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在线全文

Piezo1介导基底硬度调控软骨细胞初级纤毛形态*

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【摘要】 **目的** 研究基底硬度如何调控软骨细胞初级纤毛的形态，并进一步探究Piezo1如何介导基底硬度调控初级纤毛的形态。**方法** 将聚二甲基硅氧烷（polydimethylsiloxane, PDMS）固化剂和主剂分别以1∶10（硬）、1∶50（中硬度）和1∶70（软）的比例混合，制成厚度为1 mm且具有不同硬度的基底膜：硬基底（2.21±0.12）MPa、中硬度基底（54.47±6.06）kPa和软基底（2.13±0.10）kPa。利用3种不同硬度基底培养软骨细胞；再分别用Tubastatin A（Tub A）抑制组蛋白脱乙酰酶6（histone deacetylase 6, HDAC6）、Piezo1激活剂Yoda1和抑制剂GsMTx4处理细胞，基于免疫荧光技术分析HDAC6、Yoda1和GsMTx4对软骨细胞形态和初级纤毛长度的影响。**结果** 硬基底会增加软骨细胞铺展面积，免疫荧光显示与中等基底和软基底相比在硬基底上细胞骨架和细胞核面积显著增加（$P<0.05$），并且初级纤毛显著伸长（$P<0.05$），但是并不影响初级纤毛的出现率。软骨细胞HDAC6的活性随着基底硬度变软依次升高，抑制软骨细胞HDAC6的活性后，细胞骨架面积和细胞核面积以及初级纤毛长度在硬基底上增加更为显著（$P<0.05$）。经测试，加入Piezo1激活剂和抑制剂可以调控软骨细胞HDAC6的活性，并且初级纤毛的长度在加入激活剂Yoda1后显著增加（$P<0.05$），而在经抑制剂GsMTx4处理后，初级纤毛长度在硬基底上显著缩短（$P<0.05$）。**结论** 培养皿基底硬度和Piezo1可能都通过调控HDAC6活性进而影响软骨细胞初级纤毛形态。

【关键词】 软骨细胞 基底硬度 初级纤毛 细胞骨架 HDAC6 Piezo1

Piezo1 Mediates the Regulation of Substrate Stiffness on Primary Cilia in Chondrocytes

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【**Abstract**】 **Objective** To investigate how substrate stiffness regulates the morphology of primary cilia in chondrocytes and to illustrate how Piezo1 mediates the morphology regulation of primary cilia by substrate stiffness. **Methods** Polydimethylsiloxane (PDMS) curing agent and the main agent (Dow Corning, Beijing, China) were mixed at the ratio of 1∶10 (stiff), 1∶50 (medium stiffness), and 1∶70 (soft), respectively, to prepare substrate films with the thickness of 1 mm at different levels of stiffness, including stiff substrate of (2.21±0.12) MPa, medium-stiffness substrate of (54.47±6.06) kPa, and soft substrate of (2.13±0.10) kPa. Chondrocytes were cultured with the substrates of three different levels of stiffness. Then, the cells were treated with Tubastatin A (Tub A) to inhibit histone deacetylase 6 (HDAC6), Piezo1 activator Yoda1, and inhibitor GsMTx4, respectively. The effects of HDAC6, Yoda1, and GsMTx4 on chondrocyte morphology and the length of primary cilia were analyzed through immunofluorescence staining. **Results** The stiff substrate increased the spread area of the chondrocytes. Immunofluorescence assays showed that the cytoskeleton and the nuclear area of the cells on the stiff substrate were significantly increased ($P<0.05$) and the primary cilia were significantly extended ($P<0.05$) compared with those on the medium-stiffness and soft substrates. However, the presence rate of primary cilia was not affected. The HDAC6 activity of chondrocytes increased with the decrease in substrate stiffness. When the activity of HDAC6 was inhibited, the cytoskeletal area, the nuclei area, and the primary cilium length were increased more significantly on the stiff substrate ($P<0.05$). Further testing showed that Piezo1 activator and inhibitor could regulate the activity of HDAC6 in chondrocytes, and that the length of primary cilia was significantly increased after treatment with the activator Yoda1 ($P<0.05$). On the other hand, the length of primary cilia was significantly shortened on the stiff substrate after treatment with the inhibitor GsMTx4 ($P<0.05$). **Conclusion** Both substrate stiffness and Piezo1 may affect the morphology of chondrocyte primary cilia by regulating HDAC6 activity.

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【Key words】 Chondrocyte Substrate stiffness Primary cilia Cytoskeleton HDAC6 Piezo1

膝关节骨性关节炎(osteoarthritis, OA)是一种常见软骨退行性疾病,通常会导致中老年人生活质量下降、医疗费用增加和致残率升高[1]。关节软骨基质力学微环境异常是引起炎症和OA退行性病变的重要原因之一[2]。硬度是细胞基质微环境的重要物理因素之一,它能够调控细胞增殖和分化[3]。譬如,硬基质促进间充质干细胞分化为成骨细胞[4]。软骨细胞是关节软骨唯一细胞类型,其周围包裹着以Ⅵ型胶原为主、厚度约2 ~ 4 μm的细胞周基质(pericellular matrix, PCM)[2]。PCM的力学特性、三维拓扑和动态应变等构成了软骨细胞力学微环境。随着关节软骨生长、老化以及OA发生,软骨细胞PCM硬度在1 ~ 205 kPa之间发生变化[3]。在OA发展过程中的PCM硬度大约会降低30% ~ 50%[2]。此外,关节软骨基质硬度在很大程度上决定着软骨细胞代谢稳态、炎症响应和软骨退变[5~7]。然而,软骨细胞感知和响应基质硬度变化的分子机制尚不清楚。

软骨细胞通过细胞机械力感受器感知基质力学微环境,这些机械力感受器主要包括整合素、细胞骨架成分、力敏感离子通道和初级纤毛[8]。近几年,初级纤毛在软骨细胞力转导和炎症信号过程中所发挥的作用备受关注[9]。软骨各层应力、应变和流体剪切力变化可引起初级纤毛出现率和长度随着远离关节表面而增加[10]。初级纤毛的缺失会导致关节软骨结构和力学特性显著改变[11],而纤毛功能破坏会导致承重软骨发生异常[12-13]。化学和机械力刺激能够调整初级纤毛形成长度,进而影响细胞状态[14]。此外,初级纤毛结构中包含OA重要信号通路的生长因子受体,如转化生长因子β(transforming growth factor β, TGFβ)家族、Wnt和Hedgehog。OA中Hedgehog激活导致肥大标志物X型胶原异常表达和基质金属蛋白酶(matrix metalloproteinases, MMPs)上调[15]。此外,OA恶化增加软骨中纤毛出现率和长度[16]。缺失初级纤毛的软骨细胞无法启动细胞内钙信号、ECM合成和ATP释放以响应压缩刺激[17~19]。近期研究发现,初级纤毛内组蛋白脱乙酰酶(histone deacetylase 6, HDAC6)在纤毛内富集,并通过纤毛微管蛋白去乙酰化和聚合调控纤毛再吸收[20~22]。用Tubastatin A(Tub A)抑制HDAC6能有效防止循环拉伸高应变刺激下初级纤毛的解体[20],而且还可以减少实验性OA软骨损伤水平[23-24]。因此,HDAC6活性是调控初级纤毛出现率和形态的关键因素。

初级纤毛通过TRP家族的各种Ca^{2+}通道感知机械载荷、信号分析和细胞外渗透压变化[25]。近几年,力敏感离子通道TRPV4(transient receptor potential vanilloid channel 4)和Piezo1在软骨细胞感知和响应基质力学微环境过程中所发挥的力转导作用备受瞩目[9,26-27]。近期研究表明,机械力激活TRPV4,其功能性上调HDAC6表达和初级纤毛长度,最终削弱IL-1β(interleukin 1β)介导的炎症信号[9]。然而,基质硬度的变化能够影响初级纤毛与中心粒的定位[28]。本课题组前期研究发现,基质硬度可通过调控HDAC6活性影响软骨细胞初级纤毛的形态[29]。Piezo1的激活也能够影响初级纤毛的形成[30]。此外,本课题组早期研究证实Piezo1在软骨细胞感受基质硬度中发挥重要力转导作用[31],而且在介导软骨细胞炎症响应的信号通路中也发挥重要作用[32]。因此,本研究主要观察Piezo1是否通过介导HDAC6活性调控基底硬度对软骨细胞初级纤毛形态的影响,以助于进一步深入认识细胞感受和响应基质力学微环境的机制,为组织工程优化诱导性生物材料提供了新的思路。

1 材料与方法

1.1 PDMS基质制备

将PDMS固化剂和主剂(Dow Corning,中国北京)分别以1∶10(硬)、1∶50(中硬度)和1∶70(软)的比例混合,然后将PDMS混合液在台式离心机中离心以除去气泡,倒入35 mm培养皿或共聚焦皿中,置于70 ℃下固化6 h,形成厚度为1 mm且具有不同硬度的基底膜:硬基底(2.21±0.12) MPa、中硬度基底(54.47±6.06) kPa和软基底(2.13±0.10) kPa。在接种细胞之前,利用离子溅射仪(SBC-12;深圳市科扬科技有限公司)对PDMS基底进行表面亲水性处理。向PDMS基底中加入0.036 mg/mL的Ⅰ型胶原稀释液(杭州欣友生物技术有限公司),在4 ℃条件下静置24 h后,用DMEM清洗并在超净台用紫外线照射40 min,以备接种细胞。接种细胞24 h后开始进行实验。

1.2 原代软骨细胞培养

本实验程序已经太原理工大学动物伦理委员会批准,动物实验按照国际动物福利指南和标准进行。将5 ~ 6日龄C57BL/6小鼠浸没在75%酒精中至麻醉状态并处死,用剪刀镊子剥离出髋关节和膝关节处的透明关节软骨,并立即放入含1%青霉素/链霉素的DMEM培养基中。将软骨块置于3 mg/mL胶原酶D消化溶液中,并在细胞培养箱(37 ℃,体积分数5%CO_2)中孵育45 min[33]。更

换新的胶原酶D消化溶液再次孵育45 min。然后，将软骨块置于含有0.5 mg/mL胶原酶D溶液的新培养皿中，过夜消化。最后，通过40 μm细胞过滤器过滤分离细胞，将细胞重悬于含10%胎牛血清和1%青霉素/链霉素的DMEM中，并接种于不同硬度的基底上，在37 ℃和体积分数5%CO_2条件下培养。

1.3　激活剂和抑制剂使用方法

使用浓度为10 μmol/L的HDAC6高效选择性抑制剂Tub A(HY-13271A; MedChemExpress, 美国)，以及浓度为5 μmol/L的Piezo1激活剂Yoda1(Tocris, 英国)和浓度为1 μmol/L的Piezo1抑制剂GsMTx4(Abcam, 美国)分别处理细胞。Tub A处理: 利用相对分子质量335.40计算细胞浓度，用DMSO将细胞浓度稀释至10 mmol/L。处理前，将10 mmol/L的Tub A用细胞完全培养基稀释至10 μmol/L。Yoda1处理: 利用相对分子质量355.27计算细胞浓度，用DMSO将细胞浓度稀释至20 mmol/L。处理前，将20 mmol/L的Yoda1用细胞完全培养基稀释至5 μmol/L。GsMTx4处理: 利用相对分子质量4 095.85计算细胞浓度，用ddH_2O稀释细胞浓度至1 mmol/L，处理前，将1 mmol/L的GsMTx4用细胞完全培养基稀释至1 μmol/L。根据需要使用HDAC6荧光测定试剂盒(ab284549; Abcam, 英国)测量HDAC6活性。

1.4　细胞免疫荧光染色和主要试剂

将细胞置于2%胎牛血清的培养基中培养12 h。软骨细胞用体积分数4%多聚甲醛固定20 min，用0.5% Triton X-100/PBS通透5 min，并用5%BSA封闭1 h。使用溶于1%BSA的抗乙酰化α-tubulin(1∶2 000, T7451, Sigma Aldrich, 英国)和Arl13b(1∶2 000, 17711-1-AP, Proteintech, 英国)免疫标记初级纤毛，在4 ℃下孵育过夜。然后，再用适当的Alexa Fluor 488和Alexa Fluor 647(CWBIO, 江苏)在室温避光孵育1 h。孵育完成后将样品洗涤5次。然后，加入Alexa Fluor 488鬼笔环肽(Thermo Scientific, 美国)在室温下避光孵育20 min。将样品洗涤5次并用DAPI复染。最后，用共聚焦显微镜(Leica, 德国)进行成像。Image J软件对免疫荧光结果进行量化。

1.5　细胞初级纤毛长度与出现率测量

在DAPI通道上，通过增加Image J的ROI功能，选择图片内的细胞核，并对每个细胞核进行编号。在乙酰化α-tubulin或Arl13 b和DAPI合并通道上计数总细胞数和纤毛细胞数以及初级纤毛长度。重复步骤，计数80个左右细胞。初级纤毛出现率= 总纤毛细胞数/总细胞数。

1.6　统计学方法

数据表示为$\bar{x} \pm s$。使用单因素方差分析进行组间比较。当涉及两个变量时，使用双因素方差分析，并采用 Tukey 多重比较进行验证，$P < 0.05$为差异有统计学意义。

2　结果

2.1　基底硬度对软骨细胞形态和初级纤毛的影响

免疫荧光染色结果(图1)显示，培养24 h后3种基底中

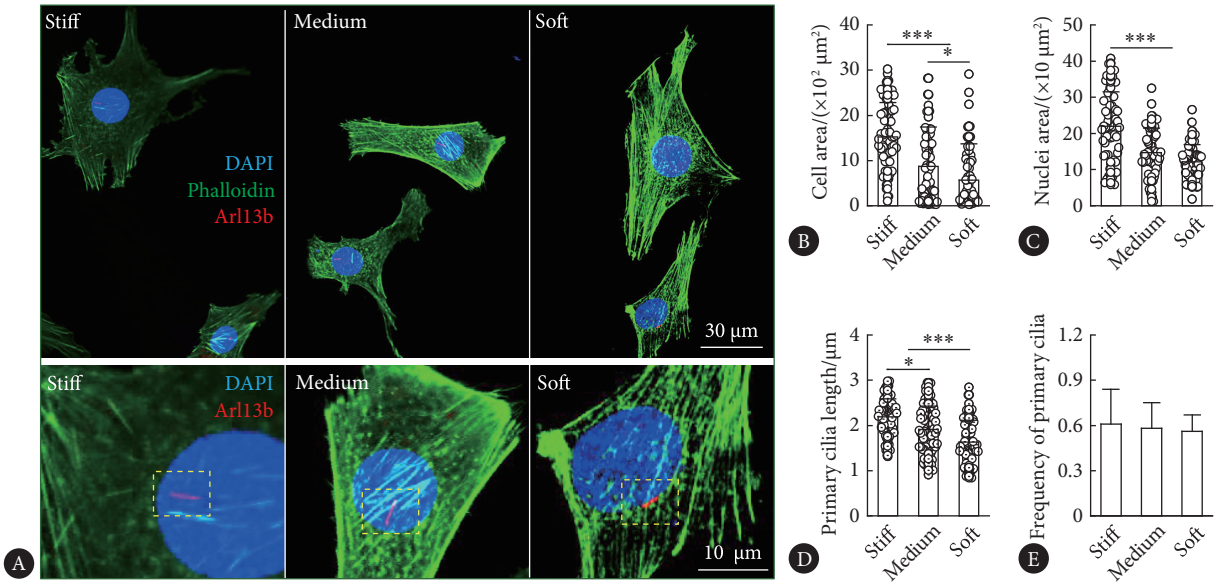

图 1　基底硬度对软骨细胞形态和初级纤毛的影响.

Fig 1　The effect of substrate stiffness on the morphology and the primary cilia of chondrocytes

A, Staining of cytoskeleton, nucleus, and primary cilia on substrates with varying stiffness. B, Cell area analysis. C, Nuclear area analysis. D, Analysis results of primary cilia length in cells. E, Frequency of presence of primary cilia in chondrocyte. n=60. $^{*}P$<0.05, $^{***}P$<0.001.

软骨细胞的细胞骨架和初级纤毛形态各异(图1A)。培养24 h后硬基底中软骨细胞更加铺展,中等基底次之,软基底中呈现细胞面积更小的现象。进一步定量分析发现,基底培养皿中的软骨细胞面积和细胞核面积均大于中硬度基底和软基底($P<0.05$)(图1B、1C),初级纤毛长度也更长($P<0.05$)(图1D),但纤毛出现率在3种硬度基底中的差异无统计学意义($P>0.05$)(图1E)。

2.2 HDAC6活性对软骨细胞初级纤毛长度的影响

10 μmol/L的Tub A处理软骨细胞24 h后,不同硬度基底培养皿中的软骨细胞表现出不同的HDAC6活性。相比于中硬度基底和硬基底,软基底培养皿中的软骨细胞HDAC6活性更高($P<0.05$)(图2A)。定量分析结果表明,Tub A处理24 h后,硬基底上的软骨细胞细胞骨架面积增加($P<0.05$),中硬度基底和软基底上的细胞骨架面积无明显变化($P>0.05$)(图2B),但在3种基底上的软骨细胞细胞核面积和初级纤毛长度都有着所增加($P<0.05$),硬基底上的软骨细胞核面积和初级纤毛长度的变化比中硬度基底和软基底更为明显($P<0.001$)(图2C~2E)。

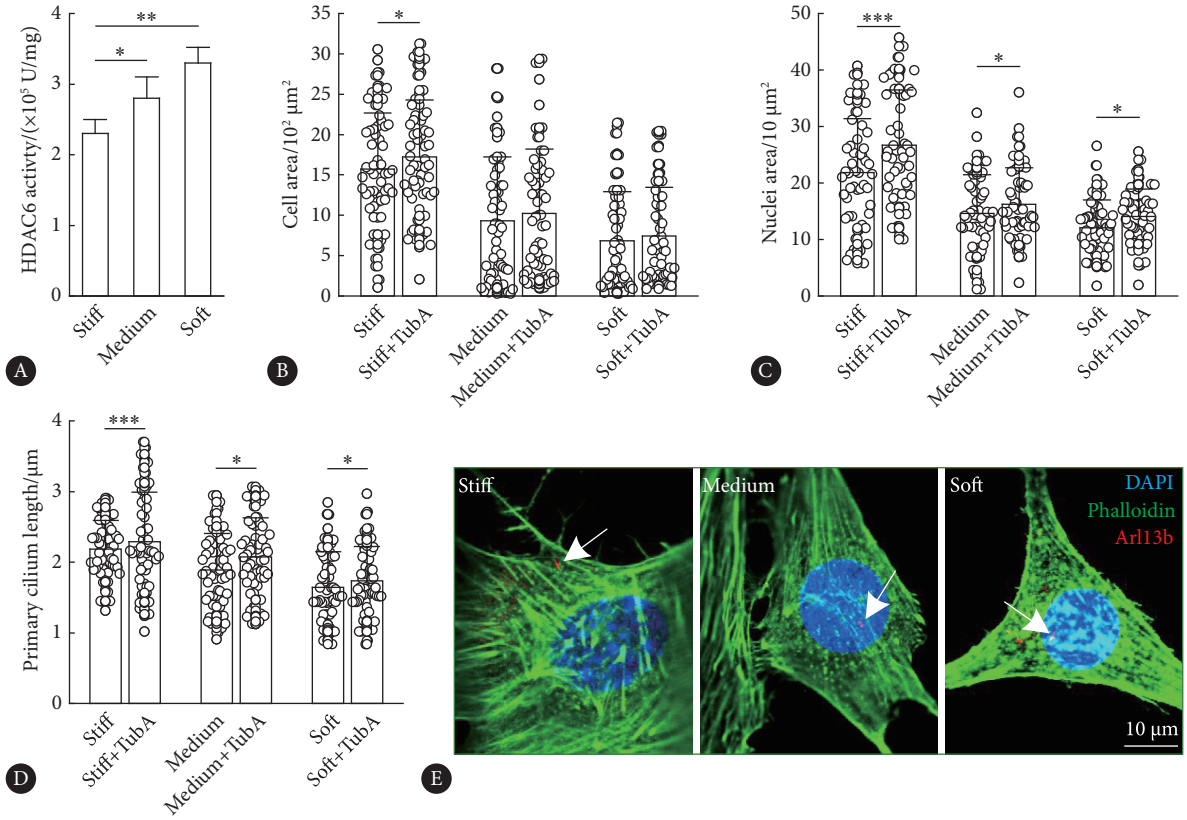

图 2 HDAC6活性对不同基底硬度中软骨细胞形态和初级纤毛的影响

Fig 2 The effect of HDAC6 activity on the morphology and the primary cilia of chondrocytes on substrates with varying stiffness

A, Effect of substrate stiffness on HDAC6 activity. B, The effect of HDAC6 activity on cell area. C, The effect of HDAC6 activity on nuclear area. D, The effect of HDAC6 activity on primary cilia length. E, Immunofluorescence staining was performed to display the morphology of primary cilia on substrate with varying stiffness. n=63. The white arrows are pointed at the primary cilia. $^{*}P<0.05$, $^{***}P<0.001$.

2.3 Piezo1激活和抑制对初级纤毛长度的影响

实验结果表明,在培养皿中经过Yoda1处理的软骨细胞与未处理的软骨细胞相比HDAC6活性降低($P<0.01$),而经过GsMTx4处理的细胞并未出现明显差异($P>0.05$)(图3A)。免疫荧光实验结果表明,经Yoda1处理后,初级纤毛长度显著增长($P<0.001$),但经GsMTx4处理的初级纤毛长度显著缩短($P<0.05$)(图3B、3C)。

在3种硬度基底中,经过Yoda1处理的软骨细胞初级纤毛长度有增长($P<0.001$)(图4A,4B),而在经过GsMTx4处理的细胞中,只在硬基底上的细胞初级纤毛长度出现缩短($P<0.05$),而在较软的基底中无明显变化($P>0.05$)(图4C)。此外在单独的激活和抑制中,初级纤毛长度依然呈现硬基底大于中软基底的趋势(图4B、4C)。

3 讨论

本研究探究了不同基底硬度对软骨细胞初级纤毛的影响,以及HDAC6与Piezo1的参与。首先关注了基底硬度对软骨细胞形态的影响,发现硬基底促进软骨细胞铺

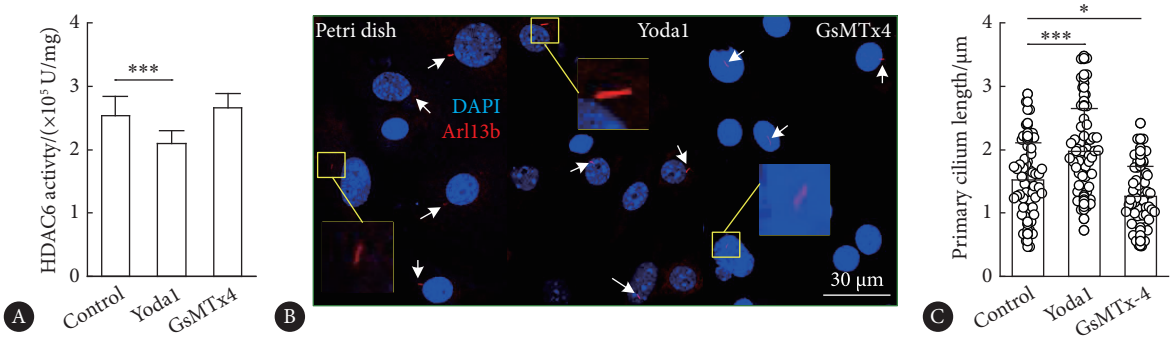

图 3 Piezo1对HDAC6活性和初级纤毛长度的影响

Fig 3 The effect of Piezo1 on HDAC6 activity and the length of primary cilia

A, The effect of Piezo1 on HDAC6 activity. B, Cellular immunofluorescence staining shows that Yoda1 and GsMTx4 affect the length of primary cilia. The white arrows are pointed at the primary cilia. The larger boxes represent a 5-fold magnification of the corresponding smaller ones. C, The effect of Piezo1 on the length of primary cilia. n=63. The white arrows are pointed at the primary cilia. $^{*}P<0.05$, $^{***}P<0.001$.

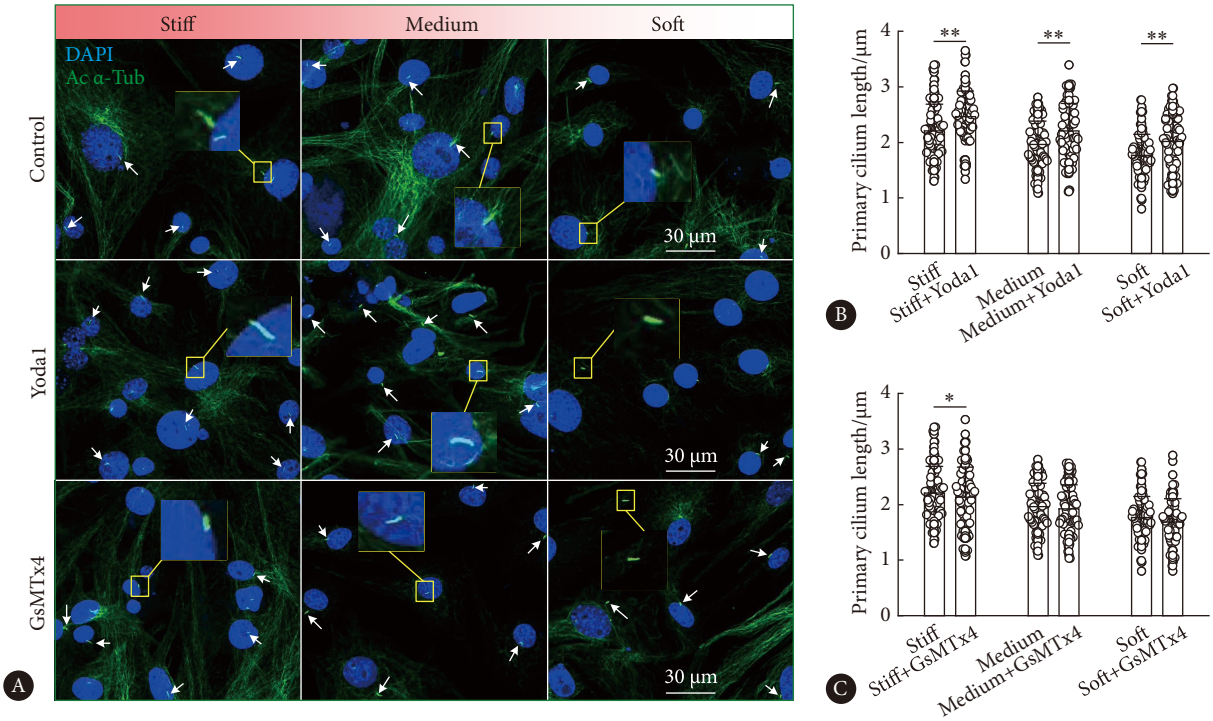

图 4 Piezo1激活与抑制对不同硬度基底上初级纤毛长度的影响

Fig 4 The effect of Piezo1 activation and inhibition on the length of primary cilia on substrates with varying stiffness

A, Immunofluorescence staining shows the effect of Yoda1 and GsMTx4 on the length of primary cilia on substrates with varying stiffness. The white arrows are pointed at the primary cilia. Within each image, the larger box represents a 5-fold magnification of the corresponding smaller one. B, The effect of activator Yoda1 on he length of primary cilia on substrates with varying stiffness. C, The effect of inhibitor GsMTx4 on the length of primary cilia on substrates with varying stiffness. The numbers of samples in B and C are the same. n=69. $^{*}P<0.05$, $^{**}P<0.01$.

展，而且使细胞骨架呈现出多边形，这与之前的研究一致。此外，本研究也发现，硬基底能够促进初级纤毛长度增加。初级纤毛和细胞骨架面积随硬度增加呈现相同增长趋势。但基底硬度不影响初级纤毛的出现率，所以基底硬度只影响软骨细胞初级纤毛的长度而不影响初级纤毛出现率。笔者认为软骨细胞骨架张力可能调控初级纤毛形态。早期研究证实，肌动蛋白牵引力能降低不同硬度基底中初级纤毛的出现率和长度[27]，但微管蛋白对初级纤毛的影响目前还尚不清楚。也有文献报道，控制细胞铺展和调节初级纤毛的基体(basal body)位置都会影响初级纤毛伸长[34]。在未来的工作中，对初级纤毛与细胞骨架之间的关系进行深入研究是必要的。

HDAC6调节微管蛋白的乙酰化和去乙酰化平衡，而乙酰化α-tubulin正是初级纤毛的重要组成部分[29]。本研究验证了在硬基底上，HDAC6较低的活性代表着α-tubulin更高水平的乙酰化。这一结果与本研究得出在硬

基底上初级纤毛越长的结论是一致的,HDAC6的活性越低,反而乙酰化微管蛋白的水平就越高,并且α-tubulin的乙酰化对于初级纤毛的组装是必不可少的,所以初级纤毛通过主动伸长的方式来调整自身的最适宜状态。并且经过TubA的抑制的细胞出现更多的树突触角,并促使细胞更为铺展。除了HDAC6之外,初级纤毛的生长和解聚都与Aurora A的活性有关。Aurora A是极光激酶家族成员之一,是一个重要的有丝分裂调节因子。调节Aurora A活性可以实现抑制初级纤毛的生长[35],但究其根源是激活基体的Aurora A能够使HDAC6磷酸化,最终导致α-tubulin的去乙酰化[36]。甚至有丝分裂主要的调节因子PIK1也可以直接磷酸化HDAC6调控初级纤毛的解聚[37]。因此,后续的研究有必要深入探究基底硬度调控Aurora A和PIK1的分子机制。

虽然有报道Piezo1的活化影响初级纤毛的出现[30],但这之间与基底硬度的具体关系我们并不清楚,所以本研究探究了Piezo1介导基底硬度对初级纤毛的影响。本研究证实,Piezo1的激活与抑制影响HDAC6的活性,使α-tubulin高水平乙酰化并以此来调控初级纤毛的长度,并在硬基底上更为显著。本课题组早期发现,硬基底能上调Piezo1的蛋白表达,而且在硬基底中,Piezo1显著增强钙信号[38]。这可能就解释了硬基底Piezo1对HDAC6活性和初级纤毛长度影响较为显著的原因。本研究是在二维基底中探究Piezo1对初级纤毛的影响,为了能够更好地模拟软骨细胞生理和病理基质微环境,后续研究三维基质力学微环境通过HDAC6与Piezo1调控软骨初级纤毛的机制将十分必要[39]。总之,在基质力学微环境中,基底硬度起着重要的作用。揭示基底硬度对软骨细胞重要力感受器初级纤毛的调控机制将为损伤和骨关节炎软骨的靶向精准治疗提供参考。

* * *

作者贡献声明 郭华庆负责论文构思、数据审编、正式分析、调查研究、研究方法、验证、初稿写作和审读与编辑写作,兰敏华和张艳君负责调查研究、研究方法和研究项目管理,张强负责调查研究和研究方法,刘艳丽负责正式分析、调查研究和研究方法,张全有负责论文构思、经费获取、研究方法、研究项目管理、提供资源、监督指导和审读与编辑写作,陈维毅负责研究项目管理和监督指导。所有作者已经同意将文章提交给本刊,且对将要发表的版本进行最终定稿,并同意对工作的所有方面负责。

Author Contribution GUO Huaqing is responsible for conceptualization, data curation, formal analysis, investigation, methodology, validation, writing--original draft, and writing--review and editing. LAN Minhua and ZHANG Yanjun are responsible for investigation, methodology, and project administration. ZHANG Qiang is responsible for investigation and methodology. LIU Yanli is responsible for formal analysis, investigation, and methodology. ZHANG Quanyou is responsible for conceptualization, funding acquisition, methodology, project administration, resources, supervision, and writing--review and editing. CHEN Weiyi is responsible for project administration and supervision. All authors consented to the submission of the article to the Journal. All authors approved the final version to be published and agreed to take responsibility for all aspects of the work.

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