 11.5 (E11.5). They then grow downward on both sides of the tongue until E14 and then reorient inwards, allowing their tips to face. At E14.5, the medial edge epithelium (MEE) covering these tips make contact and adhere.[1] Adhesion of MEE depends on correct differentiation of periderm cells on their surfaces[2] and the presence of appropriate cell adhesion and cell matrix molecules.[3–5] MEE cells then intercalate[4] to form the midline epithelial seam, producing confluent palatal mesenchyme. MEE cells disappear soon after.[6] By E15.5 in the mouse, the palate is completely fused.

Cleft palate (CP) might appear when any of these processes fails. CP is one of the most frequent orofacial clefts, occurring in about 1.7:1000 live born babies.[7] It has a strong genetic origin, the most frequent causes being mutations of *TGF-β_3*, *MSX1*, *IRF6*, and *RUNX2* genes and those of the *FGF* family.[8] Risk factors for CP[9] include maternal exposure to tobacco smoke, alcohol, drugs, pesticides, and nutritional deficiencies. Importantly, inherited and de novo single gene variants causing primary defects in regulators of epithelial cell adhesion explain a substantial proportion of CP.[10]

Folate plays an essential role in the human body as a major coenzyme in one-carbon metabolism, including DNA synthesis (deoxythimidine monophosphate (dTMP)), repair, and methylation.[11] A low folate status may perturb dTMP and methylation pathways resulting in birth defects such as neural tube defects (NTDs)[12] and tongue defects,[13,14] while the frequency of CP increased in children whose mothers did not take folic acid (FA) supplements during pregnancy.[15] Periconceptional FA supplements also led to a significant reduction in the occurrence of NTDs.[16] In mice, we demonstrated that a long period of dietary FA deficiency in females caused CP in their progeny, whereas an FA deficiency affecting only the gestation period, while not producing CP, altered many of the mechanisms leading to palate closure.[17]

Studies in humans and animal models have addressed the effect of a dietary supplement of FA on CP, but it has been difficult to separate its effect from that of other B vitamins administered during pregnancy. The administration of FA supplements in a mouse model of retinoic acid-induced CP[18] and in a mouse strain with a high frequency of spontaneous CP[19] had a protective effect. However, whether the administration of isolated FA supplements prevents the CP caused by genetic mutations is unknown, as is the effect of an FA supplement on the mechanisms leading to palatal shelf adhesion, which is a crucial step in palate fusion.

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To investigate these aspects, we selected a mouse model of genetic CP, the transforming growth factor β_3 null mutant mouse (*Tgf-β₃* −/−). This exhibits congenital CP in 100% of cases.[20] Their CP appears to be caused by heavily altered palatal shelf adhesion, lack of correct differentiation of periderm cells, and the total absence or alteration of cell adhesion and cell matrix molecules in the MEE.[2–4] In addition, palatal mesenchyme cell proliferation is reduced just prior to the contact of palatal shelves, impeding their proper growth and apposition and decreasing the possibilities of MEE adhesion.[21] Interestingly, CP severity depended on the mouse strain used. The great majority of C57BL/6J *Tgf-β₃* −/− mice showed complete CP, whereas 100% of the MF1 (Manchester strain) *Tgf-β₃* −/− mice showed incomplete CP, with only partial adhesion of palatal shelves in the middle third of the palate.[2] Indeed, molecules present in the MEE involved in palatal shelf differentiation and adhesion, such as collagen IV, laminin, and fibronectin, are more altered in C57BL/6J than in MF1 *Tgf-β₃* −/− mice,[5] possibly explaining this difference. The use of these two strains allowed us to measure, for the first time, the effect of FA supplementation on the severity of a genetically induced CP and palatal shelf adhesion. Our results demonstrate that this supplementation significantly reduced the severity of the CP in both strains by improving palatal mesenchyme cell proliferation, MEE differentiation, and palatal shelf adhesion.

METHODS

Animals and diets

Eight-week-old wild-type (*Tgf-β₃* +/+) and *Tgf-β₃* heterozygous (*Tgf-β₃* +/−) C57BL/6J and MF1 mouse females had free access to either a control diet (2 mg FA/kg diet) that met the FA requirements for mice[22] or an FA-supplemented (FAS) diet (40 mg FA/kg diet), as previously reported,[23] for 2–16 weeks. Both diets were purchased from Harlan Laboratories, Inc., Indianapolis, IN. Males and wild-type females were fed with control diet only.

Mice were kept in the animal house of the School of Medicine at the Universidad Complutense of Madrid at 22 ± 2°C, with light–dark cycles of 12/12 h. The animal facilities and the experimental protocol used in the studies reported herein were reviewed and ethically approved by the Animal Experimentation Committee of the Complutense University of Madrid.

Mating was allowed for one night only, and the day on which a vaginal plug was detected was designated day 0. Female mice showing signs of pregnancy were sacrificed by cervical dislocation at E13.5 for culture experiments, E14.5 for immunohistochemistry and bromodeoxyuridine (BrdU) staining, and E17.5 for macroscopic and histological observations. Embryos were removed by cesarean section, placed in cold sterile phosphate-buffered saline (PBS), and decapitated. Embryos to be used for culture experiments were extracted under sterile conditions.

Analysis of FA levels

Some studies have shown that variations in maternal folate levels are correlated with those of the embryo.[24] Therefore, we measured FA levels in the mothers' livers under different experimental conditions. Livers from *Tgf-β₃* +/− pregnant females previously fed the control ($n = 3$) or FAS diet (3 livers per week on diet) were collected and placed in liquid nitrogen. Livers were prepared through extraction and subsequent enzyme treatment according to the previously described trienzyme extraction method.[25] Total hepatic folate levels were determined using the microbiological method with *Lactobacillus casei* ssp. *Rhamnosus* (ATCC 7469) as described by Horne and Patterson,[26] with the modifications introduced by Tamura.[27] Briefly, after incubation of the samples in Folic Acid Casei Medium in sterile 96-well plates at 37 °C for 24 h, the absorbance at $\lambda = 620$ nm was measured using an automatic microplate reader. The standard stock solution was prepared by dissolving FA in 0.01 mol/L NaOH (20 mmol/L), and concentrations were determined in pH 7.0 buffered solutions at ultraviolet absorption $\lambda = 282$ nm for FA and a molar extinction coefficient (ε) of 27,000 M^{-1} cm^{-1}.[28] Suitable volumes of the stock solution were diluted in potassium phosphate buffer to construct an 8-point calibration curve (0–0.5 pg/μL), which was included in each assay in addition to the target samples.

Macroscopic and histological study

Intraoral images from buffered 10% paraformaldehyde-fixed E17.5 *Tgf-β₃* +/+ and *Tgf-β₃* −/− control and FAS mouse heads were obtained using a Nikon SMZ800 dissecting microscope (Nikon Corp., Tokyo, Japan) and photographed with a Leica EC3 (Leica Geosystems AG, St. Gallen, Switzerland) digital camera. Heads were embedded in paraffin and 7-μm-thick coronal sections were stained with hematoxylin–eosin, observed using a Leica DMR microscope, and photographed with a Leica DFC 320 digital camera.

Palatal shelf organ cultures

To investigate in vitro palatal shelf adhesion, palatal shelves have to be extracted before palatal shelf adhesion starts, which occurs by E14.5. Palatal shelves from E13.5 C57BL/6J *Tgf-β₃* +/+ ($n = 7$) and *Tgf-β₃* −/− control ($n = 7$) and FAS ($n = 7$) embryos were extracted microsurgically and placed apposed for 36 h in culture, as described Gato et al.[3] They were then fixed in buffered 10% paraformaldehyde for 2 h, embedded in paraffin, and sectioned along the anterior–posterior axis. Seven-μm-thick sections were stained with hematoxylin and eosin, studied using a Leica DMR microscope, and photographed with a Leica DFC 320 digital camera.

Assessment of the CP length

Measurements of the lengths of the anterior and posterior palate clefts were made in 7-μm-thick sections from E17.5 MF1 *Tgf-β₃* −/− control ($n = 9$) and FAS ($n = 9$) fetuses. For the anterior cleft, consecutive sections were counted, starting from the first where the primary and secondary palate appeared to be separated, and ending at the section where both palatal shelves were separated immediately anterior to the adhered zone. To measure the posterior cleft, sections were counted starting from the first where both palatal shelves were separated immediately posterior to the adhered zone and ending at the posterior end of the palatal shelves. The length of each cleft was the result of the number of sections in each case multiplied by 7 μm (thickness of each section). The total length of the secondary palate was measured starting at the beginning of the anterior cleft and ending at the end of the posterior cleft in the *Tgf-β₃* −/− palates and at the same start and end points in *Tgf-β₃* +/+.

Assessment of palatal shelf adhesion

In vivo palatal shelf adhesion was analyzed by measuring the dorsal–ventral height of the palate in one out of ten coronal sections from E17.5 MF1 *Tgf-β₃* +/+ ($n = 9$) and *Tgf-β₃* −/− control ($n = 9$) and FAS ($n = 9$) mouse heads using a Leica IM50 measuring program. In vitro palatal shelf adhesion was measured with the same program in one out of ten transverse sections from E13.5 C57BL/6J *Tgf-β₃* +/+ ($n = 7$) and *Tgf-β₃* −/− control ($n = 7$) and FAS ($n = 7$) mouse palatal shelf cultures. The length of MEE adhesion was assessed as described in Martínez-Sanz et al.,[5] and statistical analysis was performed using the mean of the lengths obtained for each specimen.

Cell proliferation assay

The "5-bromo-2′-deoxy-uridine Labeling and Detection Kit II" (Roche Diagnostics Corp., Indianapolis, Ind.) was used to detect cell proliferation in E14.5 C57BL/6J *Tgf-β₃* −/− control ($n = 5$) and FAS ($n = 5$) mouse heads, just prior to the contact of opposing palatal shelves. Cell labeling and immunohistochemistry to detect proliferating cells were performed as described in Del Río et al.[21]

Immunohistochemistry

To assess the presence and distribution of cell matrix, cell adhesion molecules and epidermal growth factor (EGF) in the embryonic palate, E14.5 C57BL/6J *Tgf-β₃* +/+ ($n = 5$) and *Tgf-β₃* −/− control ($n = 5$) and FAS ($n = 5$) mouse heads were fixed overnight in 10% buffered paraformaldehyde. Because we aimed to study the presence of these molecules immediately before palatal shelf adhesion, only specimens where palatal shelves had closely approached were selected. Standard paraffin embedding was then performed. Epitopes were unmasked in 5-mm-thick sections using 0.2% pepsin solution (Sigma-Aldrich, Inc., St. Louis, MO) in HCl 0.1 N (for anti-collagen IV) or 1 mM EDTA (Sigma-Aldrich) (for anti-fibronectin and-laminin). Sections were preincubated in 1% PBS–bovine serum albumin for 10 min. They were then incubated with either 1:100 polyclonal rabbit immunoglobulin G (IgG) anti-human collagen IV (ICN Biomedicals Inc., Aurora, OH), 1:50 polyclonal rabbit IgG anti-mouse laminin (Sigma-Aldrich), 1:75 monoclonal mouse IgG antihuman fibronectin (BD Transduction Laboratories, Franklin Lakes, NJ) for 2 h at room temperature or with 1:150 monoclonal mouse IgG antihuman cytokeratin-17, 1:50 polyclonal goat IgG anti-mouse EGF (R&D Systems, Inc., Minneapolis, MN) overnight at 4 °C. Labeling was developed for the same amount of time for all experimental conditions using either the Rabbit/Mouse EnVision Peroxidase System, (Dako Corp., Carpinteria, CA) for anti-collagen IV, -laminin, -fibronectin, and -EGF and 3,30-diaminobenzidine (DAB kit) as chromogen (Dako Corp.). Secondary antibody for developing labeling with anti-cytokeratin-17 was a fluorescein-conjugated goat anti-mouse secondary antibody AlexaFluor-488 dye (Invitrogen Corp. Carlsbad, CA) at 1/200 for 1 h. Some of the sections were counterstained with propidium iodide (Invitrogen) (0.01/200 dilution) for 5 min and all were mounted in Fluoro-gel (Electron Microscopy Sciences, Hatfield, PA). Negative controls were performed using mouse or rabbit IgG (controls) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at the same concentrations and conditions used for the respective experimental studies.

Immunolabeling in sections was visualized using a Leica DMRB microscope and photographed using a Leica DFC 320 digital camera or a 75-mW confocal laser scanning microscope (Leica TCS SPE, Leica Bensheim, Germany) equipped with an argon–krypton ion laser (488/568/647 nm). Digitized images were recorded and overlapped.

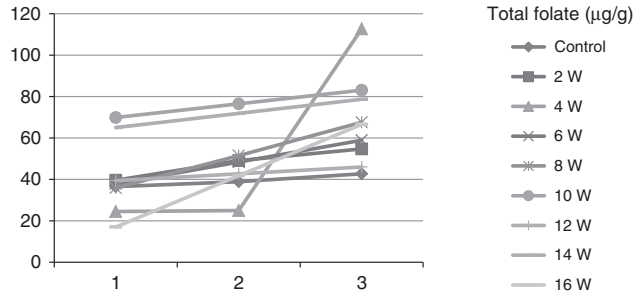

Fig. 1 Liver folate concentrations in *Tgf-β₃* +/− female mice fed the control or folic acid supplemented diet. Compared to the controls, total folate levels in the 2–16-week folic acid-supplemented groups increased significantly ($p < 0.05$). No significant differences were observed among the folic acid-supplemented groups

Assessment of cell proliferation and EGF labeling

Proliferating and anti-EGF immunolabeled cells were counted in the palatal mesenchyme of sections from the control and FAS embryos on a fixed area of mesenchyme, following the procedure described in Del Río et al.[21] Briefly, BrdU-positive cells in sections from E14.5 *Tgf-β₃* −/− control ($n = 5$) and FAS ($n = 5$) mouse palates were measured. The number of sections measured in each experimental condition was between 150 and 220. Once the image was captured, by using the Metamorph Image Analyzer (version 7.01, Universal Imaging Corp., Sunnyvale, CA) a fixed area of the mesenchyme was selected. Once the areas were selected, the observer fixed the threshold color of the BrdU-positive cells, and the Metamorph counted the number of spots having the color range selected. The measures for each group are the median values of the number of BrdU-positive cells from all sections of the group. Sections from the control and each experimental condition were measured in the same session to avoid differences in the basal parameters.

Statistical analysis

The normality of data distribution and the homogeneity of variances were studied first with the Shapiro Wilk and Levene tests, respectively. After checking that both conditions were met, the comparison of means was statistically analyzed by a one-way

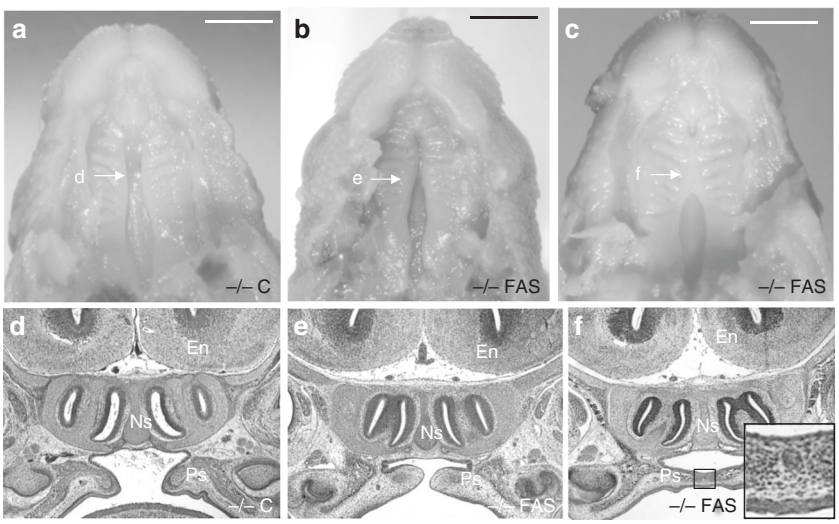

Fig. 2 Cleft palate appearance in C57BL/6 J *Tgf-β₃* −/− control (**a**, **d**) or folic acid-supplemented (**b**, **c**, **e**, **f**) mouse palates. Sections in **d**, **e**, **f** correspond to the levels indicated in **a**–**c**, respectively. Notice the closeness between the palatal processes in **e** and the adhesion in the middle third of the palate (**f**) in folic acid-supplemented mice. Inset is a high magnification of the squared area in **f** and shows an epithelial rest (arrow) of the midline epithelial seam in the adhered zone of the palate. En encephalon, Ps palatal shelf, Ns nasal septum. Scale bar is 1 mm in **a**–**c** and 500 µm in **d**–**f**

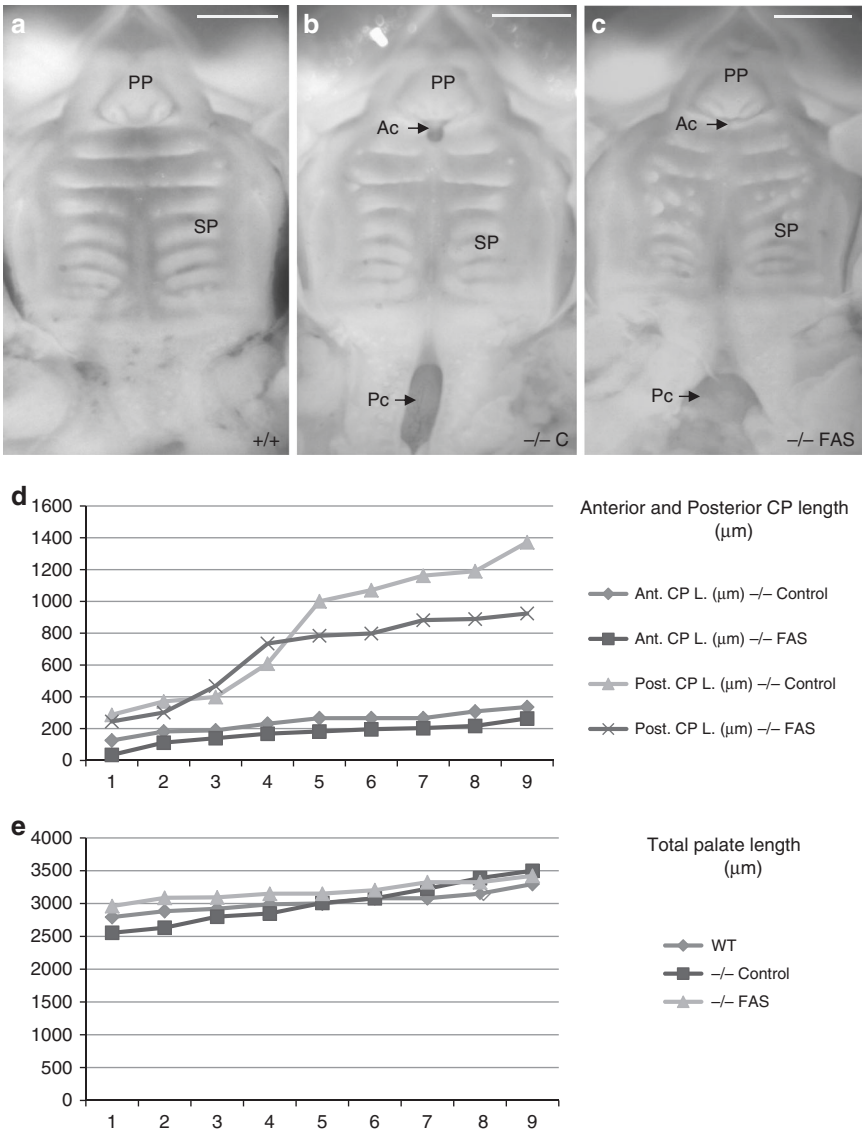

Fig. 3 Cleft palate appearance in MF1 *Tgf-β₃* +/+ (**a**) and *Tgf-β₃* −/− control (**b**) and folic acid-supplemented (**c**) mouse palates. Compared to the *Tgf-β₃* −/− controls (**b**), the anterior cleft (arrow) is greatly reduced in the folic acid supplemented (**c**) palates, very much resembling the aspect observed in the wild type (**a**). **d**, **e** Measure of the length of the anterior and posterior (**d**) clefts and total length of the palate (**e**) in MF1 *Tgf-β₃* −/− control and folic acid-supplemented mice. The length of the anterior cleft is reduced significantly ($p < 0.05$) in the folic acid-supplemented mice. Ac anterior cleft, Pc posterior cleft, PP primary palate, SP secondary palate. Scale bar in **a**–**c**: 1 mm

analysis of variance (ANOVA). When ANOVA resulted in differences, multiple comparisons between means were studied by Bonferroni tests. Differences were considered significant at $p < 0.05$ (SPSS 24.0, IBM Corp., Armonk, NY).

RESULTS

Levels of folate in the liver of *Tgf-β₃* heterozygous mice fed a FAS diet

With respect to controls, total folate levels increased significantly in the liver of mouse females under a FAS diet for 2–16 weeks. Levels in the supplemented mice were all statistically similar, independent of the weeks of FAS prior to or during pregnancy (Fig. 1).

Reduced severity of the CP presented by *Tgf-β₃* null mice under FA supplementation

At E17.5, most of the control C57BL/6J *Tgf-β₃* −/− fetuses ($n = 29$) exhibited complete CP with highly separated palatal shelves (96.6%; Fig. 2a, d), only one specimen showed incomplete CP with palatal shelf adhesion in the middle third of the palate (3.4%). The progeny ($n = 29$) of C57BL/6J *Tgf-β₃* +/− mouse females fed an FAS diet included 5 (17.24%) *Tgf-β₃* −/− specimens showing either complete CP with very close edges (Fig. 2b, e) or incomplete CP with palatal shelf adhesion in the middle third of the palate (Fig. 2c, f). The rest of the mice showed a CP similar to that presented by the *Tgf-β₃* −/− controls.

Since most C57BL/6J *Tgf-β₃* −/− exhibited a complete CP, it was not possible to measure the reduction of the cleft and to quantify the benefit of an FAS diet. For this purpose, we used the MF1 strain (Fig. 3a–e), with all *Tgf-β₃* −/− mice bearing an incomplete CP with partial palatal shelf adhesion.[2] Compared to the MF1 *Tgf-β₃* −/− controls (Fig. 3b), FAS mutants showed a reduction in the anterior cleft (compare Fig. 3b, c), almost appearing the same as the wild type (Fig. 3a). We found a significant decrease in the length of the anterior cleft from FAS mice, with no differences in the length of the posterior cleft (Fig. 3d) or the total length of the palate (Fig. 3e). No differences

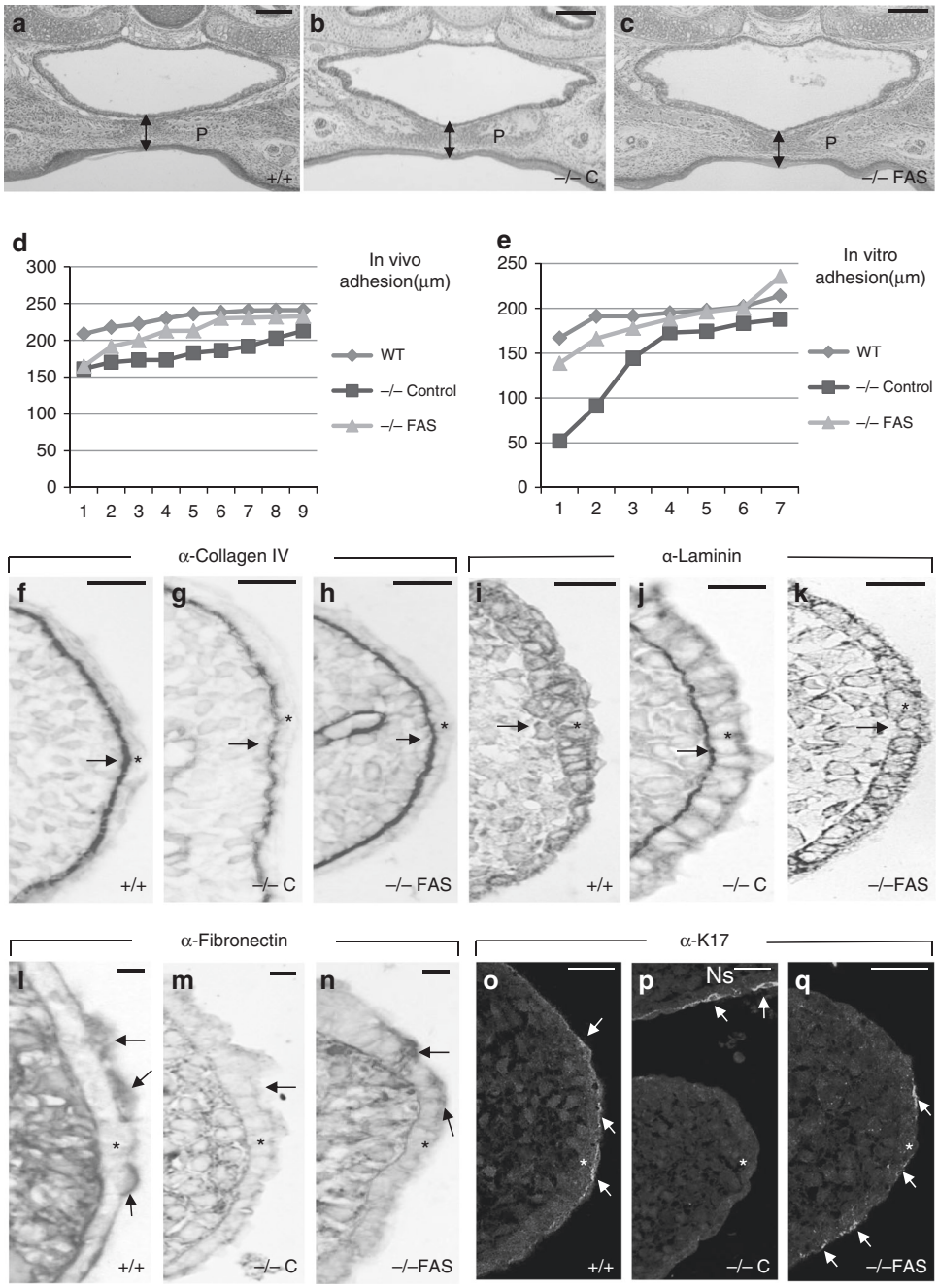

Fig. 4 **a**–**e** In vivo (**a**–**d**) and in vitro (**e**) palatal shelf adhesion in MF1 (**a**–**d**) and C57BL/6 J (**e**) wild-type (**a**, **d**, **e**) and *Tgf-β*$_3$ −/− control (**b**, **d**, **e**) and folic acid-supplemented (**c**–**e**) mice. Compared with the palatal shelf adhesion observed in the wild type (double head arrow in **a**), this adhesion is significantly ($p < 0.01$) reduced in the *Tgf-β*$_3$ −/− controls (same double head arrow in **b**, and **d**, **e**). Palatal shelf adhesion increases significantly ($p < 0.01$) in the folic acid-supplemented mice with regard to the *Tgf-β*$_3$ −/− controls (same double head arrow in **c**, and **d**, **e**). **f**–**q** Immunolabeling of C57BL/6J wild type (**f**, **i**, **l**, **o**) and *Tgf-β*$_3$ −/− control (**g**, **j**, **m**, **p**) and folic acid-supplemented (**h**, **k**, **n**, **q**) palates with antibodies against collagen IV (**f**–**h**), laminin (**i**–**k**), fibronectin (**l**–**n**), and cytokeratin-17 (**o**–**q**). Arrows point to the presence of all four proteins in the palate or in the nasal septum (Ns). Asterisk: Medial edge epithelium. Scale bar in **a**–**c**: 100 μm; in **f**–**k**, **o**–**q**): 25 μm; in **l**–**n**: 10 μm

were observed in the reduction of the cleft regarding the weeks during which the mouse females were FAS.

Improved palatal shelf adhesion in FAS *Tgf-β*$_3$ null mice

Compared with the E17.5 MF1 *Tgf-β*$_3$ +/+ mice (Fig. 4a), in vivo palatal shelf adhesion, measured as the height of the palate in the midline, was significantly reduced in the *Tgf-β*$_3$ −/− controls (Fig. 4b, d), but similar in the FAS *Tgf-β*$_3$ −/− palates (Fig. 4c, d).

Since palatal shelf adhesion is affected by the contact between palatal shelves,[3] we analyzed palatal shelf adhesion in vitro, where palatal shelves are forced into contact through the initial apposition of their opposing MEE. Comparison of the height of the adhered MEE between wild-type and *Tgf-β*$_3$ −/− control and FAS palate cultures showed a significant decrease of the adhered zone in the *Tgf-β*$_3$ −/− control, which increased in the supplemented mice and was comparable with that observed in the wild type (Fig. 4e).

The presence of collagen IV, laminin, and fibronectin was examined (Fig. 4f–n). Collagen IV, which is discontinuous and scarce at the basal surface of the *Tgf-β*$_3$ −/− MEE (Fig. 4g), formed a

continuous and abundant layer in the FAS *Tgf-β₃* −/− (Fig. 4h) and *Tgf-β₃* +/+ (Fig. 4f) mice. Laminin formed a continuous and marked layer at the basal surface of the *Tgf-β₃* −/− MEE (Fig. 4j), while its presence at this surface was scarce and discontinuous in the supplemented mice (Fig. 4k), as observed in the *Tgf-β₃* +/+ (Fig. 4i). Fibronectin is absent from the apical surface of the *Tgf-β₃* −/− MEE (Fig. 4m) but was restored at this location in the supplemented mice (Fig. 4n), as also seen in the *Tgf-β₃* +/+ (Fig. 4l).

Cytokeratin-17 is present in palate periderm cells.[29] Periderm cells are involved in opposing palatal shelf adhesion.[4] Since these cells are heavily decreased in *Tgf-β₃* −/− mice,[2] we investigated any possible variation in the presence of cytokeratin-17 in FAS *Tgf-β₃* −/− palates regarding the *Tgf-β₃* −/− control and wild-type palates. Cytokeratin-17 was present at the MEE apical surface of wild-type palates (Fig. 4o), while it was absent in the *Tgf-β₃* −/− palates (Fig. 4p). However, cytokeratin-17 was observed on the MEE apical surface of FAS *Tgf-β₃* −/− mouse palates (Fig. 4q).

Increased cell proliferation in the FAS *Tgf-β₃* null mouse palate mesenchyme

One reason for the reduction of palatal shelf adhesion in *Tgf-β₃* −/− mice is the decrease in cell proliferation in the palatal shelf mesenchyme at the time of contact between palatal shelves.[21] We compared the presence of BrdU-labeled cells in E14.5 *Tgf-β₃* −/− control and FAS mice. Proliferating cells in the palatal mesenchyme of FAS mice increased significantly with regard to controls (Fig. 5a–f). This increase occurred both in the anterior (Fig. 5e) and posterior (Fig. 5f) parts of the palate, with higher significance in the anterior part (Fig. 5e).

No change in the presence of EGF in the palatal mesenchyme of FAS *Tgf-β₃* null mice

Changes in the presence of EGF have been directly correlated with altered cell proliferation, palatal shelf adhesion, and fusion in *Tgf-β₃* −/− mice.[21,30] The number of EGF-positive cells in the pre-adhesion palatal mesenchyme did not significantly differ between FAS and control *Tgf-β₃* −/− mice (Fig. 6a–c).

DISCUSSION

In this work, we supplemented the diet of *Tgf-β₃* +/− mouse females from two different strains with FA and analyzed its effect on the CP presented by their *Tgf-β₃* −/− progeny. FA supplementation was reported to benefit CP induced by an external agent in mice,[18] rats,[31] and *Xenopus*,[32] while the same effect was observed in the A/WySnBK mouse strain, which shows a high incidence of spontaneous orofacial clefting.[19] The present work is the first to investigate the effect of an FAS in a CP caused by a genetic mutation (on the TGF-β_3 gene) in two mouse strains with different gene penetrances and CP type.

Our results have demonstrated an improvement of CP presented by the *Tgf-β₃* −/− mice from the two strains under a FAS diet. This was evidenced by a significant reduction in the length of the anterior cleft in the strain with a partial cleft and the approach of the palatal edges or their partial adhesion in the strain with complete CP. This was accompanied by a significant increase of palatal shelf adhesion, both in vivo and in vitro, with restoration of the normal deposition of collagen IV, laminin, and fibronectin in the pre-adhesion MEE, which is altered in the *Tgf-β₃* −/− embryonic palate.[5] Importantly, cytokeratin-17, which is present in wild-type MEE periderm cells and is absent in the *Tgf-β₃* −/− palates, was restored in the *Tgf-β₃* −/− FAS mice. This revealed an FAS-induced improvement in the differentiation of MEE superficial periderm cells, which are greatly involved in palatal shelf adhesion.[2] In addition, mesenchymal cell proliferation, which is greatly reduced in the *Tgf-β₃* −/− mouse palate and has a role in palatal shelf adhesion,[21] increased significantly in the FAS mice.

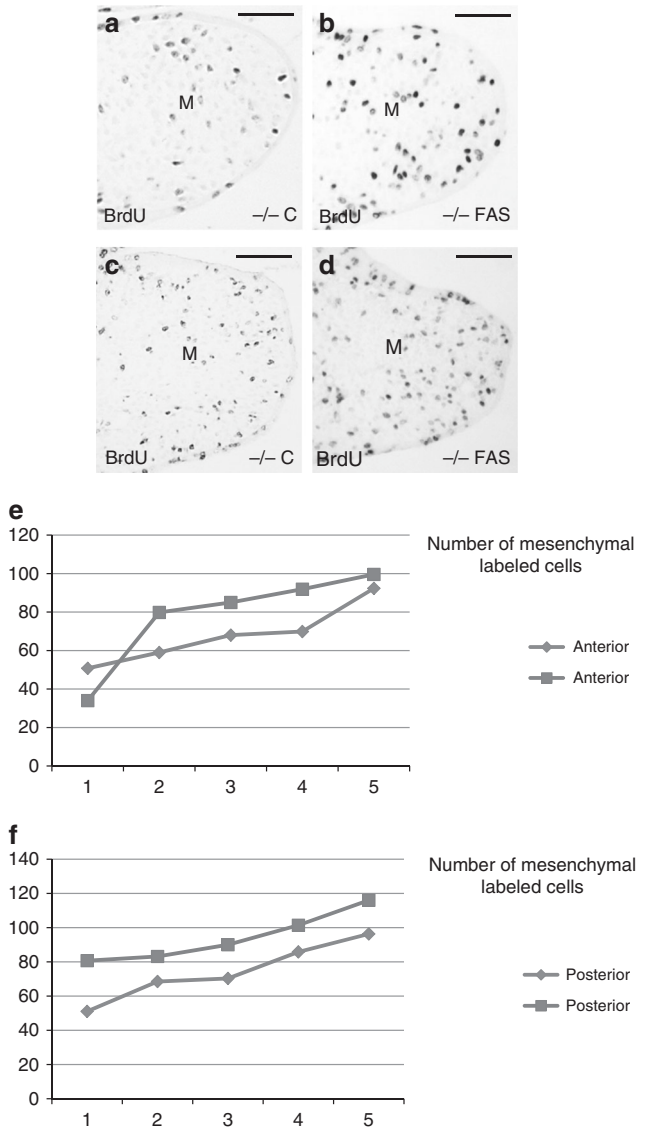

Fig. 5 Cell proliferation in the palatal mesenchyme of *Tgf-β₃* −/− control (**a**, **c**) and folic acid-supplemented (**b**, **d**) palates in their anterior (**a**, **b**) and posterior (**c**, **d**) parts. **e**, **f** There is a significant increase of mesenchymal cell proliferation both in the **e** anterior ($p < 0.01$) and **f** posterior ($p < 0.05$) parts of the folic acid supplemented palates. M mesenchyme. Scale bar in **a**–**d**: 50 µm

These results have demonstrated the ability of FA to ameliorate the effects due to the absence of the *TGF-β₃* gene. The FA induction of mesenchymal cell proliferation observed in FAS *Tgf-β₃* −/− mouse palates is consistent with increased rates of cell proliferation observed after FA treatment during embryonic development.[31] Specifically, FA supplementation enabled cells to progress through the G1/S restriction point, resulting in cell growth similar to normal levels for primary cultures of mouse embryonic palatal mesenchymal cells, which had been delayed by methylenetetrahydrofolate reductase gene silencing.[33] Interestingly, the increase in palatal mesenchymal cell proliferation caused by the FAS was higher in the anterior than in the posterior part of the palate, helping to explain the reduction of the cleft in the anterior region and not in the posterior one.

FA action could exert a direct effect on the mechanisms responsible for palatal shelf adhesion. Indeed, the interaction between FA and receptors mediating cellular adhesion has been recently established in other systems.[34] The level of folate has

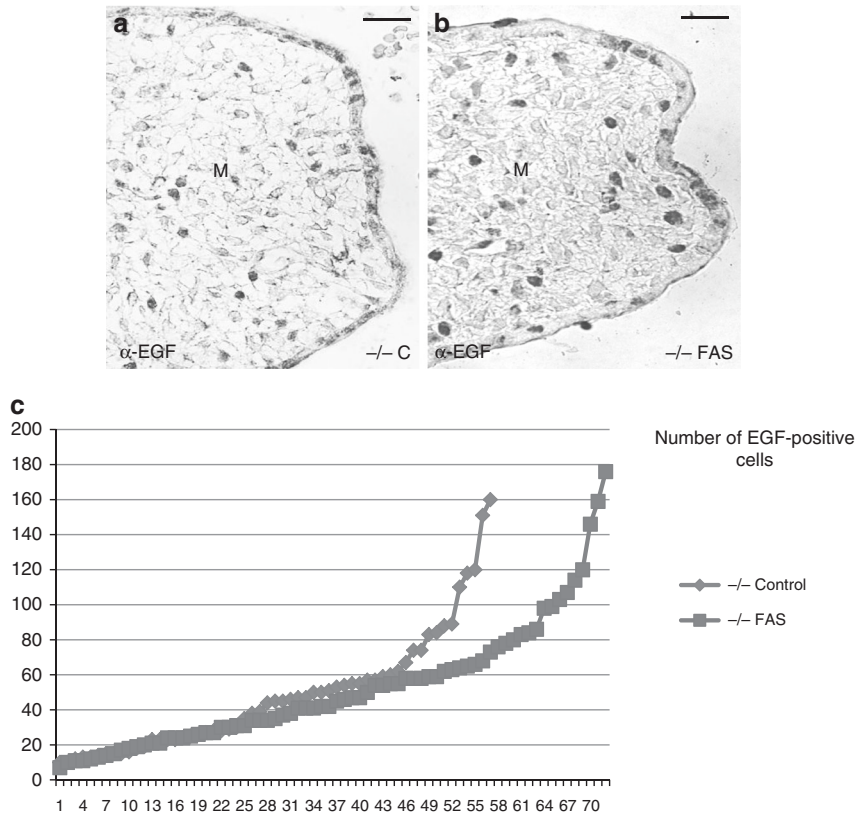

Fig. 6 Immunolabeling of *Tgf-β₃* −/− control (**a**) and folic acid-supplemented (**b**) palates with an anti-epidermal growth factor antibody. No significant differences were observed in the number of mesenchymal epidermal growth factor-positive cells between the control and folic acid-supplemented mice (**c**). M mesenchyme. Scale bar in **a**, **b** is 50 µm

been correlated with expression of several collagen genes,[35] and specifically high doses of FA have been shown to induce the synthesis of collagen IV.[36] Interestingly, cell adhesion to laminin, which is increased in *Tgf-β₃* −/− MEE basal cells and impedes proper palatal shelf fusion,[30] has also been reported to depend on the presence of folate.[37] In our model, FAS of *Tgf-β₃* −/− mice resulted in the reduced presence of laminin at the basal membrane of MEE cells, very much resembling its presence in the wild-type mouse.

Alternatively, the action of FA could be exerted on genes that interact with or are downstream to *Tgf-β₃* and play a role in palate development. FA has been reported to induce the expression of TgfβRII in mouse palatal mesenchymal cells,[18] and this could increase the activity of other members of the TGF-β family. Indeed, *Tgf-β₁* has been reported to modify MEE cell behavior and palatal shelf fusion, even in the absence of *Tgf-β₃*.[6] Interestingly, *Irf6*, which is downstream to *Tgf-β₃*,[38] regulates periderm cell differentiation and could be responsible for rescuing the expression of cytokeratin-17 observed in superficial MEE cells of FAS mice in the current study.

EGF also interacts with *Tgf-β₃* during palate development. In fact, the pattern of expression and distribution of EGF is greatly modified in the palates of *Tgf-β₃* null mice,[21] and both the abnormal palate mesenchymal cell proliferation and shelf adhesion observed in *Tgf-β₃* −/− mice are normalized when EGF activity is blocked.[21,30] However, the beneficial effect of the FAS diet on these mechanisms was not mediated by EGF, as we did not observe any modification in the number or distribution of EGF-positive cells in the palates of FAS mice.

The effect of an FAS maternal diet on palatal shelf adhesion observed here in mice may be operating in humans and could explain the reduction in CP appearance in children from women with an FAS diet during pregnancy.[39] We used high doses of FA (20× the FA included in a mouse control diet) because, although it has caused cardiac congenital malformations in mice,[40] high doses of FA used in humans seems to reduce the incidence of CP without producing other congenital malformations.[39] Our work also demonstrates that, at least in this experimental model, supplementation with FA during pregnancy is sufficient to benefit palate development and reduce the severity of CP, since we did not observe any benefit in supplementing FA for more weeks than those of the gestation period. This is linked to the fact that similar amounts of folate were observed in the liver of females supplemented for 2–16 weeks and may respond to the capacity of tissues to store folate.

The results reported here demonstrate the ability of FA to reduce the severity of the CP caused by a genetic mutation in an experimental model. This is important because the mutation or altered expression of certain genes is known to play a role in the etiology of the majority of CPs in humans. Our study reinforces the need for FA supplementation during pregnancy. Furthermore, we have provided insight into the improvement in mechanisms involved in palatal shelf adhesion related to this specific mutation, which contributes to our understanding of why a higher rate of CP is observed in children from women who did not take a FA supplement during pregnancy.[15] However, the action of FA in different mouse models of genetic CP may affect mechanisms other than palatal shelf adhesion (i.e., palatal shelf growth or reorientation), and these should be further explored to determine the role of FA in a broader spectrum of developmental anomalies known to cause genetic CP.

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AUTHOR CONTRIBUTIONS

Y.L.-G., E.M., L.N., E.M.-S., I.P.-L., M.I.A., and T.P. took care of the animals and/or acquired the data. Y.L.-G., A.d.R., M.C.B., J.M., and C.M.-Á. analyzed the results obtained. C.M.-Á. designed the experiments and wrote the manuscript. All authors approved the manuscript before submission.

ADDITIONAL INFORMATION

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