



小鼠腭发育模型中舌体影响腭板抬升模式的表征分析*

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【摘要】目的 研究哺乳动物的腭发育中腭板抬升模式在前后区域差异的决定机制。**方法** 以小鼠腭发育为模型,将抬升前(E13.5早期)、抬升中(E13.5晚期)和抬升后(E14.5早期)的腭板进行冠状向多平面切片,HE染色对比腭和舌的形态变化及空间关联,肌球蛋白重链1(myosin heavy chain 1, MYH1, 存在于慢肌纤维中,负责肌肉的收缩和运动)免疫荧光染色观察腭不同抬升阶段舌肌发育成熟特征;通过腭器官体外培养试验,HE染色观察E13.5早期无舌情况下腭板抬升模式变化;通过胞外基质肌腱蛋白C(Tenascin-C)表达检测(免疫荧光染色),验证舌对前后份腭板抬升模式差异的影响。**结果** 切片HE染色结果显示,在腭抬升期从后到前份,舌冠状向高度下降,侧向倾斜加强且变扁平,但舌矢状向伸长,腭板的抬升模式从缓慢重塑转变为快速翻折,MYH1在此时期大量表达于舌内外肌束。体外腭器官培养发现:无舌条件下,腭板后份抬升未形成新的舌侧凸起,Tenascin-C的区域表达与上抬前一致,腭后份抬升类似于前份抬升模式。**结论** 舌体可能是腭板后份形态重塑抬升模式形成的重要影响因素。

【关键词】 唇腭裂 腭板抬升 胞外基质重塑 舌

Characterization of the Effect of Tongue on Palatal Shelf Elevation Patterns in a Mouse Model of Palatogenesis

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[Abstract] Objective To investigate the mechanisms underlying regional heterogeneity in the elevating patterns of palatal shelf during mammalian craniofacial development. **Methods** Using a mouse model of embryonic palatal development, we acquired coronal multi-plane slices of the palatal shelves before elevation (early E13.5), during elevation (late E13.5), and after elevation (early E14.5). Hematoxylin and eosin (HE) staining was performed to compare the morphological changes and spatial correlations between the palate and tongue. Immunofluorescence staining of myosin heavy chain 1 (MYH1), a marker found in slow muscle fibers and responsible for muscle contraction and movement, was performed to observe the tongue muscle development characteristics at different stages. We also observed changes in the palatal shelf elevating patterns at early E13.5 in the absence of the tongue through HE-stained *in vitro* palate organ culture. Further immunofluorescence staining of tenascin-C, an extracellular matrix protein, was performed to evaluate the effect of the tongue on the elevating pattern of the palatal shelf along the anterior-posterior axis. **Results** HE staining results of the coronal multi-plane slices showed that during the elevation period, from the posterior toward anterior, the coronal height of the tongue decreased, lateral inclination and flattening increased, but the sagittal length of the tongue increased. The elevating pattern of the palatal shelf changed from slow remodeling to rapid flipping, and MYH1 was abundantly expressed in both the internal and external muscle bundles of the tongue during this period. According to findings from *in vitro* cultivation of palatal organs, the posterior part of the palatal shelf elevated without forming new lateral lingual protrusions in the absence of the tongue. The regional expression pattern of tenascin-C was consistent with that observed before elevation. The posterior palate exhibited an elevation pattern similar to that of the anterior region. **Conclusion** The tongue may play a crucial role in shaping the posterior morphological remodeling and distinct elevation patterns of the palatal shelf.

[Key words] Cleft lip and palate Palatal shelf elevation Extracellular matrix remodeling Tongue

哺乳动物腭发育过程中,继发腭发生了剧烈的形态变化,其从垂直转到水平向生长,最终分离口鼻腔。由于

该过程发生迅速,其具体的调控机制仍然难以阐明^[1]。过去数十年,人们针对腭板形态变化驱动的原因提出了几个假设:WALKER和FRASER认为腭板上抬是腭突舌侧壁萌发新突起和原腭突尖端的迅速萎缩协调进行,并描述腭板从后向前以拉链形式融合^[2]。COLEMAN认为腭板前部直接发生90°“翻折”,而后部和中部则以阿米巴样间质重塑的方式完成抬升。具体地,腭间充质内的蛋白多

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糖,如透明质酸,发挥重要的渗透压调节及体积膨胀作用^[3-4]。FERGUSON及CHIQUET则认为腭板抬升过程伴随着显著的细胞骨架方向性收缩,提示腭架上抬存在某种内在驱动力^[2,5]。尽管如此,人们似乎忽略了外部因素对腭抬升的影响。譬如,舌体与腭突需要在口腔局限的空间内精确协调三维位置关系。Pierre-Robin综合征的患者往往因为小下颌畸形导致腭发育中舌体无法充分下降,引起腭突发育空间不足,形成腭裂^[6]。以上现象提示舌在腭抬升中可能产生重要影响,但目前对此缺乏认识。

本研究拟以小鼠为模型,在体内系统追踪腭板抬升时舌与腭突空间互作的基础上,通过体内有舌和体外无舌腭器官培养进行舌腭形态结构对比分析,并辅以观察肌腱蛋白C(Tenascin-C)和肌球蛋白重链1(myosin heavy chain 1, MYH1)的区域表达特征变化,阐述舌对腭板抬升模式的具体影响。本研究结果将有助于研究者从舌体这一外部结构思考腭抬升的影响因素,完善对腭发育机制的认识。

1 材料与方法

1.1 实验小鼠及胚胎组织处理和发育进度划分

本实验所用的8周龄雄性和雌性ICR小鼠购自斯贝福(北京)生物技术有限公司,在SPF实验室饲养。饲养条件:22℃室温,昼夜12h循环,充足食物和纯净水。雌雄小鼠按2:1于晚8点合笼后,次日早8点检查阴栓,查到者记为受孕0.5d,即胚胎期0.5d(E0.5)。小鼠双侧腭突E13.5时期位于舌体两侧,垂直向下延伸,之后协同舌体下降转而水平向生长,在舌背上方水平位接触(E14.5)并融合(E15.5)。为细致观察此过程中腭与舌的空间位置及形态变换特征,本研究根据腭突形态变化进展,进一步将腭发育阶段细化为E13.5晚期(双侧腭突形态不一致,非垂直位也非水平位)以及E14.5早期(双侧腭突形态一致,处于水平位但未接触)。因此,本研究选择在胚胎发育期E12.5、E13.5早期(当日上午8:00)、E13.5晚期(当日晚上8:00)、E14.5早期(次日上午8:00)以及E14.5晚期(次日晚上8:00)处死孕鼠(每个时间点3只,样本量选择根据“3R”原则),剪取胚胎头部。所获样本在PBS中漂洗后,置于体积分数为4%多聚甲醛(Solarbio, P1110)中4℃固定过夜。次日采用乙醇梯度脱水后,完成浸蜡包埋。所有样本均于冠状位石蜡切片,厚度为8μm,用于后续HE染色和免疫荧光检测。本实验所涉及的动物使用严格按照兰州大学实验动物使用指南进行,并经兰州大学口腔医院动物实验伦理委员会批准(审批号:LZUKQ-2021-041)。

1.2 腭器官体外培养

腭器官体外培养方法参照本团队既往流程^[7]。具体步骤:在无菌条件下剪取E13.5早期的小鼠胚胎头部,在体视显微镜下使用显微操作器械从胚鼠口角剪至耳后,双侧剪开后分离下颌及舌体,暴露双侧腭突。进一步在眼球位置完成水平向切割,分离颅面。最后在腭后份缘做切口离断多余组织。所得上颌及腭突组织为一体,置于上衬微孔滤膜的金属网状支架上,整体转移至6孔板中培养。6孔板内添加DMEMF12培养基(Gibco™, 11320033),内含10%FBS血清(Gibco™, A5256701),以及1%青霉素及链霉素(Beyotime, C0222)。培养期间维持组织处于气液交界面,每24h换液一次。分别在37℃和体积分数为5%CO₂的培养箱中培养24h及48h后收样。每组样本设置3个重复。所获腭器官置于体积分数为4%多聚甲醛中4℃固定过夜。次日采用乙醇梯度脱水后,完成浸蜡包埋,以7μm厚度完成石蜡切片,进行后续HE和免疫荧光染色。

1.3 HE染色

样本石蜡切片在60℃条件下烤30min后进行脱蜡至水:先将切片置于二甲苯中10min×2次;然后依次置于100%、95%、85%和75%乙醇,每级放置5min,再用蒸馏水浸洗5min。染色:苏木素染色1min,盐酸乙醇分化3s,自来水返蓝30s,伊红染色5min。脱水、透明后,中性树脂封片待其自然晾干,置于显微镜下拍照观察。

1.4 免疫荧光染色

通过Tenascin-C标记染色观察腭突内部细胞外基质的分布,通过MYH-1标记染色观察舌体内部肌纤维的发育及分布。样本石蜡切片脱蜡复水之后进行热修复抗原:将切片浸入柠檬酸盐缓冲液(pH=6.0),微波炉高火加热20min后拿出,冷却至室温;冷却后PBS(pH7.2~7.6)洗涤5min×2次。血清封闭:切片滴加封闭液放入湿盒中,室温封闭60min。孵育一抗:滴加一抗(Tenascin-C antibody, 1:200, HUABIO ET1608-50; MYH-1 antibody, 1:100, proteintech 25182-AP), 4℃冰箱孵育过夜;次日PBS浸洗5min×4次后滴加荧光二抗[Multi-rAb™ CoraLite® Plus 488-Goat Anti-Rabbit Recombinant Secondary Antibody (H+L), 1:500, proteintech, Cat No. RGAR002],室温孵育1h, PBS浸洗3次。滴加DAPI(Beyotime, C1002)避光孵育5min,对标本进行染核, PBS浸洗4次洗去多余DAPI;防荧光淬灭封片剂封片、正置生物荧光显微镜(奥林巴斯-BX53)观察。

1.5 统计学方法

荧光强度比较分析实验重复3次,两组间的表达强度

差异比较使用单因素方差分析(One-Way ANOVA), $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 腭上抬过程中腭与舌的空间位置及形态变换

根据E13.5早期、E13.5晚期、E14.5早期以及E14.5晚期收集胚胎样本进行头颅冠状位切片。根据腭突前-后轴, 选择软腭、硬腭后份、硬腭中份及硬腭前份的切片进行HE染色, 据此对比分析腭突与舌如何完成空间和形态上的协调。结果显示(图1): 在腭发育的更早时期(E12.5), 舌由后向前几乎占据整个口腔, 舌截面呈圆角矩形, 舌背与颅底之间仅存微小缝隙, 而腭突垂直处于舌体两侧, 圆钝而短小。在E13.5早期, 舌背与颅底之间间隙明显增大, 舌体截面也从软腭后份的正梯形过渡至硬腭中后份的圆角矩形, 再至硬腭前份的倒梯形; 而腭突进一步垂直向伸长, 但腭突后份依旧圆钝, 前份腭突变尖且向

舌腹下方延伸。之后在E13.5晚期, 舌背与颅底之间间隙进一步加大, 舌体截面从后向前逐渐脱离颞舌肌的附着, 舌体也协同腭突上抬出现同侧倾斜(图1中绿色箭头指示), 由后向前倾斜幅度加大; 双侧腭突在此时期上抬进程明显不一致, 在软腭与硬腭后份位置处, 原腭突尖端收缩, 而腭突舌侧壁膨隆(图1中蓝色箭头指示), 硬腭中份及前份的腭突上抬更快, 并未出现如后份般形变过程。在E14.5早期, 舌体截面较之前发育时期变得扁平, 居中无偏斜; 双侧腭突形态对称, 均处于舌背上方, 水平相对但未接触。在E14.5晚期, 舌体截面从后向前较E14.5早期又变立体, 但与腭突远离; 双侧腭突水平接触, 开始融合形成腭中嵴上皮缝。由此可见, 舌体形态在腭架抬升中发生着下降、倾斜和扁平化行为, 综合协同并为腭突提供充足的上抬空间。腭突后份上抬采用缓慢的形变收缩(阿米巴样运动)方式, 而腭突前份上抬采用快速的翻折方式。

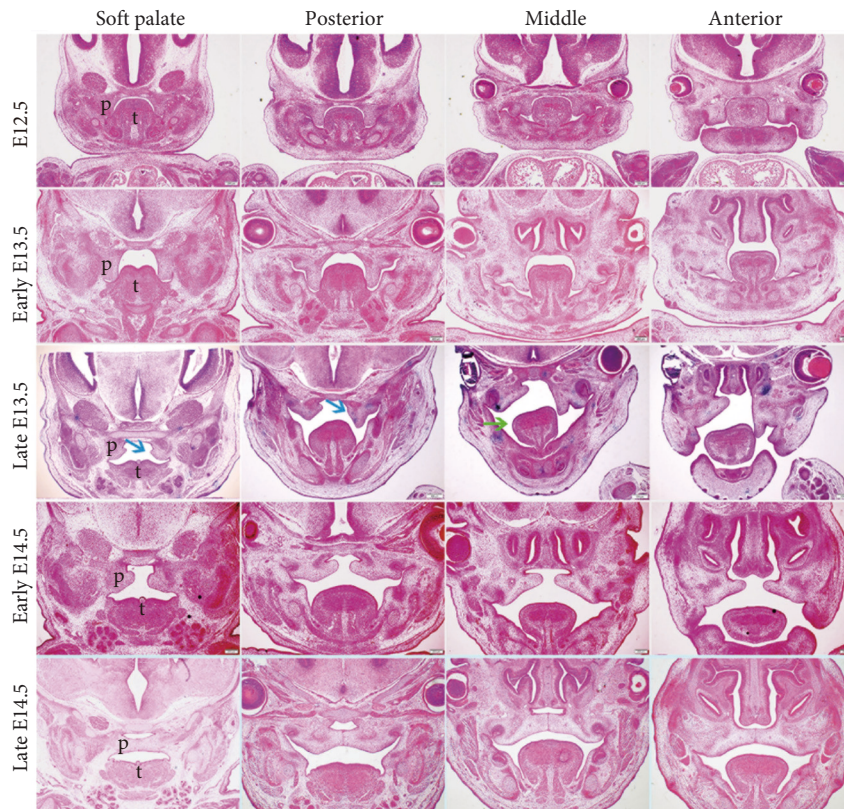


图 1 腭发育不同时期小鼠胚胎头部冠状位切片 (苏木精-伊红染色 $\times 40$)

Fig 1 Hematoxylin and eosin staining of coronal slices of mouse embryonic heads during different stages of palatal development (original magnification $\times 40$)

E12.5: early palate development; early E13.5: pre-elevation; late E13.5: mid-elevation; early E14.5: post-elevation; late E14.5: contact stage; p: palatal shelf; t: tongue. The green arrow indicates the inclined tongue, and the blue arrow indicates the newly formed protrusion on the lingual wall of the palatal shelf.

2.2 舌肌的同期分化成熟协调腭上抬

本研究检测了舌体内快速性骨骼肌MYH1的成熟度

及分布, 结果显示(图2): 在腭发育的早期(E12.5), 舌体内肌纤维开始出现, 但不成熟, 舌横肌与颞舌肌发育早于

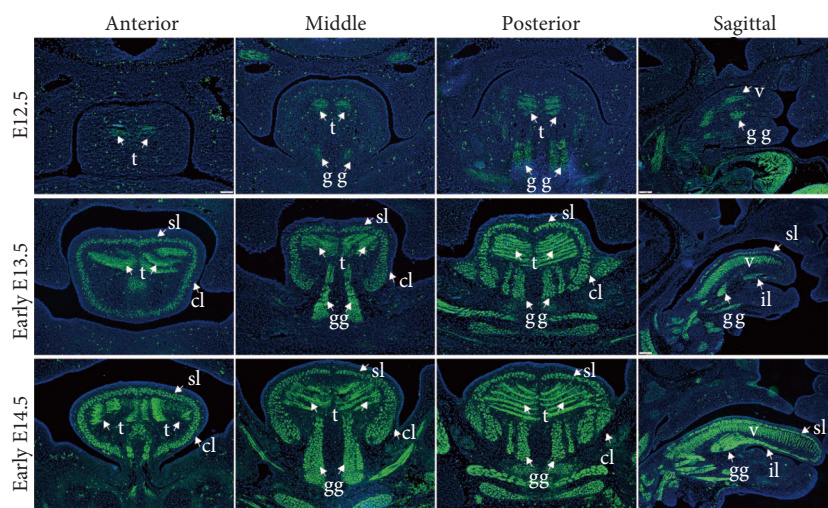


图 2 腭发育不同时期小鼠胚胎头部冠状位和正中矢状位切片MYH1免疫荧光染色。×100

Fig 2 MYH1 immunofluorescence staining of coronal and mid-sagittal sections of mouse embryonic heads at different stages of palatal development (original magnification × 100)

cl: combined longitudinal muscle; sl: superior longitudinal muscle; il: inferior longitudinal muscle; t: transverse muscle; v: vertical muscle; gg: genioglossus muscle; the other abbreviations are explained in the note to Fig 1.

其他肌群;而在腭架抬升前后(E13.5晚期~E14.5早期),舌垂直肌与舌横肌和舌上、下纵肌纤维相互垂直交错,更加密集和成熟,这使舌体变薄变宽。从矢状向观察:腭架抬升前后(E13.5晚期~E14.5早期),舌肌纤维分布开始贯穿整个舌体,舌体前后向长度明显变长,突出口腔,舌背进一步下降。这些现象提示:腭上抬的顺利进行需配合舌体内外肌群的分化成熟协同进行,以此通过舌体形变和位移为腭上抬提供空间。

2.3 无舌模式下腭抬升模式发生改变

腭抬升时需舌体通过一系列形态变化让出空间,这提示舌体可能对腭上抬产生阻挡干扰的作用。为此,本研究剪取E13.5早期的胚鼠头部,通过腭器官体外培养,

以便在去除舌体干扰情况下分析腭上抬的特征。结果显示(图3):无舌条件下的腭突在培养24 h后,前份已完成上抬,中份处于上抬中,而后份仍处于垂直位却向中线倾斜靠拢;继续培养48 h后,腭突前中份均完成上抬,后份处于上抬中,但并未出现原尖端皱缩,新凸起在舌侧壁膨隆的情况。以上现象提示:后份的腭突在无舌条件下,其抬升模式从间充质重塑可能转为类似于前份的翻转模式。

2.4 细胞外基质蛋白Tenascin-C的表达模式证实舌体是前份腭架抬升模式差异形成的影响因素

后份腭架抬升是以间质重塑的模式进行的,其间伴随着细胞外基质蛋白的重新分布,其中以肌腱蛋白Tenascin-C最具代表性。本实验免疫荧光染色发现

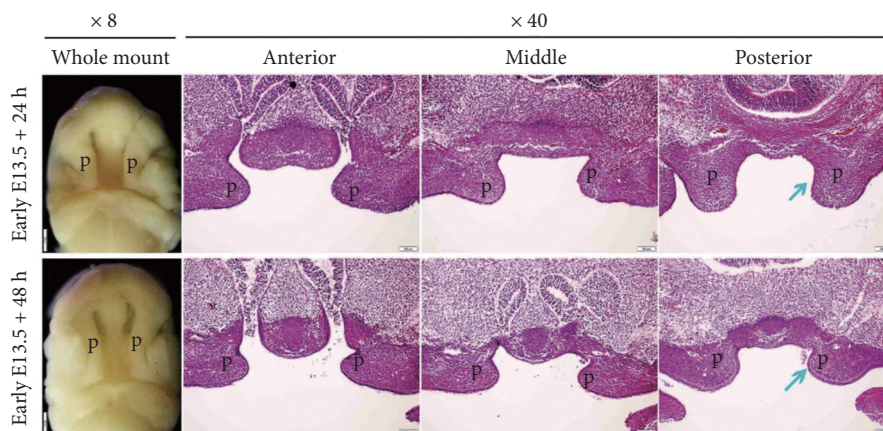


图 3 无舌腭器官培养

Fig 3 The palatal organ culture without tongue

The abbreviations are explained in the note to Fig 1. After 24-h and 48-h culturing of early E13.5 palatal organs, whole embryo stereoscopy and section hematoxylin eosin staining were used to observe the morphology. Blue arrows point to the deformed sites on palatal shelf.

(图4): 在E13.5早期, Tenascin-C在腭突的后份舌侧较颊侧间充质表达丰富; 而在前份颊侧沟区(腭突颊侧的上皮弯折)的表达则表现为颊侧显著高于舌侧, 且前份明显高于后份。进一步, 本研究将体外无舌腭突(E13.5早期+48 h)和体内有舌腭突(E14.5早期)上抬完成后, Tenascin-C表达区域分布进行比较发现: 腭突前份在无舌条件下

(E13.5早期+48 h), Tenascin-C的分布颊侧区高于舌侧区, 与体内有舌条件一致(E14.5早期); 而腭突后份在无舌条件下(E13.5早期+48 h), Tenascin-C的分布舌侧区高于颊侧区, 与体内有舌条件(E14.5早期)分布不一致。以上结果提示: 后份腭突在无舌条件下间质重塑行为为减弱, 上抬模式改变。

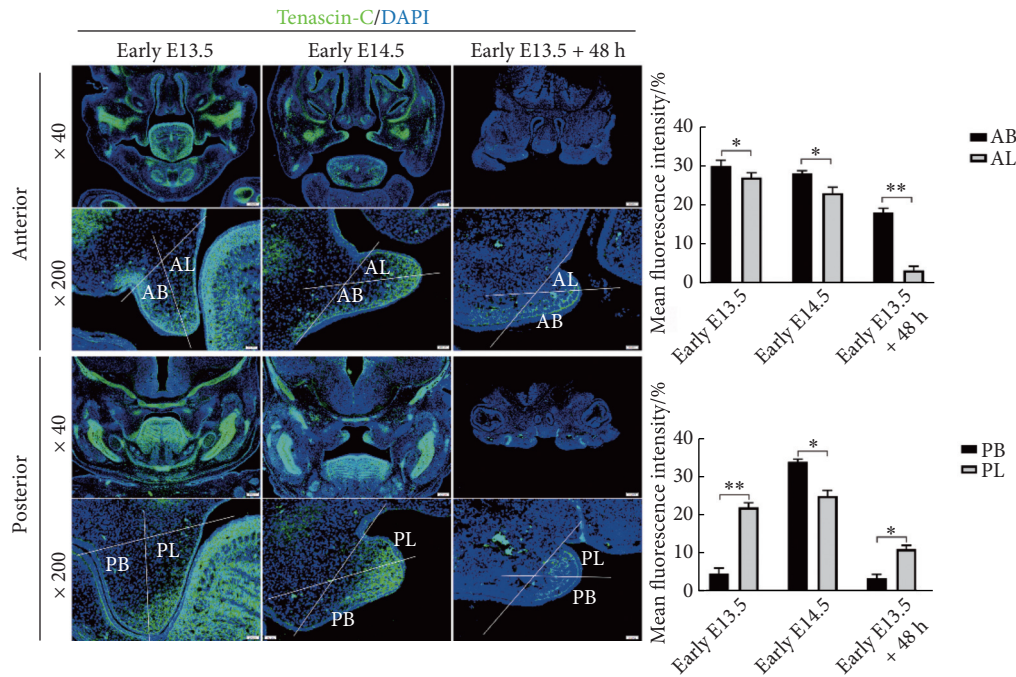


图 4 免疫荧光染色检测Tenascin-C在体内和体外腭突抬升前后的表达模式

Fig 4 Immunofluorescence staining showed the Tenascin-C expression patterns before and after palatal protrusion elevation *in vivo* and *in vitro*

AB: anterior buccal side; AL: anterior lingual side; PB: posterior buccal side; PL: posterior lingual side. The other abbreviations are explained in the note to Fig 1. The palatal shelves were divided into buccal and lingual halves in anterior (AB & AL) and posterior (PB & PL) regions. Quantification of mean fluorescence intensity of Tenascin-C were conducted three times to get the average values. * $P < 0.05$, ** $P < 0.01$. $n = 3$.

3 讨论

腭上抬过程迅速, 通常在短短十几小时内发生剧烈的形态变化^[8]。过去数十年关于腭抬升的控制机制未获得统一认识。目前学者们大都认为: 哺乳动物腭突上抬在前后份是存在差异的, 以磨牙牙胚为界, 前份腭突是以从垂直向快速翻转至水平向的模式实现上抬, 而后份腭突则以缓慢间质重塑形式完成上抬^[2,3,9-10]。后份腭突上抬时需要原腭突尖端收缩, 同时在舌侧壁上形成新凸起。后份腭突这种阿米巴样运动方式可能通过腭突内部自身因素控制^[5]。譬如, 透明质酸和糖胺聚糖等胞外基质蛋白伴随着腭上抬大量合成, 腭间充质因内部渗透压提升而吸水膨胀^[11]; 腭上抬时也可明显观察到细胞骨架沿间质重塑方向规律性重排, 可能通过骨架收缩为腭上抬提供驱动力^[5,12-13]。然而, 近年来发现以上观点并不能合理解

释腭突上抬在前后份存在差异的原因。LAN等^[4]和CHEN等^[14]分别用*Hand2-Cre*(表达于下颌)和*Osr2-Cre*(表达于上颌)敲除胚鼠*Has2*(透明质酸合成酶2)后对比发现, 尽管*Hand2-Cre; Has2^{ff}*与*Osr2-Cre; Has2^{ff}*小鼠均发生腭裂, 但腭上抬失败现象仅发生于*Hand2-Cre; Has2^{ff}*小鼠, 舌形变及下降明显不足, 这提示舌体这一外部因素在协调腭上抬中产生重要影响。

在腭上抬中, 舌与腭发生密切的形态协调。伴随着腭板抬升过程, 舌体内部及外部肌纤维的分化成熟及分布特征如何, 研究者仍对此缺乏清晰的认识。通过腭上抬时间点的精细划分, 本研究观察到舌体横截面形态在不同时间点存在明显异常, 这种差异也存在于腭前后份。伴随腭上抬, 舌体变得扁平, 舌背高度下降, 舌前份还发生倾斜, 而舌长度变长, 吐出口腔; 腭上抬完成后, 舌体又恢复立体且居中的位置。舌体内部及外部存在着丰

富的肌纤维,包含内部的联合纵肌(combined longitudinal muscle, cl)、舌上纵肌(superior longitudinal muscle, sl)、舌下纵肌(inferior longitudinal muscle, il)、舌横肌(transverse muscle, t)、舌垂直肌(vertical muscle, v),以及外部的颞舌肌(genioglossus muscle, gg)与颞舌骨肌(geniohyoid),这些肌束对舌形态维持起到重要作用^[15-17]。舌外肌群包括颞舌肌、舌骨舌肌、茎突舌肌和腭舌肌,其中颞舌肌呈扇形分布于舌大部分区域,起点是下颌骨的颞棘,终点是舌骨和舌背,两侧颞舌肌同时收缩,使舌伸向前下,单侧收缩可使舌尖伸向对侧^[18-19]。舌内肌群包括舌上纵肌、舌下纵肌、舌横肌、舌垂直肌,这些肌束在三维方向上互相垂直交织组合,改变舌形态。舌肌属于横纹肌,其中的MYH1又称横纹肌肌球蛋白重链1,是一种由Myh1基因编码的蛋白质,可通过ATP的水解将化学能转化为机械能。根据既往研究,MYH1特异性表达于舌体内外分化成熟的肌纤维内,其表达量及空间分布特征常用来追踪舌肌发育进程^[6,15]。本研究发现MYH1的表达量与舌体形变以及腭上抬密切相关,且舌快速且剧烈的形态变化属口腔空间有限、舌体为腭突挪让空间的结果,而舌内外肌肉协同发育成熟也为其顺利形变提供有力辅助。Pierre Robin综合征是一组病因复杂、临床表现多样的先天性发育畸形,其特征为下颌骨发育不全(小颌畸形或颌后缩)、舌后坠及其所致的上气道梗阻,伴发腭裂是因口腔狭窄,限制并迫使舌向上发育造成腭上抬空间不足^[6,20-21]。本团队既往研究也发现:腭突的最后份和最前份区域,因舌体微小、空间充足,腭突在早期即呈现水平向生长,无需上抬过程^[8]。Hand2-Cre; SmoC/C表现为无舌畸形而腭发育正常,在无舌体阻碍情况下,双侧后份腭突相互靠拢,并未出现明显的间质重塑^[22]。通过腭器官培养,本研究同样发现:在无舌体阻碍情况下,后份腭突并未出现如体内般间质重塑,腭突将以更直接、类似于前份腭突翻转形式快速上抬,这显示出舌体阻碍在决定腭抬升中的重要作用。

腭后份上抬过程中所发生的细胞外基质重塑可被一些标记基因追踪,如Tenascin-C、POSTN以及Coll1a1^[5,23-24]。Tenascin-C是一种多模块细胞外基质糖蛋白,具有多种分子形式(180~250 kDa)^[24]。在组织器官发育中,Tenascin-C能与纤维连接蛋白、胶原蛋白、骨膜蛋白、蛋白聚糖结合,维持组织强度并促进多种转录因子表达。大量研究表明组织受机械压力刺激时Tenascin-C表达会明显上调,且肌纤维坏死在再生过程中可释放Tenascin-C促进肌肉干细胞增殖。本研究观察到舌肌纤维的增生和分化成熟(MYH1为标记)均伴随着Tenascin-C的丰富表达,这提示舌体内部的Tenascin-C可能在感知来自腭突的机械力,促

进舌肌快速发育,甚至在协调舌肌收缩功能中发挥作用。在腭突后份重塑过程中,Tenascin-C表现为从原始尖端到新突起的方向性重排,这反映了腭突内部收缩力和腭突阿米巴样运动的方向。本研究观察到无论是体内还是体外,腭突前份区域的Tenascin-C在抬升前后颊侧区的分布均比舌侧区更丰富,这再次印证了腭突前份翻转上抬模式。然而,腭突后份区的Tenascin-C在上抬前舌侧区的分布较颊侧区丰富,但因有舌条件下内部间充质重塑,Tenascin-C在上抬后出现颊侧区多于舌侧区的现象;而这种现象在去除舌体培养的腭器官内并未出现,上抬后舌侧区Tenascin-C的表达依然多于颊侧区,这进一步证明了舌体在后份间质重塑抬升模式形成中的决定性作用。

然而需注意的是,体外腭器官培养条件始终有别于体内,在未来可进一步采用基因打靶构建的小鼠无舌发育模型为舌体腭板抬升模式提供更充足的证据。

* * *

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