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体外培养 GFP 裸小鼠颅骨细胞的表形分析

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【摘要】　目的　本研究建立的转绿色荧光蛋白基因近交系裸小鼠(GFP 裸小鼠)已在示踪研究胶质瘤微环境方面起了重要作用,但在治疗自体颅骨成形术后骨吸收并发症的效果及机制方面未见报告,本文旨在分析体外培养 GFP 裸小鼠颅骨细胞的表形,为治疗颅骨吸收准备工具细胞奠定基础。**方法**　取生后 3 d 的 GFP 裸小鼠,在无菌条件下解剖双侧顶骨,连同骨膜,将颅骨剪成 1 mm^2 左右的小片,置于含有胎牛血清的 1640 培养基中,在 5% CO_2 培养箱中作短期传代培养,收集从骨片上长出的细胞作相关检测。**结果**　对原代(P0)和继代(P1、P2)细胞观察表明,在 60 mm 皿底长满 90%的传代时间约 6~8 d,抽样细胞计数约 2.3×10^6 ~ 2.5×10^6 个/皿,形态以纤维形为主,也有星形和树突状;在荧光显微镜下所有细胞全部发绿色荧光,形态与白光镜下一致;标志蛋白检测表明,在整个细胞群中同时存在 $BMP\text{-}6^+$的成骨祖细胞和 $CD206^+$、$CD68^+$的巨噬细胞。**结论**　基于颅骨再生,除了成骨祖细胞作为起始细胞,还必须有巨噬细胞参与维持环境稳态,培养成功的 P0,P1 和 P2 三代细胞因同时满足这个需要,有望作为工具细胞进一步用于颅骨再生的研究。

【关键词】　绿色荧光转基因裸小鼠;BMP;CD68;CD206;颅骨细胞培养
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Morphological analysis of GFP nude mice skull cells cultured *in vitro*

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【Abstract】　Objective　The inbred transgenic nude mice with green fluorescent protein gene (GFP nude mice) line that we established have played an important role in tracing studies of the glioma microenvironment, but no study has yet reported its mechanism and effect in treating bone resorption complications after autologous cranioplasty. Our purpose in this article is to analyze the phenotype of GFP nude mouse skull cells cultured *in vitro* to lay the foundation for preparing

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tool cells for the treatment of skull resorption. **Methods** We selected GFP mice within three days after birth. Under sterile conditions, bilateral parietal bones with periosteum were isolated and cut into small 1 mm^2 pieces, and then placed in 1640 medium with fetal bovine serum. We then incubated the samples in a 5% CO_2 incubator. Those cells that crawled out of the bone slices were collected for short-term subculture and relevant analyses. **Results** Observation of primary (P0) and secondary (P1, P2) cells indicates that the passage time in which cells grow to cover 90% of the bottom of a 60 mm dish is about 6~8 days, with a sampling calculation of $2.3 \times 10^6 \sim 2.5 \times 10^6$ cells per dish. The cell morphology is mainly fibrous, but also star-shaped and dendritic. All cells emit green fluorescence under a fluorescent microscope, which is consistent with that under a white light microscope. Marker protein detection showed that BMP-6^+ osteoblasts and $CD206^+$, $CD68^+$ macrophages coexist in the entire cell population. **Conclusions** Based on our skull regeneration studies, macrophages must also be involved in maintaining environmental homeostasis, in addition to the osteoblastic progenitor cell role as a starting cell. Successfully cultured P0, P1, and P2 third-generation cells meet this need, and are all expected to be further used as a tool cell in skull regeneration research.

【**Keywords**】 green fluorescent transgenic nude mice; BMP; CD68; CD206; skull cell culture

转绿色荧光蛋白(green fluorescent protein, GFP)基因的近交系裸小鼠(GFP 裸小鼠)是由苏州大学黄强课题组研发的[1],已在脑胶质瘤移植实验中发现,GFP 裸小鼠的脑组织和细胞因高表达 GFP,在示踪宿主源性肿瘤微环境(Tumor microenvironment, TME)方面,不需要加荧光示踪剂而能直接起到清楚的示踪作用[2-7],能很好地证明 TME 中的巨噬细胞,树突状细胞,少突胶质细胞和血管内皮等细胞,在变成肿瘤相关细胞的过程中,通过细胞融合而恶变,重构成更加异质的 TME;但在我们正在进行的颅骨成形研究的 GFP 裸小鼠骨细胞中,是否也能在示踪新骨形成方面起重要作用仍是未知。本文提出这个问题,是由于发现 GFP 新生裸小鼠的颅骨细胞,在短期培养中,见到成骨前体细胞有强大的扩增和分化能力,除了能在荧光显微镜下定性 GFP 的局部位置和荧光强弱,还能在拉曼/多光子显微镜下进行量化,有望为下一步用于颅骨成形研究中对成骨和破骨细胞进行甄别和调控。

1 材料和方法

1.1 实验动物

出生后 3 d 的 GFP 裸小鼠(Foxn1^{nu}.B6-CAG-EGFP/SU,SPF 级)6 只,无关性别,体重约 3 g,购自并饲养在苏州大学实验动物中心[SCXK(苏)2018-0006][SYXK(苏)2017-0043],本实验经苏州科技城医院医学伦理委员会批准(IRB2018019),并严格遵循“3R”原则给与人道关怀。

1.2 主要试剂与仪器

细胞培养箱(MCO-18AC,Sanyo 日本);倒置显微镜(CKX41, Olympus 日本),荧光显微镜(DM2500, Leica 德国);BMP-6 抗体(AB15640, abcam);CD11c 抗体(AF1396,碧云天);CD68 抗体(GK600710,基因科技);CD206 抗体(141711, BioLegend);DAB 试剂盒(GK347010,基因科技);RPIM1640 培养基(AE26543277, HyClone);离心机(TDZ5-WS, BIORIDGE);RPIM1640 培养基(AE26543277, HyClone);胎牛血清(1616756, BI);青/链霉素(15140163, gibco);细胞培养板(corning, 60 mm);12 孔细胞培养板(corning);15 mL 离心管(corning);移液枪(Thermo);超净台(苏州净化)。

1.3 实验方法

1.3.1 细胞培养方法

参照陈洁等[8]的方法,加以改良,简要过程如下:(1)乳鼠脱颈处死,75%乙醇消毒全身,置于超净台上,剪开颈背及头顶皮肤,取双侧顶骨,保留骨膜,放入含有青/链霉素的 PBS 溶液中,剪成 1 mm^2 大小的骨片,直接接种于含有 10%胎牛血清及青/链霉素的 RPMI1640 培养基中,在 5% CO_2 培养箱中培养;(2)待细胞移行出骨片贴培养皿壁生长后用消毒镊子夹出骨片,继续培养到布满皿壁 90%时,用 0.25%胰酶消化 3 min,得到原代培养细胞 P0;(3)P0 按 1∶1传代,每 3 d 换液 1 次,待细胞长满 90%时,收获到的细胞为 P1;(4)P2 参照 P1 培养和收集。P0,P1 和 P2 均在液氮中保存备用。

1.3.2 细胞免疫化学染色

被检细胞放入加有圆形载玻片的 12 孔板内,待细胞爬满玻片后,用 PBS 润洗 3 次,4%多聚甲醛溶液室温固定 20 min,后续简要过程如下:(1)0.5% TritonX-100 室温通透 20 min,PBS 清洗三次。(2)3%过氧化氢溶液清除内源性过氧化物酶,室温 15 min,PBS 清洗。(3)10%山羊血清室温封闭 30 min。

(4)吸出血清,孵一抗,4℃过夜。(抗体工作浓度:BMP-6,1∶200;CD11C,1∶150;CD68,1∶100;CD206,1∶100)(5)PBS 冲洗 3 次,室温孵二抗 30 min,后用 PBS 冲洗。(6)DAB 光镜下显色 2~5 min,弃去 DAB,PBS 冲洗数次。(7)苏木精染核 15 s,自来水冲洗玻片。(8)无水乙醇脱水 3 min 共 3 次,中性树胶封片。

2 结果

2.1 P0~P2 细胞和离体骨瓣状态

游离骨片置于培养皿底 7 d,在骨片中央和边缘能见到细胞陆续移行出来,沿皿壁生长,随着培养时间延长,细胞数量不断增多,排裂紧,在低倍镜下呈鹅卵石样,在高倍镜下以长纤维和星形为主,白光和荧光镜下所见到的形态基本一致。到 6~8 d 细胞布满皿底的 90%时收获(P0),接着又继续传了二代(P1 和 P2),传代时间分别为 8 d 和 15 d,如果再往下传,细胞生长趋势是越来越慢,因此结束细胞传代实验,并把收获的细胞于液氮冻存。

从细胞形态看,随着传代次数增多,向终末分化细胞增加,甚至老化会加重。如果将取出的骨片继续培养(骨片传代),仍可见到细胞从骨片边缘移行出来继续生长,说明培养液中存放的离体骨片本身也是活着的,也可传代,但这属于类器官培养,难度更大,有待今后研究。详见图 1。

2.2 标志蛋白表达

(1)骨形态发生蛋白(BMP-6)的免疫复合物为棕色,在镜下见阳性细胞散在分布于细胞群各处,所占比例不高(图 2A 红箭头),但表形很有特征性,免疫复合物主要在细胞质,组成细胞骨架(图 2A 黄箭头),也有些沉积于染色质(图 2A 绿箭头)。更具特征性的是在胞外也有沉积,跨越多个细胞核,容易误判在多核细胞中表达(图 2A 蓝箭头);

(2)巨噬细胞甘露糖受体 1(CD206)的深棕色免疫复合物也只存在于细胞群中的少数细胞中,特征是只在细胞浆表达,复合物呈颗粒状(图 2B);

(3)CD68 的表达与 CD206 相似,但 CD11c 未见表达(图 2C 和图 2D)。

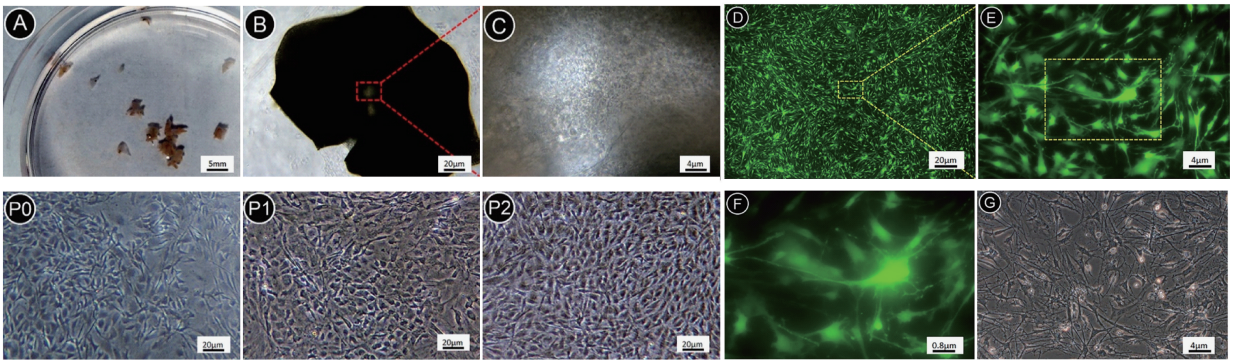
3 讨论

3.1 对正常成骨细胞系的评价

这是指已具永生化潜能的细胞,就颅骨而言:有 Kodama 等[9] 和 Sudo 等[10] 分别于 1981 和 1983 年报告了新生小鼠颅骨细胞体外连续传代培养至数十代,以碱性磷酸酶阳性表达,矿化细胞聚集,形成骨的雏形为建系标准;其中 Sudo 等[10] 建立的 MC3T3 细胞系至今已成为商品被广泛应用。可是 1997 年 Nakayama 等[11] 从 p53 缺陷小鼠的颅骨中建立 MMC2 细胞系时指出,在他之前的细胞系几乎都是反复传代,获得永生化基础上建立的,这种永生化与未检测的相关基因突变有关。他报告的 MMC2 成骨细胞系的染色体已是异倍体,所幸小鼠致瘤试验阴性,他的结论是 MMC2 的永生化,除了与 p53 缺陷相关,不排除还有其它异常基因参与。2004 年,Kadowaki 等[12] 从新生的 GFP 转基因小鼠的颅骨中分离出成骨细胞,连续传 26 代后建立了自发绿色荧光的 C3 细胞系,再通过腺病毒转染 BMP-2 于 C3 细胞,用于 4 mm 直径圆形缺损颅骨的修补,在荧光镜下明确显示,荷 BMP-2 的 GFP^+ 细胞是形成新骨的主体。同样,除了通过荧光示踪更加直接证明 C3 细胞的成骨作用,但 C3 基因型是否有改变不得而知。总之,永生化细胞系如果作为治疗的工具细胞,用于改善颅骨成形术的预后,应注意致癌事件的发生。

3.2 对短期培养的成骨细胞评价

这里指来自生长发育中的新生小鼠颅骨细胞,培养早期的扩增能力不亚于成骨细胞系,不存在致瘤性,用于细胞接种时,根据所处的环境可向不同方向分化,评价其优劣的标准是看这个群体中的前体细胞占有率和亚群细胞的类型。从本文报告的结果来看(图 2),至少有与骨再生高度相关的成骨前体细胞和巨噬细胞(macrophages,M)两种亚群细胞标志蛋白阳性,不妨探讨一下潜在的研究价值。已知骨再生有膜内成骨和软骨内成骨,前者见于骨折间隙小的扁平骨,后者见于间隙大的长骨[13],无论那种,M 都必须参与修复[14-16]。在修复初期,M 的作用是吞噬骨折部位的坏死组织和细胞碎片,后期募集间充质干细胞和血管祖细胞[17],这种功能转变称“极化”,炎症性 M 又称为经典活化巨噬细胞(M1);在再生中激活的 M 称选择性激活巨噬细胞(M2),M1/M2 的出生地均在骨髓(M0),根据骨折当时建立稳态环境需要决定对 M0 是否进行征集。本文检测到的 $CD68^+$ 细胞可定性为 M1 细胞[18],同样 $CD206^+$ 细胞可定性为 M2 细胞[19],结合还检测到的 $BMP\text{-}6^+$ 细胞可定性为成骨前体细胞[20]。有鉴于此,本文的 P0,P1 和 P2 有望作为工具细胞用于骨

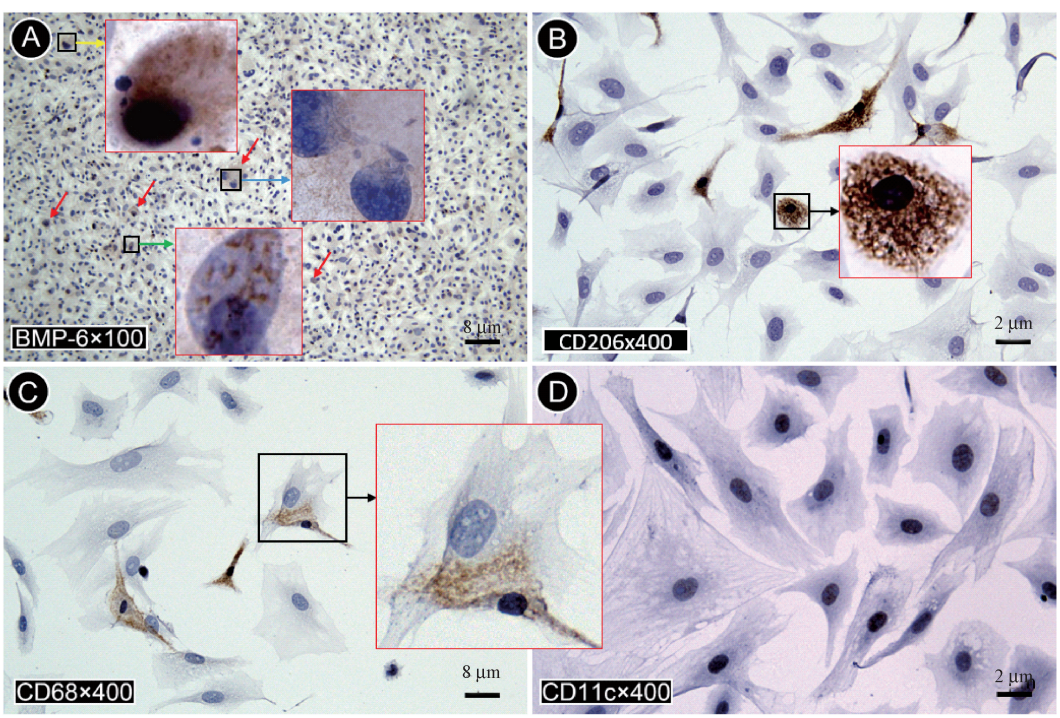


注：A（肉眼）：用于骨细胞培养的小鼠颅骨碎片；B（倒置显微镜）：培养 1 d 的骨片及骨细胞，骨片周围白色区域可见骨细胞开始增殖，随着培养时间的延长，增殖区域逐渐扩大，并离开骨片向培养皿底部伸展；C（倒置显微镜）：B 红框的放大，是培养 2 d 的骨细胞；P0～P2（倒置显微镜）：为连续传代细胞，P0（初代）、P1（一代）和 P2（二代）增殖能力好，细胞形态如鹅卵石样；D、E、F 为同一视野不同倍数荧光下的细胞形态，E 和 G 是在相同倍数荧光与日光下的比较，两者形态基本一致，只是 E 的胞浆显示更清晰，在进一步放大的情况下，F 能清晰显示伪足和伪丝。

图 1　新生小鼠颅骨细胞培养

Note. A (naked eye), Mice skull fragments for osteocyte culture. B (inverted microscope), The white area around the bone plate showed that the bone cells began to proliferate. As the culture time increased, the proliferation area gradually expanded and extended from the bone plate to the bottom of the dish. C (inverted microscope), Amplification of red B-frame was the bone cells cultured for 2 days. P0～P2 (inverted microscope) is a continuous passage cell, P0 (Zero generation), P1 (First generation), P2 (Second generation) They have good proliferators, and their cells are like pebble-like; The morphology of D, E and F cells under different multiples of fluorescence in the same field of vision. E and G were compared under the same multiple fluorescence to daylight. The two forms were basically the same, except that the cytoplasm of e showed more clearly, and in the case of further magnification, F could clearly show pseudo-foot and pseudo-filament.

Figure 1　Culture of skull cells in newborn mice



注：免疫组化染色（红框）。A：BMP-6 棕色免疫复物只存在于少数细胞中（红箭头），放大后，可确定存在于细胞质内，组成细胞骨架（黄箭头），少数存在于染色质（绿箭头），更受关注的是，细胞间质中也有（蓝箭头）；B：CD206 深棕色免疫复合物仅存在于细胞浆，也只在少数细胞中表达；C：CD68 的表达情况与 CD206 类似；D：CD11c 未见表达。

图 2　标志蛋白的免疫细胞化学染色

Note. Immunohistochemical staining (red frame). A, BMP-6 brown immune complex is only present in a few cells (red arrow). After enlarging, it can be determined to be present in the cytoplasm, constituting the cytoskeleton (yellow arrow) and a few in the chromatin (green arrow). More importantly, the complex are also (blue arrow) in the interstitial cells (blue arrow). B, Dark brown immune complex of CD206 is present only in cytoplasm and express only in a few cells. C, The expression of CD68 is similar to that of CD206. D, CD11c did not express.

Figure 2　Immunocytochemical staining of marker proteins

缺损修复实验。不得不指出的是,代表 M0 的 $CD11c^+$细胞[21]未被检测到,究其原因可能与骨片离体培养,脱离了机体的骨髓环境有关。

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