Glycoprotein A repetitions predominant (GARP) positively regulates transforming growth factor (TGF) β3 and is essential for mouse palatogenesis

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Glycoprotein A repetitions predominant (GARP) (encoded by the Lrc32 gene) plays important roles in cell-surface docking and activation of TGFβ. However, GARP’s role in organ development in mammalian systems is unclear. To determine the function of GARP in vivo, we generated a GARP KO mouse model. Unexpectedly, the GARP KO mice died within 24 h after birth and exhibited defective palatogenesis without apparent abnormalities in other major organs. Furthermore, we observed decreased apoptosis and SMAD2 phosphorylation in the medial edge epithelial cells of the palatal shelf of GARP KO embryos at embryonic day 14.5 (E14.5), indicating a defect in the TGFβ signaling pathway in the GARP-null developing palates. Of note, the failure to develop the secondary palate and concurrent reduction of SMAD phosphorylation without other defects in GARP KO mice phenocopied TGFβ3 KO mice, although GARP has not been suggested previously to interact with TGFβ3. We found that GARP and TGFβ3 co-localize in medial edge epithelial cells at E14.5. In vitro studies confirmed that GARP and TGFβ3 directly interact and that GARP is indispensable for the surface expression of membrane-associated latent TGFβ3. Our findings indicate that GARP is essential for normal morphogenesis of the palate and demonstrate that GARP plays a crucial role in regulating TGFβ3 signaling during embryogenesis. In conclusion, we have uncovered a novel function of GARP in positively regulating TGFβ3 activation and function.

GARP is a type 1 transmembrane protein with 20 leucine-rich repeats in the extracellular domain, a single transmembrane domain, and a short 14-amino acid cytoplasmic tail (1–5). GARP is highly expressed by platelets, activated regulatory T cells (Tregs) (6–9), mesenchymal stromal cells (10), hepatic stellate cells (11), and transformed tumor cells (12, 13). Multiple studies have established that GARP plays a role in activating TGFβ by mediating the surface expression and integrin-mediated activation of latent TGFβ1 (1, 2, 8, 14–18). GARP associates with latency-associated peptide to form an alternative cell-surface platform for latent TGFβ presentation (1, 2). GARP participates in the regulatory function of activated Tregs, demonstrated by impaired suppressive activity upon GARP silencing (4, 6) and inefficient support by GARP KO T cells for the generation of inducible Tregs (19). Aberrant over-expression of GARP in the tumor microenvironment also promotes Treg generation and immune escape (12, 13). Recently, we further identified that gp96 plays an essential role in surface GARP–TGFβ complex expression by chaperoning GARP in the endoplasmic reticulum (5). Further, GARP has been shown to function in promoting immune tolerance and tumor progression in cancer (5, 12, 15, 17). Thus, GARP is a key posttranslational regulator of TGFβ biogenesis and immune tolerance.

The TGFβ superfamily is a crucial cytokine family for both development and immunity, performing various functions in cell proliferation, differentiation, and cancer (20–22). There are three TGFβ isoforms. GARP reportedly binds only to TGFβ1 and TGFβ2 but not TGFβ3 (1). Genetically modified mice lacking Tgfb1 are born with autoimmune, endocrine, reproductive, vascular, and developmental abnormalities and die at 3–4 weeks of age (23). Tgfb2-null mice show perinatal mortality and multiple developmental defects, including cardiac, lung, craniofacial, limb, spinal column, eye, inner ear, and urogenital defects (24). Tgfb3 KO mice have a cleft palate caused by defects in medial edge epithelial (MEE) seam degeneration and palate fusion, leading to death within the first day after birth (25–27). Although all three TGFβs are expressed in the palate during mouse palate development, only inhibition of TGFβ3, but not TGFβ1 or TGFβ2, results in the palate fusion defect (28, 29), demonstrating the isform-specific role of TGFβ family members in vivo. However, the regulation of TGFβ3 biogenesis and signaling remains largely unknown.

To determine the GARP function in vivo, we have developed a reversible GARP KO mouse model using the flexible accelerated STOP TetO (FAST) knockin system (30), the GARP FAST...
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Figure 1. Ablation of GARP/Lrrc32 causes cleft palate in mice. A–H, H&E histological analysis (A–C and E–G) and whole-mount (D and H) of palatogenesis of wild-type (A–D) and GARP KO mice (E–H). Formation and elevation of the palatal shelf (P) are shown for both the wild-type (A) and KO (E) at the rostral region of E14.5 embryos. A midline epithelial seam is seen in the WT palate (b, arrowhead) but absent from GARP KO mice (p, asterisk). From E15.5 to E16.5 embryos, palatal fusion is completed throughout the entire palate in the wild type (C and D) but not observed in the mutant (G and H). Instead, a large gap can be seen in GARP mutants (asterisk), demonstrating a cleft palate phenotype. T, tongue.

Results

Knockout of Lrrc32 causes postnatal lethality and a cleft palate

Successful disruption of GARP was accomplished by inserting a stop cassette and TetO element into the endogenous promoter region of the Lrrc32 locus. Genomic PCR and flow cytometry analysis on platelets of day 0 live pups confirmed the absence of GARP expression in the homozygous GARP FAST neonates (supplemental Fig. S1).

Immediately postpartum, the ratios of wild-type, heterozygous, and homozygous offspring (total n = 94) from intercrossing between heterozygotes followed the expected Mendelian pattern (24.5% +/+ , 23.4% −/− , 52.1% +/− ; supplemental Table S1), demonstrating that the loss of GARP does not cause prenatal mortality. However, no offspring with homozygous mutated alleles survived 24 h after birth. The dead GARP-null offspring showed no visible milk in their stomachs. Further gross anatomical analysis and IHC of the KO mice showed no apparent abnormalities in the major organs, including the heart, lung, spleen, and liver (data not shown).

Importantly, cleft palates were observed in all homozygous mutated pups (Fig. 1). No developmental defects were identified in heterozygous mice. Thus, we subsequently focused on analyzing palate development in GARP mutant embryos and wild-type littermate controls to specify the cause of the pathology. At embryonic day 13.5 (E13.5), when the palatal shelves form from the maxillary processes, proliferate, and undergo elevation to reach the horizontal position, the palates in mutants and controls were indistinguishable (data not shown). The differences between GARP KO and wild-type embryos became evident at E14.5, when the palatal shelves begin to fuse (Fig. 1). At E14.5, the formation and elevation of the palatal shelf was comparable between wild-type and GARP mutant mice at the rostral region (Fig. 1, A and E). However, at the caudal region, where palatal fusion has begun, a midline epithelial seam is seen in WT palate (Fig. 1B, arrowhead) but is absent from GARP mutants (Fig. 1F, asterisk). From E15.5 to E16.5, palatal fusion is completed throughout the entire palate in the wild type (Fig. 1, C and D) but not observed in the mutants (Fig. 1, G and H). Instead, a large gap can be seen in GARP mutants (Fig. 1, G and H, asterisk), demonstrating a cleft palate phenotype. Together, these observations suggest that GARP is required for epithelial fusion during palatogenesis.

Palatal edge epithelial cell proliferation, apoptosis, and TGFβ signaling in the absence of GARP

We next analyzed the possible mechanism underlying the defective palatogenesis caused by GARP deletion. First, by staining for and quantifying phospho-histone H3, we found no difference in palatal edge epithelial cell proliferation between mutant and WT embryos (supplemental Fig. S2), suggesting that the proliferation abnormality is not the cause for the cleft palate.

MEE cell apoptosis has been shown to be important for palate fusion (32). We therefore used TUNEL assays to determine whether apoptotic signaling is compromised in KO palates. Indeed, we observed a significant decrease of apoptosis at the MEE seam in the mutant palate (Fig. 2). Because GARP is known to enhance TGFβ activation, and the TGFβ-SMAD2/3 signaling pathway plays an essential role in palatogenesis (32–34), we next focused on discerning the possible TGFβ signaling defect in the developing palates of KO mice. Indeed, decreased pSMAD2 was observed in E14.5 mutant palates, revealing clear defective canonical TGFβ signaling in the absence of GARP (Fig. 3).

Expression and co-localization of GARP and TGFβ3 in palatal edge epithelial cells

The isolated phenotype observed in GARP KO mice is conspicuously similar to Tgfb3 KO mice (25, 26). This suggests the possibility that GARP and TGFβ3 function together in vivo to regulate palatogenesis. If so, then GARP and TGFβ3 are expected to co-express at the MEE region. To address this pre-
diction, we performed GARP IHC using a sheep anti-mouse GARP antibody and discovered that GARP was expressed at the MEE region in WT E14.5 palates but not in the corresponding KO palates (Fig. 4A). Consistently, TGFβ3 was also detected in WT MEE cells (Fig. 4B). However, as IHC cannot differentiate various forms of TGFβ3, we did not observe a clear difference in TGFβ3 levels between WT and KO palates, proving that GARP does not affect total TGFβ3 expression. Altogether, these results suggest that strategically expressed GARP in MME cells is required for TGFβ3 activation and palatogenesis.

**GARP interacts with proTGFβ3 and is required for its cell-surface expression**

Up to this point, the literature has suggested that GARP regulates both TGFβ1 and TGFβ2 but not TGFβ3 (1). We next revisited this question by performing extensive in vitro biochemical studies. We co-overexpressed proTGFβ3 and HA-tagged mouse GARP in HEK293 cells, followed by immunoprecipitation analysis. As expected, TGFβ3 was co-pulled down with GARP (Fig. 5, A and B). In addition, we overexpressed proTGFβ3 in a mouse mammary carcinoma cell line, 67NR, which was made to express either mouse full-length GARP or the soluble extracellular domain of GARP fused with the Fc domain of IgG (GARP-Fc), which can interact with protein A/G beads (supplemental Fig. S3). Thus, using protein A/G beads, we were able to immunoprecipitate GARP-Fc with TGFβ3 from the cell lysates and the supernatants of cells that express both GARP-Fc and TGFβ3. These results demonstrate a direct interaction between the two proteins.

Because GARP is a cell-surface TGFβ docking receptor (1, 2, 4–6), we next investigated whether GARP is important for the surface expression of TGFβ3. Similar to what was previously done with TGFβ1 (35), we tagged human TGFβ3 with a His tag right after the furin cleavage site. The tagged TGFβ3 (His-TGFβ3) was expressed in HEK293 cells with or without GARP. We demonstrated that surface His-TGFβ3 is only detected in cells that co-express GARP (Fig. 5, C and E), despite similar levels of secreted TGFβ3 from GARP+/H11001 and GARP-/H11002 cells (Fig. 5D). Finally, by confocal microscopic analysis, co-localization of GARP and His-TGFβ3 on the cell surface was demonstrated (Fig. 5E). Altogether, we identified TGFβ3 as a novel GARP ligand and that GARP is required for cell surface expression of TGFβ3.

**Figure 2. Decreased apoptosis in GARP KO palates.** A, a TUNEL assay was used to detect apoptotic cells at the MEE region in E14.5 WT and corresponding KO palates. Arrowheads indicate TUNEL-positive apoptotic cells. B, quantification of apoptotic cells in WT and KO mice at the indicated time points. HPF, high-power field. The values are expressed as mean ± S.D. (n = 3). *, p < 0.05; **, p < 0.01. The data are representative of two independent experiments.

**Figure 3. Ablation of GARP results in reduction of SMAD2/3 phosphorylation in palatal edge epithelial cells.** IHC was performed to determine the expression of pSMAD2/3 at the MEE region in E14.5 WT and the corresponding KO palates. The immunohistochemical staining was performed with antibody against pSMAD2/3. Slides were counterstained with hematoxylin. Representative images are shown. Arrowheads points to pSMAD2/3-positive cells. The staining intensity scores for pSMAD2/3 are expressed as mean ± S.D. (n = 4). *, p < 0.05.
Discussion

TGFβ is a pleotropic cytokine whose activity is subject to extensive regulation to control its expression level, activation status, spatial and temporal availability, and downstream signaling. GARP is a non-signaling receptor that is primarily responsible for cell-surface docking of TGFβ onto Tregs and platelets. Not surprisingly, the research focus on GARP has been in the area of immune regulation. However, there is evidence for the notion that GARP also functions in cell differentiation and embryonic development. As early as in 1996, Roubin et al. (36) reported that GARP transcripts are expressed in various tissues in mouse embryos. Importantly, the highest levels were detected in E13.5 and E15.5 embryos (36), with unclear significance. This study is the first to establish a role for GARP in mouse embryonic development.

Although 100% lethality was observed with GARP KO neonates, analysis of embryos and day 1 neonates did not reveal other malformations in any other organs except for a cleft palate. The isolated defect in palatogenesis in GARP KO mice is identical to the one observed in TGFβ3 mice. Thus, although GARP is broadly expressed in embryos, its most important function during embryogenesis appears to be selectively facilitating the function of TGFβ3. Indeed, we found that both GARP and TGFβ3 are co-expressed in MEE cells at E14.5. More importantly, we showed that GARP directly interacts with TGFβ3 and is responsible for cell-surface expression of TGFβ3, challenging a previous study claiming that GARP binds to the latent forms of TGFβ1 and TGFβ2 but not TGFβ3 (1).

Sequencing alignment of all three TGFβs (supplemental Fig. S4) showed that the Cys-4 of proTGFβ1, which is important for interaction with GARP (2), is conserved among proTGFβ1, proTGFβ2, and proTGFβ3. In addition, the RGD motif is present in proTGFβ1 and proTGFβ3 but not in proTGFβ2. This suggests that TGFβ1 and TGFβ3 share similar mechanisms of activation by GARP and αV integrins (αVβ6 or αVβ8).

It should be noted that GARP-null embryos show no clear phenotype in TGFβ1- or TGFβ2-null embryos. Does this mean that GARP is more important for the function of TGFβ3 than that of TGFβ1 and TGFβ2 in vivo? The answer is not immediately clear, but it is plausible that the loss of GARP in control
Interestingly, a recent study observed no pups for CMV-cre/GARP\(^{fl/fl}\) mice and suggested an "embryonic lethal phenotype" (39). As neither embryos nor dead pups have been analyzed for defects of CMV-cre/GARP\(^{fl/fl}\) mice/embryos, whether the two kinds of GARP KO mice have the same lethal defects need to be further determined by analyzing CMV-cre/GARP\(^{fl/fl}\) mice. Nevertheless, the study further confirms the essential role of GARP in embryonic development.

Cleft palate in humans occurs in about one in 700 live births worldwide. Although mutations in a few genes have been suggested to contribute to cleft lip and cleft palate (40), our knowledge of genetic factors that contribute to the more common isolated cases of cleft palate is still incomplete. In addition to family genetics, a cleft palate is present in many different chromosome disorders, such as Patau syndrome. A study has reported the de novo deletion of chromosome 11q13.4-q14.3 in a boy with microcephaly, including a cleft palate, ptosis, and developmental delay (41). A 2014 review further summarized that seven cases among 32 patients with deletion of 11q13-q23.2 have a cleft palate (42). Based on this study and the knowledge that Lrrc32 locates in chromosome 11q13.5, we suggest that GARP mutation in patients may constitute a novel mechanism of cleft palate clinically.

In summary, we have uncovered, genetically, an important developmental function of GARP in regulating palatogenesis. Mechanistically, it is the first time that GARP is linked to regulate TGF\(\beta\) function in vivo. Contrary to a previous report, we demonstrated that GARP binds directly to TGF\(\beta\) and that it is important for TGF\(\beta\) expression on the cell surface. Our study points to another mechanism of human craniofacial deformities. The GARP-targeted strategy will also be useful for probing other unique aspects of TGF\(\beta\) function, such as in immune regulation, in the future.

**Experimental procedures**

**The GARP-FAST mouse model**

All animal procedures in this study were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee. We developed a reversible GARP KO mouse model using the flexible accelerated STOP TetO knockin system (inGenious Targeting Laboratory) (30). The GARP-FAST mouse model was generated by inserting the protomoter region of Lrrc32 with a stop cassette and a TetO-responsive element, which collectively deactivate the endogenous Lrrc32 protomoter and enable the use of the tetracycline transcription system to induce/silence GARP expression.

Male and female GARP-FAST\(^{+/+}\) mice were intercrossed to generate GARP-FAST\(^{+/−}\) (KO) mice, whose genotypes were confirmed by genomic PCR (primers: FAST-SQ1, 5’-ACA CCT CCC CCT GAA CCT GAA AC-3’; SC1, 5’-GCG ACA AAT ACC GAG CCA AAG CTC-3’; SQ1, 5’-AGC CTC TTG AGT TCC AGA ATA CCA C-3’). Noon of the day of plug appearance was counted as day 0.5. Embryos at different gestation stages were collected surgically and investigated.

**Sample preparation and H&E staining**

All samples for histological analysis were fixed in 4% formaldehyde and processed into optimal cutting temperature...
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compounds—embedded sections. Sections (6 μm) were mounted on poly-L-lysine—coated slides. For general morphology, sections were stained with hematoxylin and eosin using standard procedures.

**Apoptosis assay**

Following treatment with proteinase K for 30 min at room temperature, apoptotic cells on histological slides were assayed by the TUNEL procedure using a terminal deoxynucleotidyltransferase in situ apoptosis detection kit (R&D Systems) according to the manufacturer’s protocol.

**Immunohistochemistry**

Immunohistochemical staining was performed with antibodies against GARP (1:100 dilution, Roche), pSMAD2/3 (Abcam, 1:100), and TGFβ3 (R&D Systems, 1:100). Slides were counterstained with hematoxylin and visualized using a standard bright-field microscope. The staining intensity was graded with the sample identity blinded as described previously (12): 0, negative; 1, faint; 2, moderate; 3, strong but less intense than 4; and 4, intense.

**Immunoblot analysis and immunoprecipitation**

A human TGFβ3 expression vector (pLVE-hTGFβ3-RES-Red) was purchased from Addgene and transiently overexpressed in HEK293 cells or HEK293 cells stably expressing HA-tagged mouse GARP. Two days after transfection, the cell pellets were collected. The cell pellets were lysed in radioimmune precipitation assay buffer and subjected to immunoblot analysis using antibodies against mouse GARP (Roche) and TGFβ3 (R&D Systems). Pulldown of GARP-HA was performed using an anti-HA antibody (Proteintech) and protein A/G beads (Bio-Rad) following the the manufacturer’s protocol. Briefly, the cells were lysates were incubated with the beads and antibody overnight at 4°C. The immunoprecipitated protein was released by treating the beads with 2× SDS Laemmli buffer for immunoblot analysis.

**Surface detection of TGFβ3 expression**

A nine-histidine (His) repeat was inserted between Arg-300 and Ala-301 in human TGFβ3. The tagged TGFβ3 (His-TGFβ3) was cloned into a MigR1 retroviral vector with GFP as a reporter. His-TGFβ3 was expressed in HEK293 cells or 293-GARP cells, followed by surface staining with an anti-His antibody (1:100, Proteintech) and a secondary anti-mouse-allophycocyanin antibody. TGFβ3 surface expression was then detected by a flow cytometer (BD FACScVerse). For microscopy analysis, the above cells were fixed with 4% formaldehyde for 10 min and permeabilized with cold methanol for 5 min, followed by anti-His and GARP antibody staining. The images were taken by an Olympus FV10i confocal microscope.

**ELISA**

The TGFβ3 ELISA was performed using an ELISA kit (R&D Systems, DY243) according to the manufacturer’s instruction.

**Statistical analysis**

Results are expressed as mean ± S.D. Comparisons among groups were made by Student’s t test. A p value of 0.05 or less was considered statistically significant.

**Author contributions**—B. X. W., A. L., L. L., C. W., S. K., and S. L. performed the experiments and data analyses. B. X. W., X. L., and Z. L. designed the study and wrote the manuscript.

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