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Subclinical features in non-syndromic cleft lip with or without cleft palate (CL/P): review of the evidence that subepithelial orbicularis oris muscle defects are part of an expanded phenotype for CL/P*

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Structured Abstract

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Objectives – Non-syndromic cleft lip with or without cleft palate (CL/P) is a common, complex birth defect with a wide phenotypic spectrum. This review summarizes the evidence that subepithelial (occult) defects of the superior *orbicularis oris* (OO) muscle represent the mildest form of the lip portion of CL/P.

Experimental Design – The rate of OO defects was assessed via ultrasound in non-CL/P relatives of individuals with CL/P and compared with controls. Descriptive histology of OO muscles from cadavers was carried out. BMP4 was sequenced in non-CL/P individuals with OO defects vs. controls.

Results – 1) Non-CL/P relatives of individuals with overt CL/P have a significantly increased frequency of OO defects compared with controls with no family history of CL/P; 2) Preliminary histological studies of cadaver OO muscles show a pattern of disorganized muscle fibers in an individual with OO discontinuities as seen on ultrasound compared with another individual with no OO defect. That is, the defects seen on ultrasound appear to have an anatomical basis; 3) Sequencing BMP4 found a significant increase in potentially damaging mutations in individuals with OO defects vs. controls.

Conclusions – Taken together, these data provide significant support for the hypothesis that subepithelial OO muscle defects are a mild manifestation of the lip portion of the CL/P phenotype. Given that subepithelial OO muscle defects are relatively straightforward to identify via ultrasound, such defects show great promise for providing more accurate recurrence risk estimates to relatives in cleft families. Furthermore, inclusion of OO defects in the CL/P phenotypic spectrum should improve the power of genetic studies.

Key words: cleft lip; cleft lip and palate; orbicularis oris muscle; phenotype; subepithelial cleft lip

Introduction

Non-syndromic (isolated) cleft lip with or without cleft palate (CL/P) is one of the most common birth defects in humans, with birth prevalence

ranging from 1/2000 in populations of African descent, to about 1/1000 in populations of Caucasian descent, to almost 1/500 in populations of Asian and Native American descent (1). CL/P is considered to be etiologically complex, with substantial familiality that does not follow standard Mendelian patterns (2), and an estimated three to 14 etiologic genes based on family recurrence patterns and genome-wide linkage scans (3, 4). Some of these genes have now been identified, including IRF6 (5–9), MSX1 (10, 11), and FGF1 (12) among others.

Additional progress in identifying CL/P genes will require a combination of state-of-the-art molecular and statistical genetic approaches, applied in large family studies in order to have adequate power. Further, another potentially fruitful approach to increase the power of CL/P genetic studies (13, 14) will be to expand the phenotypic features that are considered affected in CL/P families (15). To date genetic studies of CL/P have assigned affection status to family members with overt (visible) CL/P. There is a broad range of defects encompassed in overt CL/P, ranging from so-called microform clefts (16, 17), to notches in the vermillion border, to complete unilateral or bilateral clefts of the lip and palate (18, 19).

The purpose of this review was to summarize the evidence that subepithelial (non-visible) defects of the *orbicularis oris* (OO) muscle represent the mildest form of cleft lip and that such defects are part of the phenotypic spectrum of CL/P.

Defects in the orbicularis oris muscle

Minimal but visible (microform) versions of cleft lip can appear as a ridge of tissue, resembling a scar on the upper lip. Histological studies show that these defects extend to the muscle fibers of the superior OO muscle (20, 21).

In a histological study of philtrum development, Martin *et al.* (22) found defects in the OO muscle in two 18-week fetuses with no obviously visible cleft lip, suggesting that the CL/P phenotype might also include occult subepithelial clefts. Martin and Martin (23) then developed a method using high-resolution ultrasonography to visualize the OO muscle non-invasively, and reported apparent defects of the OO in first-degree relatives of CL/P individuals. Using this method, Martin *et al.* (24) designed a blinded follow-up study to examine the OO muscle in a small sample of non-cleft first degree relatives (n = 62) of individuals with CL/P and healthy controls (n = 52), and found a significant increase in the frequency of subepithelial OO defects in the non-cleft relatives (40%) vs. the controls (11%).

This finding was subsequently confirmed by Neiswanger *et al.* (25) in the Pittsburgh Oral-Facial Cleft Study (POFC), a large on-going study of expanded phenotypic features in CL/P multiplex families that is designed to identify genes contributing to CL/P (15). Non-overt-cleft individuals from multiplex families (i.e. those with two or more members affected with overt CL/P) were examined, as well as controls with no known family history. Families and controls were drawn from three POFC populations (Pittsburgh, PA; Guatemala; and Madrid, Spain).

The ultrasounds of the OO muscles from family members and controls were classified as normal, affected, or unknown by three independent raters blinded as to case or control status. A muscle was considered normal if a continuous hypoechogenic band of uniform thickness could be visualized, with no obvious discontinuities. Alternatively, a muscle was considered to have a defect if one or more discontinuities were present. A discontinuity typically manifested as an echogenic break, indicating a missing segment of muscle or an abrupt thinning. Muscles with isolated areas of slight thinning but no other defects were rated as normal. Fig. 1 depicts the ultrasound plane used to visualize the OO muscle. Fig. 2A shows an ultrasound of a normal OO muscle, Fig. 2B shows an ultrasound of an OO muscle with bilateral discontinuities. The Neiswanger *et al.* (25) definition of OO muscle defects was therefore more conservative than the definition used by Martin *et al.* (24) which included muscles with thinning. See Fig. 1 for examples of images taken from videotaped ultrasound sequences of normal and defective muscles (25).

Of 823 total study subjects, 782 (95%) could be rated as normal or not; the remainder were scored as unknown and not included in the study. In the 525 non-overt-cleft family members [234 male, 291 female (44.6% male)] from CL/P families, 54 (10.3%) were rated with an OO defect [28 male, 26 female (51.8% male)]. Among the 257 controls [94 male, 163 female (36.6% male)], 15 (5.8%) had an OO defect [3 male, 12 female (20.0% male)]. Thus, the proportion of sub-

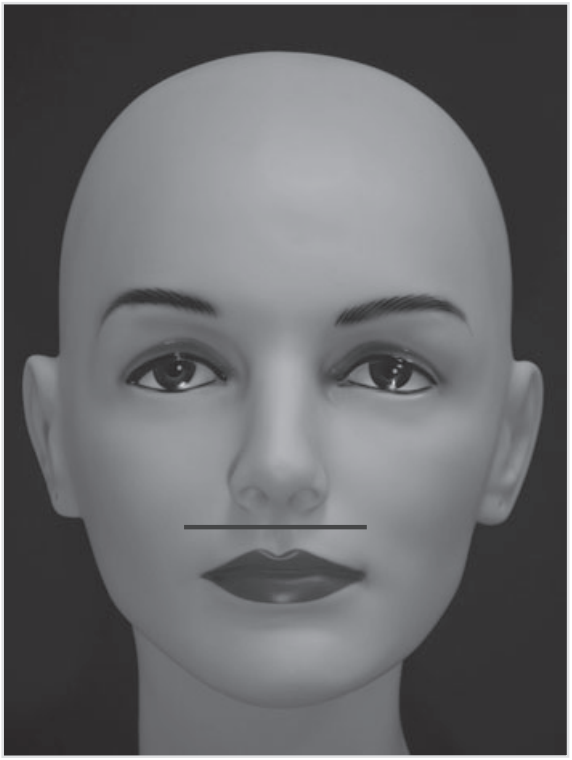

Fig. 1. Orbicularis oris muscles are visualized on high-frequency ultrasound through the plane indicated by the black line.

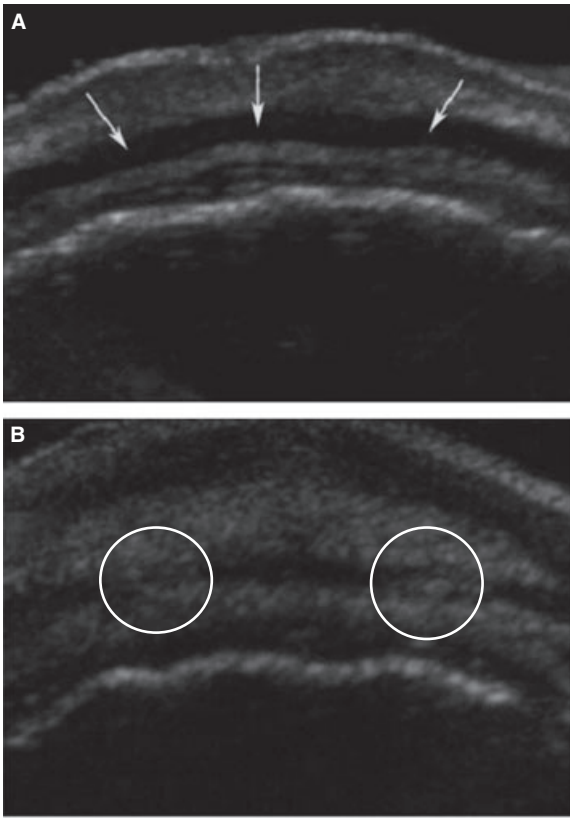

Fig. 2. (A) Ultrasound image of a normal orbicularis oris (OO) muscle, with the muscle indicated with arrows. (B) Ultrasound image of an OO muscle with discontinuities circled. For each image, the surface of the lip is at the top, the bright objects toward the bottom are teeth, and the subject's left is on the right.

epithelial OO defects was significantly increased in non-cleft relatives, compared with controls ($p = 0.04$). This increase was also significant when male relatives [28/234 (12.0%)] were compared with male controls [3/94 (3.2%); $p = 0.01$]. An increase was also seen for female relatives vs. female controls, but was not significant [26/291 (8.9%) relatives vs. 12/163 (7.4%) controls; $p = 0.56$]. Fig. 3 shows two pedigrees of POFC families with overt CL/P and OO defect members. In both cases, inclusion of the OO defect individuals makes the family patterns more consistent with Mendelian transmission of the phenotype.

In summary, the largest OO muscle study to date (25) found that non-overt-cleft relatives in CL/P families were nearly twice as likely to have a subepithelial OO defect as controls with no family history of clefting, confirming the initial results of Martin *et al.* (24) and supporting the hypothesis that OO defects represent the mildest form of cleft lip. Neiswanger *et al.* (25) also observed sex differences in the occurrence of OO defects; this observation requires further confirmation in larger samples but is noteworthy in light of the increased incidence of non-syndromic CL/P in males worldwide (26–31).

OO Muscle development and histology

Consideration of normal OO muscle development can provide insights into the development of OO defects (25). At approximately 7 weeks post-conception in humans the two maxillary prominences fuse with the medial nasal prominence (32) but lip fusion is not complete until the epithelial seams disappear through transformation of the epithelial cells into mesenchymal tissue and/or apoptosis (33). Mesoderm then migrates across the fused prominences. By 8 weeks post-conception a dense, continuous band of mesenchymal cells corresponding to the future OO muscle can be seen, with discernable OO muscle fibers present by 12 weeks (34), and the complete OO muscle architecture by 16 weeks (35). Considering the close temporal relationship of the fusion of the future lip elements necessary to form a continuous OO muscle, it is clearly possible that a slight delay in fusion could result in a subepithelial OO defect, with delayed fusion altering the migration of mesoderm into the

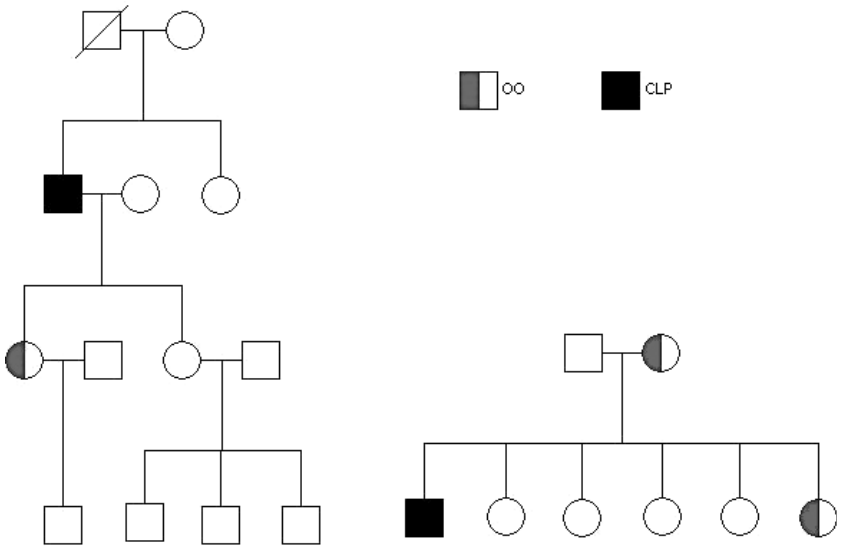

Fig. 3. Two sample families from the Pittsburgh Oral-Facial Cleft study, with individuals with overt clefts indicated in black and individuals with *orbicularis oris* muscle defects indicated in grey.

medial upper lip and resulting in a loss of muscle continuity.

POFC investigators are examining the histology of the OO muscle in order to characterize the defects visualized on ultrasound. The upper lips of 32 cadaver heads, frozen but not otherwise preserved, were visualized on ultrasound and then dissected out for histological sectioning. None of the individuals were affected with CL/P but their family histories were unknown. One of the 32 had an obvious OO discontinuity and two others had other non-normal OO features. Initial sectioning and staining of the OO muscles from the individual with obvious discontinuities and another with a normal-appearing OO muscle has been completed (C. Rogers, T. Smith, S.M. Weinberg, and M.L. Marazita, unpublished data). Initial results indicate that the muscle fibers in the OO sections from the individual with OO defects on ultrasound appear more disorganized than those from the OO-normal individual; analyses are on-going to characterize additional individuals.

The pattern of disorganized muscle fibers is consistent with histological investigations of OO muscle tissues obtained from overt CL/P individuals. Muscle fibers obtained from a stillborn infant with unilateral CL + P (36) and from surgical samples from three incomplete CL cases (37) all showed chaotic and disorganized muscle fiber patterns. As part of a fetal morphology study (35), OO muscles were also examined in 29 human fetuses (nine with CL/P). The CL/P OO muscles showed asymmetrical fiber distribution and abnormal fiber insertion points (35) compared with non-CL/P fetuses, although no significant differences were seen in muscle thickness or volume.

BMP4 and 'healed' cleft lip

As an alternative explanation to the hypothesis that delayed or poorly timed fusion leads to subepithelial OO defects, such defects could represent the remnant of an *in utero* repaired cleft lip. In a BMP4 conditional knock mouse model, Liu *et al.* (38) noted that at 12 days post-conception all BMP4-knockout embryos had bilateral cleft lips, while at 14.5 days post-conception only 22% still demonstrated a cleft lip. They hypothesized that many of the initial cleft lips were therefore 'rescued' or healed *in utero*. An analogous process may occur in humans, with a subepithelial OO defect resulting from a patch of scar tissue in the area of initial discontinuity of a cleft repaired *in utero*.

To investigate this possibility, BMP4 was sequenced in 44 individuals with subepithelial OO defects from the Neiswanger *et al.* (25) study, in 30 individuals with microform clefts, and 529 controls (S. Suzuki, J.C. Murray, and M.L. Marazita, unpublished data). Potentially damaging mutations were found in two individuals with OO defects, one with a microform defect, four with overt CL/P and in none of the controls. The frequency difference between OO defects plus microforms vs. controls was significant (4% vs. 0%, $p = 0.002$), but not between overt clefts and controls (0.5% vs. 0%, $p = 0.12$). Furthermore, each of the OO defect plus

microform case with a BMP4 mutation was a parent of a child with an overt cleft. When BMP4 was sequenced in the children, the same mutation was found, further supporting the hypothesis that OO defects are part of the phenotypic spectrum of CL/P.

Clinical implications

In conclusion, these results provide strong evidence that OO discontinuities are indeed part of the phenotypic spectrum of CL/P. Determination of subepithelial OO defects may eventually become important in a clinical setting, in particular as a way to provide more accurate recurrence risk estimates for families with members affected with CL/P. OO defects are readily screened by ultrasound for relatives with no overt CL/P, and the screening process is non-invasive and not time-consuming. Before full implementation in a clinical setting, additional studies will need to be done to assess the importance of OO defects in a consecutive series of families, including both simplex and multiplex families. The intriguing results to date were obtained chiefly in multiplex families, and it will be necessary to assess a large sample of families to determine accurate recurrence risks in all family situations. Further, the preliminary BMP4 results might lead to a possible genetic test that could be done in non-overt-cleft relatives that are found to have an OO defect on ultrasound, although again additional studies of large numbers of families will be necessary to confirm and refine the clinical applicability. Even with these caveats, ultrasonography of the upper lip shows promise as a way to confirm the existence of occult manifestations CL/P, which will aid in the clinical management of CL/P families and improve the power of genetic studies of non-syndromic CL/P.

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References

1. Wyszynski D. Locating genes for oral clefts in humans. In: Wyszynski D, editor. *Cleft Lip and Palate: From Origin to Treatment*. Oxford: Oxford University Press; 2002. pp. 255–64.
2. Marazita ML. Segregation analyses. In: Wyszynski D, editor. *Cleft Lip and Palate: From Origin to Treatment*. Oxford: Oxford University Press; 2002. pp. 222–33.
3. Schliekelman P, Slatkin M. Multiplex relative risk and estimation of the number of Loci underlying an inherited disease. *Am J Hum Genet* 2002;71:1369–85.
4. Marazita ML, Murray JC, Lidral AC, Arcos-Burgos M, Cooper ME, Goldstein T, et al. Meta-analysis of 13 genome scans reveals multiple cleft lip/palate genes with novel loci on 9q21 and 2q32-35. *Am J Hum Genet* 2004;75:161–73.
5. Zucchero TM, Cooper ME, Maher BS, Daack-Hirsch S, Nepomuceno B, Ribeiro L, et al. Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. *N Engl J Med* 2004;351:769–80.
6. Blanton SH, Cortez A, Stal S, Mulliken JB, Finnell RH, Hecht JT. Variation in IRF6 contributes to nonsyndromic cleft lip and palate. *Am J Med Genet Part A* 2005;137A:259–62.
7. Ghassibé M, Bayet B, Revencu N, Verellen-Dumoulin V, Gillerot Y, Vanwijch R, et al. Interferon regulatory factor-6: a gene predisposing to isolated cleft lip with or without cleft palate in the Belgian population. *Eur J Hum Genet* 2005;13:1239–42.
8. Scapoli L, Palmieri A, Martinelli M. Strong evidence for linkage disequilibrium between polymorphisms at the IRF6 locus and nonsyndromic cleft lip with or without cleft palate, in an Italian population. *Am J Hum Genet* 2005;76:180–3.
9. Srichomthong C, Siriwan P, Shotelersuk V. Significant association between IRF6 820GàA and nonsyndromic cleft lip with or without cleft palate in the Thai population. *J Med Genet* 2005;42:e46.
10. Jezewski PA, Vieira AR, Nishimura C, Ludwig B, Johnson M, O'Brien SE, et al. Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. *J Med Genet* 2003;40:399–407.
11. Vieira AR, Avila JR, Daack-Hirsch S, Dragan E, Felix TM, Rahimov F, et al. Medical sequencing of candidate genes for nonsyndromic cleft lip and palate. *PLoS Genet* 2005;1:e64.
12. Dode C, Hardelin JP. Kallmann syndrome: fibroblast growth factor signaling insufficiency? *J Mol Med* 2004;82:725–34.
13. Bixler D. Phenotypic definition of cleft lip and its importance to genetic studies of the trait. *Am J Med Genet* 1991;41:271–2.
14. Melnick M. Cleft lip (± cleft palate) etiology: a search for solutions. *Am J Med Genet* 1992;42:10–4.
15. Weinberg SM, Neiswanger K, Martin RA, Mooney MP, Kane AA, Wenger SL, et al. The Pittsburgh oral-facial cleft study: expanding the cleft phenotype. Background and justification. *Cleft Palate Craniof J* 2006;43:7–20.
16. Cosman B, Crikelair GF. The minimal cleft lip. *Plast Reconstr Surg* 1966;37:334–40.
17. Carstens MH. The spectrum of minimal clefting: process oriented cleft management in the presence of an intact alveolus. *J Craniof Surg* 2000;11:270–94.
18. Gorlin RJ, Cohen MM, Hennekam RCM. *Syndromes of the Head and Neck*, 4th edn. Oxford: Oxford University Press; 2001. 1283 pp.
19. Eppley BL, van Aalst JA, Robrey A, Havlik RJ, Sadove AM. The spectrum of orofacial clefting. *Plast Reconstr Surg* 2005;115:101e.
20. Lehman JA, Artz JS. The minimal cleft lip. *Plast Reconstr Surg* 1976;58:306–9.
21. Heckler FR, Oesterle LG, Jabaley ME. The minimal cleft lip revisited: clinical and anatomic correlations. *Cleft Palate J* 1979;16:240–7.
22. Martin RA, Jones KL, Benirschke K. Extension of the cleft lip phenotype: the subepithelial cleft. *Am J Med Genet* 1993;47:744–7.
23. Martin RA, Martin KA. Ultrasound imaging as a tool for possible detection of subepithelial clefts in 1st degree relatives of isolated cleft lip patients (Abstract). *J Investig Med* 1995;43 (Suppl.):136A.

24. Martin RA, Hunter V, Neufeld-Kaiser W, Flodman P, Spence MA, Furnas D, et al. Ultrasonographic detection of orbicularis oris defects in first degree relatives of isolated cleft lip patients. *Am J Med Genet* 2000;90:155–61.
25. Neiswanger K, Weinberg SM, Rogers CR, Brandon CR, Cooper ME, Bardi ME, et al. Orbicularis Oris Muscle Discontinuities as an Expanded Phenotypic Feature in Nonsyndromic Cleft Lip with or without Cleft palate, submitted. *Am J Med Genet A* (in press).
26. Fogh-Andersen P. *The Inheritance of Hare Lip and Cleft Palate; Contribution to the Elucidation of the Congenital Clefts of the Face*. Copenhagen: Nyt Nordisk Forlag Arnold Busck; 1942. p. 266.
27. Niswander JD, MacLean CJ, Chung CS, Dronamraju K. Sex ratio and cleft lip with or without cleft palate. *Lancet* 1972;2:858–60.
28. Wyszynski DF, Beaty TH, Maestri NE. Genetics of nonsyndromic oral clefts revisited. *Cleft Palate Craniof J* 1996;33:406–25.
29. Mossey PA, Little J. Epidemiology of oral clefts: an international perspective. In: Wyszynski D, editor. *Cleft Lip and Palate: From Origin to Treatment*. Oxford: Oxford University Press; 2002. pp. 127–44.
30. Blanco-Davila F. Incidence of cleft lip and palate in the northeast of Mexico: a 10-year study. *J Craniof Surg* 2003;14:533–7.
31. Hashmi SS, Waller DK, Langlois P, Canfield M, Hecht JT. Prevalence of nonsyndromic oral clefts in Texas: 1995–1999. *Am J Med Genet Part A* 2005;134A:368–72.
32. Sperber GH. *Craniofacial Development*. Hamilton, Ontario: B.C. Decker; 2001. p. 220.
33. Jiang R, Bush JO, Lidral AC. Development of the upper lip: morphogenetics and molecular mechanisms. *Develop Dynam* 2006;235:1152–6.
34. Marazita ML, Mooney MP. Current concepts in the embryology and genetics of cleft lip and cleft palate. *Clin Plast Surg* 2004;31:125–40.
35. Mooney MP, Siegel MI, Kimes KR, Todhunter J. Development of the orbicularis oris muscle in normal and cleft lip and palate human fetuses using three-dimensional computer reconstruction. *Plast Reconstr Surg* 1988;81:336–45.
36. Kernahan DA, Dado DV, Bauer BS. The anatomy of the orbicularis oris muscle in unilateral cleft lip based on a three-dimensional histologic reconstruction. *Plast Reconstr Surg* 1984;73:875–81.
37. Dado DV, Kernahan DA. Anatomy of the orbicularis oris muscle in incomplete unilateral cleft lip based on histological examination. *Ann Plast Surg* 1985;15:90–98.
38. Liu W, Sun X, Braut A, Mishina Y, Behringer RR, Mina M, et al. Distinct functions for Bmp signaling in lip and palate fusion in mice. *Development* 2005;132:1453–61.