重组人源性抗 HBsAg Fab 抗体纯化方法的比较研究

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摘 要 抗 HBsAg Fab 抗体被认为在预防和治疗 HBV 引起的肝病中具有重要的作用。为了建立稳定的、适于生产应用的重组人源性抗 HBsAg Fab 抗体的纯化工艺,本实验对不同纯化方法进行了比较研究。比较了抗 Fab 抗体亲和层析、ScFv 单克隆抗体亲和层析和离子交换层析等 3 套纯化工艺在酵母发酵生产的重组人源性抗 HBsAg Fab 抗体纯化中的效率。结果显示,抗 Fab 抗体亲和层析柱纯化酵母表达的重组 Fab,纯度为 96.8%,但回收率偏低,只有 30% ~ 40%。ScFv 单克隆抗体亲和层析纯化的重组 Fab,纯度为 97.5%,回收率达 75% ~ 85%。该工艺能很好的适应较小规模的生产应用。离子交换层析纯化的重组 Fab,纯度为 97%,回收率为 75% ~ 85%。该工艺能很好的适应较大规模的生产应用。以上结果表明,应用 ScFv 单克隆抗体亲和层析和离子交换层析纯化技术均能很好的纯化出重组人源性抗 HBsAg Fab 抗体,这两种纯化工艺不仅大大节约纯化成本,且纯化效率和回收率有很大提高。为重组 Fab 抗体的工业化生产应用奠定了基础。

关键词 HBsAg, 重组 Fab, 纯化方法, 毕赤酵母

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乙型肝炎病毒感染是全球性公共卫生问题,不仅引起急慢性肝炎,且与肝硬化和肝癌的发生有密切的关系。能和乙肝病毒表面特异性位点结合,并封闭其侵袭肝细胞活性的抗乙型肝炎表面抗原单克隆抗体和抗体片段,是唯一能用于紧急预防的生物制品[1]。Fab 抗体具有广泛的应用价值,如用于疾病的检测和治疗、体外诊断、亲和纯化等[2-4],具有全抗体相同的抗原结合活性,糖基对抗原抗体的结合活性没有影响[5]。抗 HBsAg Fab 抗体被认为具有预防和治疗 HBV 引起的肝病的作用[6]。

噬菌体展示技术的建立为大量制备人源性单克隆抗体及临床治疗应用提供了切实可行的手段,而且为制备其他的具有生物活性的酶、激素或新药提供了可能[7,8]。通过噬菌体展示技术筛选获得人源性抗 HBsAg Fab 抗体基因[9],构建到毕赤酵母(*Pichia pastoris*)中获得高效表达[10-12]。该 Fab 抗体具有较强的抗原结合活性和较高的特异性[10],具有进一步开发应用的前景。根据酵母表达的人源性抗 HBsAg Fab 抗体的特点,从经济、实用、纯化效率等为出发点,本实验设计了三套纯化方案,对酵母表达的人源性抗 HBsAg Fab 抗体的纯化方法进行了比较研究。

1 材料与方法

1.1 材料

重组人源性抗 HBsAg Fab 抗体酵母工程菌 GS115/Fab,本室保存。ScFv 单克隆抗体细胞株 14F7,上海第二医科大学实验中心提供。5mL HiTrap™ NHS activatited Sepharose HP 层析柱、5mL HiTrap™ DEAE-sepharose Fast Flow 层析柱、5mL HiTrap™ CM-sepharose Fast Flow 层析柱、30mL 层析柱等均为 Amersham Pharmacia 公司产品。DEAE-sepharose 和 CM-sepharose 离子交换填料购自 Amersham Pharmacia 公司,AKTA 快速液相蛋白层析仪(FPLC)为 Pharmacia 公司产品。其余试剂均为国产分析纯。

1.2 方法

1.2.1 重组人源性抗 HBsAg Fab 抗体的发酵生产:从新鲜 YPD 平板挑重组人源性抗 HBsAg Fab 抗体酵母工程菌 GS115/Fab 单菌落接种于 10mL BMGY 种子培养基,30℃振荡培养至 OD_{600} 达 10 左右,转接于 200 mL BMGY 培养基中,30℃振荡培养至 OD_{600} 达 10

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左右作为发酵菌种。按 10%接种量转入 5L 发酵罐的基础培养基中,进行 Fed-batch 甲醇诱导发酵 96h。收集发酵液经 6000 r/min 25min 离心,收集上清,40%饱和度的$(NH_4)_2SO_4$沉淀、将沉淀溶于 0.1mol/L TrisHCl(pH8.0)中,用透析缓冲液 4℃透析 24h 备用。

1.2.2 抗 Fab 抗体亲和层析纯化重组 Fab:按照说明书方法将鼠抗人 Fab 抗体(Sigma 公司)溶于偶联缓冲液(0.2mol/L $NaHCO_3$,0.5mol/L NaCl, pH8.3)中(终浓度 1mg/mL),装置 5mL HiTrap™ NHS activatited Sepharose HP 层析柱,3 × 10mL 冰冷 1mmol/L HCl 洗柱,流速 0.5mL/min,洗脱柱上的异丙醇。注入 5 ~ 10mL 抗 Fab 抗体溶液,25℃吸附 30min。灭活缓冲液 A(0.5mol/L 乙醇胺,0.5mol/L NaCl,pH8.3)、灭活缓冲液 B(0.5mol/L 乙酸,0.5 mol/L NaCl,pH4.0)交替洗柱,洗脱非特异性结合抗体并灭活未结合的活化基团。中和缓冲液至 pH 中性,4℃保存备用。

分别用 Fab 抗体柱再生洗脱液 A(0.1mol/L NaAc, 0.5mol/L NaCl, pH4.5)和再生洗脱液 B(0.1mol/L Tris-HCl, 0.5mol/L NaCl, pH8.0)各 25mL 洗柱,流速为 2mL/min。0.1mol/L TrisHCl, pH8.0 的平衡缓冲液(30 ~ 40mL)平衡 Fab 抗体柱,流速同上。将透析脱盐的重组人源性抗 HBsAg Fab 抗体样品直接从进样口上样 20mL,流速为 1mL/min。用 0.1 mol/L TrisHCl, pH8.0 平衡缓冲液(30 ~ 40mL)洗脱,再用 0.01mol/L TrisHCl, pH8.0 缓冲液(20 ~ 30mL)洗脱,流速为 2mL/min。用 0.1mol/L Gly-HCl 洗脱缓冲液进行样品洗脱。洗脱速度为 1.5mL/min。收集样品洗脱液,将有目的蛋白的样品洗脱液分部收集并进行 SDS-PAGE 和 Western-Blot 分析。

1.2.3 抗 ScFv 单克隆抗体亲和层析纯化重组 Fab:注射抗 ScFv 单克隆抗体细胞株 14F7 入 Balb/C 小鼠腹腔进行单克隆抗体扩增,收获的腹水经辛酸法[13]纯化。

重组 Fab 抗体与 ScFv 单克隆抗体亲和力的测定:将 Fab 柱纯化的重组 Fab 抗体、发酵上清按一定的量包被 ELISA 板,4℃放置过夜。洗涤,按不同比例加入 ScFv 单克隆抗体,37℃静置 1h。洗涤,加入辣根过氧化物酶标记的羊抗鼠 IgG,37℃静置 1h。OPD 显色,酶标仪测定 $A_{450/630}$值。

将纯化的鼠抗 ScFv 单克隆抗体溶于偶联缓冲液中(终浓度 1mg/mL),装置 5mL HiTrapTM NHS activatited Sepharose HP 柱。分别用 Fab 抗体柱再生洗脱液 A 和 B 各 25mL 洗柱,流速为 2mL/min。0.1mol/L 磷酸缓冲液(0.1mol/L Na2HPO4,0.1mol/L NaH_2PO_4,pH8.0)平衡 ScFv 单克隆抗体亲和层析柱,流速同上。将透析样品直接从进样口上样 20 ~ 30mL,流速为 1mL/min。用 0.1mol/L pH8.0 的磷酸缓冲液(30 ~ 40mL)洗脱杂蛋白,再用 0.01mol/L pH8.0 磷酸缓冲液(20 ~ 30mL)平衡洗脱,流速为 2mL/min。最后用 0.1mol/L pH2.5 的 Gly-HCl 洗脱缓冲液进行样品洗脱。洗脱速度为 1.5mL/min。收集样品洗脱液,将有目的蛋白的样品洗脱液分部收集并进行 SDS-PAGE 和 Western-blot 分析。

1.2.4 离子交换层析纯化重组 Fab:0.1mol/L 离子交换平衡缓冲液(0.1mol/L Na_2HPO_4, 0.1mol/L NaH_2PO_4, pH6.5)平衡洗脱 5mL HiTrap™ DEAE-sepharose Fast Flow 层析柱,后用 0.02mol/L 的离子交换平衡缓冲液平衡。流速 2mL/min。从进样管上样 20mL 脱盐样品。流速 1.5mL/min。收集穿流液作后续纯化。用 1mol/L NaCl 溶液洗脱阴离子交换柱。将上面结合的杂蛋白全部洗脱下来。洗脱速度为 1.5mL/min。

0.1mol/L 离子交换平衡缓冲液平衡洗脱 5mL HiTrap™ CM-sepharose Fast Flow 层析柱,后用 0.02mol/L 的离子交换平衡缓冲液平衡。流速 2 mL/min。将 DEAE 交换柱的穿流液直接从进样管上样。流速 1.5mL/min。用 1mol/L NaCl 溶液作梯度洗脱阳离子交换柱。步长为 120mL,洗脱速度为 1.5 mL/min。分部收集洗脱液,SDS-PAGE 分析以确定最佳洗脱浓度。

分别取适量的 DEAE-Sepharose CL-6B 和 CM-Sepharose CL-6B 填料,用水浸泡,充分膨胀后,分别用 1mol/L NaOH 和 1mol/L HCl 浸泡 30min 以除去杂质。每次用酸(或碱)处理后,均用水洗涤至中性。再用碱(或酸)处理使其带上需要的反离子。用 0.01mol/L 离子交换平衡缓冲液平衡后分别装于 30mL(2cm × 20cm)的层析柱。重组 Fab 抗体的纯化过程同上述 5mL 离子交换预装柱。

1.2.5 纯化重组 Fab 抗体的性质分析及活性测定:纯化的重组人源性抗 HBsAg Fab 抗体的等电点测定参照文献[20]进行。纯化的重组人源性抗 HBsAg Fab 抗体的分子量测定参照文献[12]进行。

重组人源性抗 HBsAg Fab 抗体的活性检测参照 In-Hak Choi 等[14]的 ELISA 方法进行。酶标板包被 HBsAg(100ng/mL),4℃过夜,2% BSA 封闭,洗涤,分别加入纯化的重组人源性抗 HBsAg Fab 抗体,37℃反应 1h,洗涤,加入辣根过氧化物酶标记的羊抗人

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Fab特异的二抗，反应1h，洗涤，显色，酶标仪测定$A_{540/630}$值。

2 结果

2.1 Fab抗体亲和层析纯化重组Fab

重组人源性抗HBsAg Fab抗体工程菌表达上清经过浓缩和脱盐处理后，上Fab亲和层析柱，其纯化结果的SDS-PAGE分析如图1。通过Fab亲和层析纯化，得到了纯度较高的重组Fab抗体，经薄层扫描分析，纯化的重组Fab抗体纯度可达96.8%以上。通过Fab层析柱可高效地分离纯化出所需要的重组Fab抗体，但是该方法的回收率偏低，仅为30%～40%。

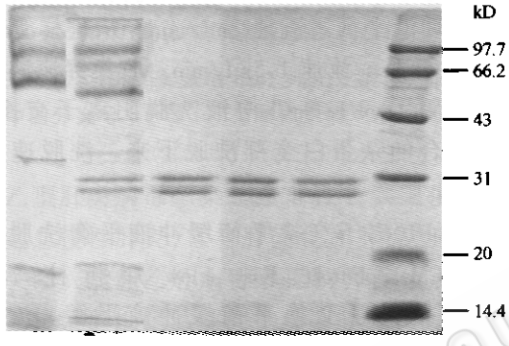

图1 Fab亲和层析柱纯化重组Fab抗体的SDS-PAGE分析

Fig.1 SDS-PAGE analysis of purified Fab fragment by Fab affinity chromatography column

2.2 ScFv单克隆抗体亲和层析纯化重组Fab

鼠抗HBsAg ScFv单克隆抗体与重组人源性抗HBsAg Fab抗体亲和力的ELISA测定结果见表1。结果显示，重组Fab抗体与ScFv单克隆抗体的亲和力非常强，完全可以用作亲和纯化的介质。

表1 重组Fab抗体与ScFv单克隆抗体亲和力的ELISA测定

Table 1 Affinity analysis of recombinant Fab antibody and ScFv mAb by ELISA

Concentration of ScFv Mab		1:1000	1:10000	1:50000	1:100000
Purified Fab		4.019	3.150	2.923	2.261
		3.232	2.538	2.407	2.597
Fermentation supernatnat		3.232	2.834	1.992	1.987
		3.032	2.049	1.868	1.506
Negative control			0.032		

ScFv单克隆抗体亲和层析纯化重组人源性抗HBsAg Fab抗体的SDS-PAGE分析如图2，该纯化方法可有效纯化重组Fab抗体，薄层扫描分析显示，用ScFv单克隆抗体亲和层析柱纯化的重组Fab抗体的纯度可达97.5%。而且用该方法纯化的回收率有了大幅度的提高，可达70%～85%。

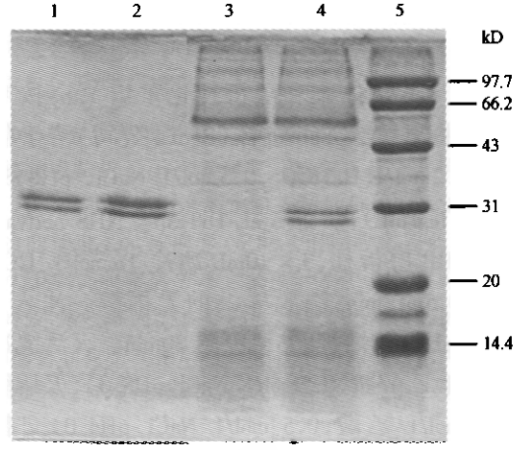

图2 ScFv单抗亲和层析柱纯化重组Fab抗体的SDS-PAGE分析

Fig.2 SDS-PAGE analysis of purified Fab fragment by ScFv Mab affinity chromatography column

2.3 离子交换层析纯化重组Fab

为了更进一步降低纯化成本，我们探索了用离子交换层析纯化重组Fab抗体的方法。重组人源性抗HBsAg Fab抗体的等电点测定值为7.5398，与理论预测的等电点值8.105相差不到一个pH单位。根据其等电点值设计了离子交换纯化方案。

首先用5mL的HiTrap™ DEAE-sepharose Fast Flow和HiTrap™ CM-sepharose Fast Flow预装柱对纯化条件进行探索，离子交换层析纯化重组Fab的SDS-PAGE分析如图3。可以看出，通过阴离子交换柱，目的蛋白全部位于上样穿流液中，比目的蛋白分子量大的杂蛋白几乎全部被阴离子交换柱吸附。将此阴离子交换穿流液上阳离子交换柱，其中的杂蛋白基本上全部从穿流液中流出。最后用NaCl对阳离子交换柱进行梯度洗脱就可得到纯的重组Fab抗体。此后，将离子交换纯化放大到30mL的离子交换柱。纯化结果的SDS-PAGE分析如图4，同样得到了很好的纯化效果。薄层扫描分析显示，通过离子交换纯化的重组Fab抗体的纯度可达97%。纯化样品的回收率为75%～85%。

2.4 纯化重组Fab抗体的性质分析及活性测定

不同纯化方法和发酵上清的Western-blot分析见图5，结果表明，所用的纯化方法均能纯化出所需要的目的产物。

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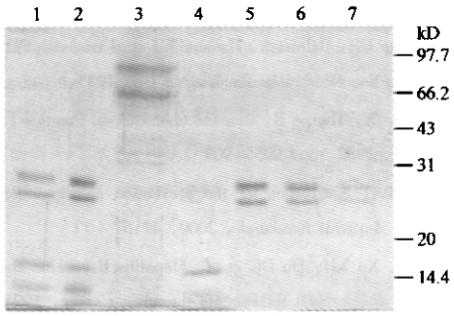

图 3　5mL 离子交换层析预装柱纯化重组 Fab 抗体的 SDS-PAGE 分析

Fig.3　SDS-PAGE analysis of purified Fab fragment by 5mL ion exchange chromatography columns

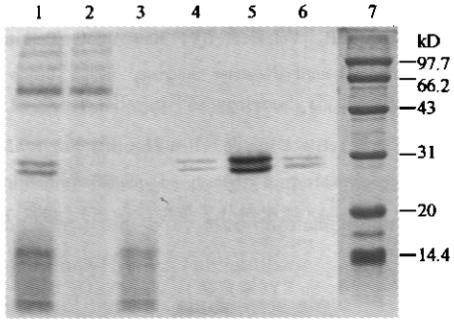

图 4　30mL 离子交换层析柱纯化重组 Fab 抗体的 SDS-PAGE 分析

Fig.4　SDS-PAGE analysis of purified Fab fragment using 30mL ion exchange column

重组人源性抗 HBsAg Fab 抗体的分子量经 MALDI-TOF-MS 测定的值为 50678.49D，与人源性抗 HBsAg Fab 抗体的一级结构求出的理论分子量(47914.65D)相比较，相差 2763.84。预示着酵母表达的重组 Fab 抗体可能存在着糖基化(大约相当于 15 个糖基)。而且，该 Fab 抗体一级结构中存在着 N-糖基化位点(Asn-X-Ser/Thr)和潜在的 O-糖基化位点。所以分子量的增加是由于糖基化造成的。

三种不同的方法纯化得到的重组 Fab 抗体的抗原结合活性见表 2 和图 6，纯化过程中重组 Fab 抗体的活性均能很好的保持，说明三种纯化方法对活性没有什么影响。纯化的重组 Fab 抗体的活性比发酵上清的高，表明通过纯化除去发酵上清中的杂质后更有利于重组 Fab 抗体与抗原的结合(其中阴性对照为转化有空载体 pPICZa-A 的 GS115 的诱导上清，没有阳性反应。阳性对照 HBIG 为人源性抗 HBsAg 的多抗。

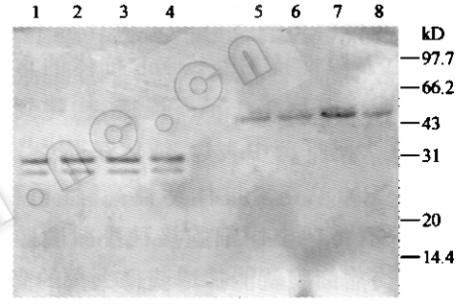

图 5　纯化的重组 Fab 抗体的 Western-blot 分析

Fig.5　Western-Blot analysis of purified Fab fragment

表 2　纯化的重组 Fab 抗体的 ELISA 分析

Table 2　Activity analysis of purified recombinant Fab antibody

	Blank control	Purified recombinant Fab antibody			Ferment supernatant	Positive control (HBIG)	Negative control
		Fab column	ScFv Mab column	Ion-exchange3 column			
Absorbence	0.0002	1.744	1.842	1.757	1.516	2.141	0.040
$A_{450/630}$	0.0004	1.890	1.867	1.874	1.675	2.057	0.034
Ratio to negative control		36.34	36.99	36.31	31.91	41.98	

(The ratio which is 2.1 times larger then the negative control was treated as positive. The value of negative control which is lower then 0.05 was treated as 0.05)

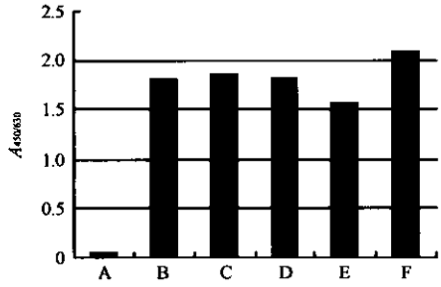

图 6　纯化重组 Fab 抗体的 ELISA 比较柱图

Fig.6　Compare of purified recombinant Fab antibody by ELISA analysis

A: negative control; B: Fab column; C: ScFv Mab column; D: ion-exchange column; E: ferment supernatant; F: positive control

3　讨论

重组 Fab 抗体的分离纯化是进一步对其性质、功能以及临床应用研究的基础，好的纯化方法要求纯度高、回收率高、活性保持好且简单快速。重组 Fab 抗体最常用的纯化方法是抗 Fab 抗体亲和层析[15-17]。

本实验采用 3 套纯化工艺对酵母表达的重组人源性 Fab 抗体的纯化进行了比较研究。抗 Fab 抗体亲和层析方法是目前采用较多的纯化重组 Fab 抗体

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的方法,利用该方法纯化出了纯度较高的重组 Fab 抗体,重组 Fab 抗体的纯度可达 96.8%,但该方法有一个最大的缺点就是成本高。国外有商品化的鼠抗人 Fab 抗体和相应的载体,但由于纯化过程中两者均需求量大,价格昂贵,所以商用的抗 Fab 抗体亲和层析仅限于实验研究用。而且,该亲和介质活性保持时间短,长时间使用则很容易导致抗 Fab 抗体的失活而造成纯化效率和回收率下降。本实验利用此法纯化重组 Fab 抗体的回收率仅为 30% ~ 40%。可能就是这个原因造成的。

为了充分利用资源,节约成本,本实验用来源于人源性抗 HBsAg Fab 的 ScFv 免疫小鼠,制备鼠抗 ScFv 的单克隆抗体。用该 ScFv 单克隆抗体作为亲和介质,对酵母表达的重组 Fab 抗体进行纯化,不论是纯度还是回收率均得到大幅提高。纯化效果比较理想。本实验用的重组 ScFv 与人源性抗 HBsAg Fab 抗体同源,由人源性抗 HBsAg Fab 抗体的重链可变区和轻链可变区基因借 Linker 连接,构建到原核表达载体中,在大肠杆菌中表达、纯化获得[18,19]。用该 ScFv 免疫制备的单克隆抗体活性高、性质稳定,且来源丰富,只需将杂交瘤细胞接种小鼠腹腔或者大量培养该杂交瘤细胞就能扩增获得大量的高亲和力的 ScFv 单克隆抗体。完全可以满足小规模的生产需要。

为了更进一步降低成本,本实验探索了离子交换纯化重组人源性抗 HBsAg Fab 抗体的纯化工艺。表达上清经两步离子交换纯化,得到了非常好的纯化效果。纯度和回收率均能达到要求。两步离子交换法进一步节约了纯化成本,样品处理量得到大幅度提高。该纯化工艺完全可以适用于大规模的重组 Fab 抗体的生产应用要求。

通过对重组人源性抗 HBsAg Fab 抗体的纯化方法的比较研究,获得了两套适于生产应用的纯化方法。通过比较研究,建立了成本最低、工艺简单的纯化方法,为重组人源性抗 HBsAg Fab 抗体的进一步生产应用奠定了基础。

REFERENCES(参考文献)

[1] Küttner G, Kramer A, Schmidtke G *et al*. Characterization of neutralizing anti-pre-S1 and anti-pre-S2 (HBV) monoclonal antibodies and their fragments. *Molecular Immunology*, 1999, **36**(10): 669 - 683

[2] Levy R, Miller RA, Tumor therapy with monoclonal antibodies. *Fed Proc*, 1983, **42**(9): 2650 - 2656

[3] Tam MR, Goldstein LC. In Manual of Clinical Microbiology, (Lennette EH, Balows A, Hausler WJ *et al* eds) 4th ED. pp905 - 909 Am. Soc Microbiol. Washington DC, 1985

[4] Johnstone A, Thorpe R, Immuno chemistry in Practice (Blachwell, OXford), 1982, pp. 905 - 909

[5] Sowyer LA. Antibodies for the prevention and treatment of viral disease. *Antiviral Reasearch*, 2000, **47**:57 - 77

[6] We YM, Xu XH, Hu DC *et al*. Hepatitis B vaccine and anti-HBs complex as approach for vaccine therapy. *Cancer*, 1995, **16**(3): 155

[7] Ditzel HJ, Binley JM, Moore JP *et al*. Nautral recombinant human antibodies to a comformation V2 and CD4-binding site sensitire epitope of HIV-1 gp120 isolated by using an epitope masking procedure. *J Immunol*, 1995, **15**(4): 893

[8] Hoogenboom HR, Volckaert GV, Raus JCM. Construction and expression of antibody-tumor necrosis factor fusion proteins. Molecular Immunol, 1991, **28**(9): 1027

[9] Gao H(高辉), Gao L(高磊), Ren ZL(任宗玲) *et al*. Screening and sequence analysis of a humanized anti-HBsAg Fab fragment. *J Cell Mol Immunol* (细胞与分子免疫学杂志), 1998, **14**(2):155 - 157

[10] Deng N(邓宁), Su KY(粟宽源), Wang XZ(王珣章) *et al*. The construction of expression vector of anti-HBsAg single chain Fab gene in pichia Pastoris, Acta scientiarum naturalium universitatis sunyatseni (中山大学学报-自然科学版), 2002, **41**(4):67 - 69

[11] Deng N(邓宁), Su KY(粟宽源), Wang XZ(王珣章) *et al*. The expression of humanized Fab fragment of the anti-HBsAg antibody in methylotropic yeast Pichia pastoris. *Chinese Journal of Biotechnology* (生物工程学报), 2002, **18**(5):546 - 550

[12] Deng N, Xiang JJ, Wang XZ *et al*. Expression, Purification and Characterization of Humanized Anti-HBs Fab Fragment. *Journal of Biochemistry*, 2003, **134** (5): 330 - 331

[13] Russo C, Callegaro L, Lanza E, Purification of IgG monoclonal antibody by caprylic acid precipitation. *J Immunol Methods*, 1983, **65**(2): 269 - 271

[14] In-Hak Choi, Jun-Ho Chun, Ik-Jung Kim. Generation of human Fab monoclonal antibodies against PreS2 of Hepatitis B Virus using antibody phage display library. *J Korean Soc Microbiol*, 1999, **34**(1): 21 - 30

[15] Maeda F, Nagatsuka Y, Ihara S *et al*. Bacterial expression of a human recombinant monoclonal antibody Fab fragment against hepatitis B surface antigen. *Journal of Medical Vurology*, 1999, **58**: 338 - 345

[16] Hu DP(胡栋平), Han HX(韩焕兴), You CX(尤长宜) *et al*. Affinity purification of engineered human monoclonal antibody HBs Fab fragments. *Acad J Sec Mil Med Univ*, (第二军医大学学报), 2000, **21**(1):50 - 52

[17] You CX(尤长宜), Luo RC(罗荣城), Ding XM(丁雪梅). Preparation of human anti-HBsAg Fab fragment by genetic engineering technology. *J Shanghai Immunology* (上海免疫学杂志), 2000, **20**(1):33 - 34, 37

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[18] Ren XR (任向荣), Xing S (熊盛), Tang YH (唐永红), *et al*. Expression and bioactivity of a human single chain Fv antibody against the HBsAg. *Chinese Journal of Immunology* (中国免疫学杂志), 2002, **18**(12):324 - 327

[19] Yan X (严兴), Rao GR (饶桂荣), Xiong S (熊盛), The purification of recombinant human anti-HBsAg scFv and the identification of its bioactivity and structure property. *Journal of South China University of Technoligy* (华南理工大学学报-自然科学版), 2002, **30**(6): 614 - 618

[20] Xia QC (夏其昌). Techniques and Development of Protein Chemistry《蛋白质化学研究技术与进展》. Beijing: Science Press, 1999

Study on the Methods of Purification of Recombinant Humanized anti-HBsAg Fab Antibody

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Abstract Anti-HBsAg Fab antibody is useful in prevention if it binds to and block surface regions of the virus, which is essential for the infection of hepatocytes. In order to establish steady and high effective methods to purify the recombinant humanized anti-HBsAg Fab antibody from fermentation supernatant of *Pichia pastoris* for industrial production, anti-Fab chromatography, anti-ScFv Mab chromatography and ion exchange chromatography were used. Results showed that the purity of recombinant anti-HBsAg Fab antibody purified by affinity chromatography of anti-Fab antibody column is 96.8%, and the recovery rate of the Fab antibody is about 30% ~ 40% and much of Fab fragment was lost. The purity of recombinant anti-HBsAg Fab antibody purified by affinity chromatography of anti-ScFv Mab column is 97.5% and the recovery rate of the Fab antibody is about 75% ~ 85%. This method can be used in smaller scale purification of recombinant Fab antibody from fermentation supernatant of recombinant yeast. The purity of recombinant Fab fragment purified by ion exchange chromatography is 97%, and the recovery rate of the Fab antibody is about 75% ~ 85%. The method of ion exchange chromatography can be used to produce the recombinant Fab antibody in large scale for industrial production. The results demonstrated that the recombinant humanized anti-HBsAg Fab antibody can be purified high effectively by the methods of anti-ScFv Mab chromatography and ion exchange chromatography from fermentation supernatant of recombinant yeast, and the methods of anti-ScFv Mab chromatography and ion exchange chromatography can be used effectively in industrial production.

Key words HBsAg, recombinant Fab, methods of purification, *Pichia pastoris*

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Establishment of Murine Embryonic Stem Cell Line Carrying Enhanced Green Fluorescence Protein and its Differentiation into Cardiomyocyte-like Cells *in vitro*

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Abstract The availability of EGFP ES cell D3 lines provided a tractable model to study cell differentiation and tissue generation *in vivo* and *in vitro*. Plasmid pEGFP N2 was introduced into the murine embryonic stem cell D3 by standard calcium phosphate precipitation. Transfected clones were screened out under the fluorescence microscope at the 488 nm emission light in the presence of G418. Strong fluorescent EGFP clones were singly picked out and further proliferated on a feeder layer of mitomycin-C treated mouse embryonic fibroblasts. One line of EGFP ES D3 cells subcultured twenty passages and still carried the EGFP DNA without the selecting pressure. It indicated that the gene might integrate into the ES genome or still dissociated in the cytoplasm. PCR analysis for EGFP DNA showed that undifferentiated EGFP ES cells at passage 8 and 18 carried the EGFP gene. Alkaline phosphatase staining, embryoid body and teratoma formation were performed to analyze the differentiation status and potential of the EGFP ES D3 cells. The cells derived from embryoid body were able to differentiate into beating cardiomyocytes with green fluorescence clearly observable under the confocal laser scanning microscopy. 30% ~ 40% of cells from embryoid bodies were capable to differentiate into cardiomyocyte-like cells, and it appeared lower than the non-transfected ES D3 cells, which could be 60% ~ 70% under the same conditions. The mechanism was currently unknown. Immunocytochemistry staining indicated that the contracting cells were cardiomyocytes based on the presence of cardiac specific molecular marker cTnT. Results showed that the stable EGFP positive ES cell line retained the typical characteristics of ES cells and possessed the pluripotential to differentiate into beating myocytes *in vitro*. The EGFP transfected cells stably yielding bright green fluorescence in real time and *in situ* rendered it was a powerful tool in cell transplantation and tissue engineering.

Key words mouse embryonic stem cells, green fluorescence protein, transfection, differentiation, cardiomycytes

Embryonic stem cells are pluripotent cells derived from the inner cell mass of pre-implantation mouse embryos. They have self-renewal ability and represent embryonic precursor cells that can differentiate into three embryonic germ layers *in vivo* and *in vitro*[1,2]. It is a tractable cellular system to investigate cellular and genetic programming of early development. In terms of clinical benefit, stem cells are generating many hopes for future regenerative medicine.

Mouse embryonic stem cells are widely used for gene mutation analysis or for incorporation of marker genes. Molecular markers are useful in studying gene expression profile during differentiation and in tracing and selecting particular subsets of cells[3,4]. The MES allows for studying genes of interest *in vitro*, but more importantly, by creating chimeras it is an extremely useful tool in analyzing genes during the embryo development *in vivo*. Eiges *et al* (2001) established a DNA transfection protocol for human ES cells. The transfected cells by ExGen 500 transfection system showed high levels

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of GFP expression limited to the undifferentiated cells, and the fluorescent cells could be separated from the differentiated cells by using a fluorescence-activated cell sorter[5]. In addition, some other GFP transfected ES cell lines are established by electroporation[6,7,8]. Here we describe the work that labeled the mouse ES cell lines with the green fluorescence protein through the standard calcium phosphate precipitation method and produced the stable EGFP ES D3 line. The EGFP ES D3 cell differentiated into cardiomyocytes-like cells *in vitro* under proper culture and treatment. The EGFP labeled clones of the ES cells will provide a useful tool for monitoring the differentiation status of the cells *in vitro* and *in vivo*.

1 Materials and Methods

1.1 Cell lines and animals

The ES cell line D3, purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, was used for all studies. Feeder cells are from ICR mouse embryonic fibroblasts (MEF), prepared from 13.5 dpc mouse embryos[9]. SNL cell line, is gifted by the College of Life Science Peking University, which is derived from the mouse STO cell line and resistant to G418. Balb/c nu mice were the recipients of the EGFP transfected ES cells to form teratoma to test the *in vivo* differentiating capacity of the EGFP ES D3 cells.

ES cells were cultured on a feeder layer of mitomycin-C treated MEF in Dulbecco's modified Eagle's medium (GIBCO-BRL), supplemented with 15% newborn bovine serum (Evergreen, Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.), 1 mmol/L glutamine (GIBCO-BRL), 0.1 mmol/L β-mercaptoethanol (Sigma), 1% nonessential amino acids stock (GIBCO-BRL), Penicillin (100 u/mL), Streptomycin (100 mg/mL) and leukemia inhibitory factor (LIF: 1000u/mL, Chemicon International Inc., Temecula, CA, USA).

1.2 Transfection of EGFP reporter gene and establishment of transgenic cell lines

The pEGFP N2 vector, a construct expressing the enhanced green fluorescent protein (EGFP) under the control of the human cymegalovirus (hCMV) promoter (purchased from Clontech) was used for transfection. The plasmid DNA was prepared according to the maximum extraction method[10]. The construct contained an SV40-driven neo selectable marker. The use of SV40 promoter in the system was sufficient to confer G418 resistance by driving the *neo* gene, although it was somewhat inefficient in mouse ES cells.

Sufficiently expanded and undifferentiated murine ES cells underwent stable transfection with pEGFP N2 plasmid DNA by the standard calcium phosphate precipitation method[10]. In following 24 ~ 48h, GFP expression was monitored under the fluorescence microscope (Diaphot, Nikon) equipped with mercury burner (HBO100W/2, Stromart:-DC, Osram). G418 (800 μg/mL) was then administered to the medium, allowing the selective propagation of transfected cells in culture, neo resistant fluorescent-labeled colonies were identified by a fluorescent microscope after one week (up to 10 colonies per well). Single transgenic colonies emitting strong green fluorescence were picked out by a micropipette, dissociated into small clumps, and transferred into a 96-well culture dish on a fresh MEF feeder. The cells continuously formed a large number of expanding undifferentiated colonies on a feeder layer of MEF. The different passages of the EGFP ES cells were examined under the fluorescence microscope.

1.3 DNA preparation and PCR analysis

Genomic DNA was extracted from undifferentiated EGFP ES cells at passage 8 and 18 respectively using standard method[10]. Genomic DNA from untransfected D3 cells was also extract as negative control in PCR. The primers for PCR were: 5′ CTGGTCGAGCTGGACGG-CGACG 3′, and 5′ CACGAACTCCAGCAGGACCATG 3′ (Clontech). PCR reaction was performed in a final volume of 50μL containing 50ng template, 1 μmol/L of sense and antisense primers, 200 nmol/L of dNTPs, 2u of Taq DNA polymerase, 1 × PCR buffer and 2.5 mmol/L $MgCl_2$ (Shanghai Sangon Co Ltd). Amplification was carried out by denaturing at 95℃ for 4min, followed by 30 cycles of 1min at 95℃, 1min at 58℃, 2min at 72℃, and finally 10min at 72℃. PCR products were separated in 1.5% agarose gels containing ethidium bromide, and bands were visualized by ultraviolet transillumination.

1.4 Analysis of EGFP labeled ES cells

1.4.1 Alkaline phosphatase assay: The Sigma Diagnostics Alkaline Phosphatase kit was used in the assay following the manufacturer's instruction. The activity of alkaline phosphatase in the ES cells was evaluated microscopically.

1.4.2 Embryoid body formation: The EGFP positive ES cells were grown in the presence of LIF to avoid the use of MEF as feeder layer. The undifferentiated cells were digested into small clumps and were induced to differentiate *in vitro* into embryoid bodies (EBs) by omitting LIF from the growth media, allowing aggregation in petri dishes. Following the formation of simple EBs by a 5-day cell aggregation procedure.

1.4.3 Differentiation *in vivo*: About 2×10^7 cells/mL EGFP positive ES cells were trypsinized and dissociated into single cells and resuspended in 0.3 mL PBS. ES cell suspension administered subcutaneously into the oxter of the male Balb/c nu mice. Teratoma was detectable after 2 weeks. Teratoma was prominent and mature after 30 ~ 50 days. The teratomas were surgically removed from the mice and fixed in 10% neutral formalin. Routine paraffin section were performed by the standard method and stained with hematoxylin and eosin. Sections were examined and pictured under microscope.

1.5 Differentiation into cardiomyocyte-like cells

1.5.1 Cardiomyocyte-like cells derived from EGFP transfected ES D3 cell line: After about 5 days of development in suspension culture, 5 EBs with similar sizes were picked out and each replated in a well of a 0.1% gelatin-coated 24-well plate in the absence of LIF. EBs were

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further spread and underwent differentiation into a monolayer cells in following days. After 7 to 8 days, cardiomyocyte-like beating activity was clearly visible, the active areas were examined and photographed under the confocal laser scanning microscopy (ZEISS LSM 510).

1.5.2 Cardiomyocytes primary culture: Cardiomyocytes were isolated from 1 ~ 5-day newborn mice as previous reports[11,12]. Briefly, the hearts of newborn mice were dissected and the basal part of the ventricle was collected. They were rinsed three times with PBS and mechanically dissected in the sterile dish. 1 mL 0.08% trypsin digestion solution was then added and the minced tissue was incubated at 37℃ for 10 ~ 15min. The tissue was gently triturated for several seconds until the tissue was dissociated into single cell or into very small clumps. Afterwards, cells and clusters were seeded into a culture flask containing coverslips, the flask was incubated at 37℃ overnight and used on the following day.

1.5.3 Immunocytochemistry: We used the ABC method to characterize the cardiomyocytes derived from the EGFP labeled ES cells. The differentiation cells and primary culture myocytes were washed with PBS twice and underwent fixation in 10% neutral formalin for 10min. A primary monoclonal antibody against Troponin T (Santa Cruz Biotechnology, Inc, cat sc-20025) was applied in a concentration of 1:200 in 10% NBS in PBS at RT for 1h to the fixed cells. The cells were then washed 3 times with PBS. Biotin-conjugated anti-mouse IgG (secondary antibodies) with 1:200 dilution in 10% NBS were added to the cells, and the mixture was incubated for 30min at RT. The cells were washed 3 times with PBS. Reagent A and B of the ABC test kit (Sino-American Biotechnology Company) were diluted 1:100 in 10% NBS in PBS. ABC compound was mixed in the same proportion of the reagent A and B and was then added to the cells and incubated at 37℃ for 30min. AEC of the AEC kit (Sino-American Biotechnology Company) was used as a chromogenic substrate. After 8 ~ 10min of staining, reaction was ended by washing the cells with bidistilled water.

2 Results

2.1 Transfection and EGFP positive colony screening

The plasmid pEGFP N2 were successfully introduced into murine D3 cells by standard calcium phosphate precipitation method. Expression of EGFP in the ES cells was detectable in as early as 24h after transfection. About 1% of the cells showed EGFP expression after 8-10 days culture in the presence of G418. These results indicated that calcium phosphate precipitation method is feasible for transfecting ES cells, and eytomegalovirus (CMV) promoter can drive the expression of EGFP gene in ES cells.

2.2 PCR for GFP DNA

The PCR results showed that three samples each contained EGFP DNA sequence (Fig. 1). The EGFP ES cells of passage 8 and passage 18 both contained the GFP gene. The PCR product was authentic GFP gene after sequencing verification of these cloned PCR product (data not shown).

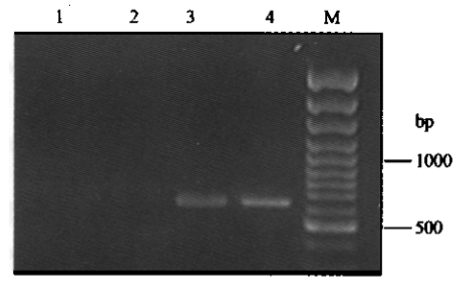

Fig. 1 GFP DNA of EGFP ES cells was amplified

1: control; 2: negative control, templates from the untransfected ES cells; 3 and 4: template from undifferentiated EGFP ES cells passage 8 and 18. The marker was GeneRuler™ 100bp DNA ladder plus (Shanghai Sangon Co Ltd), indicated to the right

2.3 The EGFP positive clone maintenance and morphological characteristics

We cloned the EGFP positive colonies by picking out the stable and strong EGFP expression clones. We further screened the picked clones by propagation up to 20 passages to choose those showed strong green fluorescence in each generation. The undifferentiated positive clones were maintained on MEF feeder layer. The EGFP colonies exhibited compact and clear edges in morphology. The EGFP positive clones grew on MEF feeder layer in the absence of G418 showed no apparent changes in proliferative capacity and in the fluorescent phenotype after long-term culture (Fig.2A).

2.4 Alkaline phosphatase assay

In mice, alkaline phosphatase staining is commonly used to evaluate pluripotency of ES cell. In the present study, similar staining was performed for characterizing EGFP positive ES cells clones. Most of the cells at passage 8 and 18 exhibited strong alkaline phosphatase (AP) activity. Whereas, those located at the edge of the ES colonies with larger sizes and differentiated morphologies showed weak activity (Fig.2F).

2.5 Differentiation *in vitro*

In vitro experiment showed that the EGFP positive clones maintained pluripotency and differentiation capacity. The ES cell clones were trypsinized to single cell or smaller clumps and seeded in culture dishes for 3 days in proper culture medium. These cells aggregated to form simple embryoid bodies (sEB) (Fig.2B). Continued culture of the sEB for 5 days longer, the two germ layers and cavities were formed and visible under the inverted microscope. These were mature EBs (maEBs). sEBs and maEBs still showed strong green fluorescence observable under the fluorescence microscope (Fig.2C).

2.6 Differentiation *in vivo*

EGFP positive ES cells were injected subcutaneously into the oxter of the Balb/c nu male mouse. Tumors were formed with a diameter of 1 ~ 2cm after about 30 ~ 50 days. Paraffin embedded sections were prepared from midplane of the tumor in about 3 ~ 5μm of

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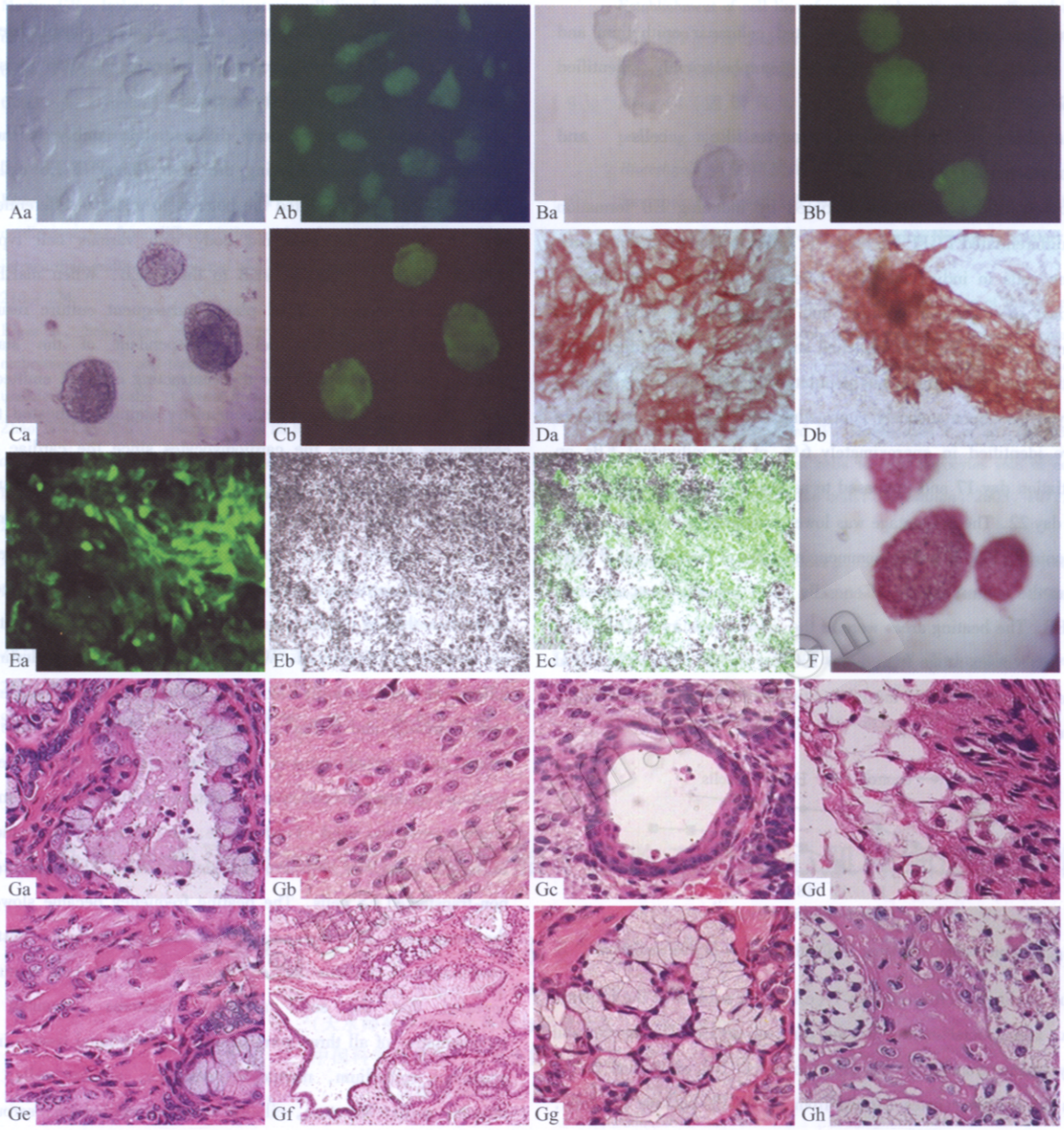

Fig.2 Identification of EGFP ES cells and the differentiation of cardiomyocyte-like cells

A: Passage 18, murine ES cells underwent stable transfection with EGFP. a. Bright field under the inverted microscope; b. Dark field under the fluorescence microscope. B、C: The transfected ES cells and their differentiated cell derivatives are shown: simple embryoid body (sEB). and mature embryoid bodies (maEBs). The B.a, C.a and B.b, C.b are bright and dark fields, respectively. Note that the undifferentiated cells, simple EBs and mature EBs are fluorescent. D: Characterization of immunostaining. Primary culture cardiomyocytes (D.a) and cardiomyocytes derived from EB (D.b) stained with antibodies against cTnT. E: Myocytes characterization under the confocal laser scanning microscopy. The cardiomyocytes derived from EB yielded bright green fluorescence under the confocal laser scanning microscopy at the wavelength of 488 nm (E.a) and the morphology of the bright field (E.b). E.c is the overlay of the E.a and E.b photos. F: The transfected EGFP ES cells exhibited strong alkaline phosphatase (AP) activity. G: Teratomas formed by the EGFP positive ES cell lines in male Balb/c nu mice. The EGFP positive ES cells after about 4 months of culture (passages 15) from about 60% ~ 70% confluent flask were injected subcutaneously into the oxter of 4-week-old male Balb/c nu mice (two or more mice). Six to eight weeks after injection, the resulting teratomas were examined histologically. (G. a) Ciliated columnar epithelium. (G.b) Neurocyte. (G.c) Typical stratified squamous epithelium. (G.d) Adipose cells. (G.e) Striated muscle. (G.f) Gutlike structures. (G.g) Serous gland. (G.h) Cartilage

thickness and stained with hematoxylin and eosin. Those sections indicated that the teratoma contained different types of tissues and cells of three germ layers. Squamous epithelium and neurocyte from ectoderm; cartilage, adipose cells, and striated muscle from

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mesoderm; and gutlike structure, ciliated columnar epithelium and serous gland from endoderm were morphologically identified (Fig.2G).

2.7 Acquiring the cardiomyocytes-like cells and immunocytochemistry test

Cardiac differentiation was initiated by inducing EB formation from undifferentiated EGFP ES cells. In order to monitor the presence of beating cells in individual EBs, EBs were seeded at low density after 7 days in suspension culture, and the locations of EBs in each well were recorded. The EBs attached and continued to proliferate and differentiate into a heterogeneous population of cells including beating cardiomyocytes. Spontaneously contracting cells appeared as clusters and were identified in approximately 6% of the individual EBs on differentiation day 17 and increased to as many as 30% ~ 40% of the EBs by day 22. The percentage was lower compared with 60% ~ 70% of the control (Fig.3). The commencing time of beating was 3 days later than the control sample. Generally 1 ~ 2 beating areas appeared in one EB. The beating areas were at the most of four in one EB. The beating frequencies range from 30 to 130 per minute. Under confocal laser scanning microscopy, the contracting cell clusters showed bright green fluorescence (Fig .2F).

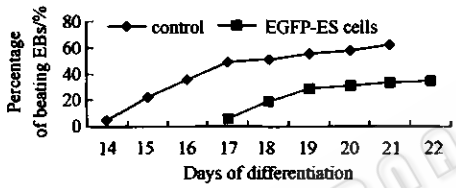

Fig.3 Percentage of beating EBs with the days of differentiation

The cardiac-specific troponin T (cTnT), a subunit of the troponin complex, is a special marker for the cardiomyocytes. The monoclonal antibody against troponin T was used to detect the expression of the cTnT in the cardiomyocytes derived from the EGFP positive ES cells. Immunostaining showed that cTnT was present only in the beating area of the culture, but not in undifferentiated EGFP ES cells or differentiated cultures without evidence of contraction (Fig.2.Eb). Similar positive result of immunostaining was also obtained from the primary culture of mouse cardiomyocytes (Fig.2.Ea). The presence of cTnT in the differentiated cells derived from the EGFP positive ES clones indicated they were indeed cardiomyocytes.

3 Discussion

From the experiment, we found that subculture of the EGFP ES cell lines can be carried out on the MEF without the presence of the G418, which was different to other reports[5-8]. PCR analysis showed that different passages EGFP ES cells cultured on the MEF still carried the GFP gene after 10 passages (from 8 to 18 passages). It seems that the transfected ES cells in our study expressed the green fluorescence protein without the selecting pressure indicating that the gene may integrate into the ES genome. The present study demonstrated that EGFP positive ES cells possessed strong alkaline phosphatase (AP) activity, most of the cells exhibited intensive alkaline phosphatase staining which is similar to other reports[2,13,14].

The *in vitro* and *in vivo* differentiation ability is the other important criteria for evaluating the pluripotency of ES cells. The EGFP labeled cells exhibited the potency to undergo differentiation *in vitro* by forming the embryoid body with various cell types and structures in steps from the sEB to the maEB. When maEBs were seeded in the gelatin coated plate, subsequent culture resulted in spontaneously contracting cells. Independent of the stages of development, sEB, maEB, or the contracting cells all emitted bright green fluorescence. Immunocytochemistry staining indicated that the contracting cells were the cardiomyocytes since the cardiac specific cTnT marker was present on these cells while the surrounding non-beating cells were negative to cTnT. The staining area was comparable to the newborn mouse cardionmyocytes primary culture. The percentage of cardiomyocytes differentiation of the EGFP ES cells was lower than that of control. And the commencing time of beating was not as early as the control. What was the mechanism underline these differences were currently unknown. We also found that both embryoid body formation and differentiation capacity of ES cells were closely related to the growth status of the ES cells and the serum in the culture medium. Stable differentiation percentage could be obtained only when proper serum was used. Furthermore, no difference was found in the beating frequency of the myocytes derived from EGFP ES cells and non-transfected ES cells. The beating frequency became slower when the temperature and freshness of the medium dropped. Histological examination of teratoma derived from the EGFP ES cells in nude mice revealed that the solid areas of teratoma contained tissues representative of all three germ layers. Differentiated tissues including squamous epithelium, neurocyte, cartilage, adipose cells, striated muscle, gutlike structure, ciliated columnar epithelium, serous gland were observed. This was similar to some related report[6,8,13,14].

To sum up, the above results completely revealed that the EGFP ES cells possess the typical characteristics of undifferentiated embryonic stem cell. And green fluorescence existed in the differentiated cells. The availability of EGFP ES cell lines provided a tractable model to study cell differentiation and tissue generation *in vivo* and *in vitro*. Transplantation of GFP-positive ES cells to murine embryos and injury mouse models are underway, which may pave ways to some new findings.

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REFERENCES

[1] Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature*, 1981, 292:154 - 156

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[2] Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *PNAS of the USA*, 1981, **78**:7634 – 7638

[3] Gossler A, Joyner AL, Rossant J. Mouse embryonic stem cells and reporter constructs to detect developmentally regulated genes. *Science*, 1989, **244**:463 – 465

[4] Klug MG, Soonpaa MH, Koh GY *et al*. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. *The Journal of Clinical Investigation*, 1996, **98**: 216 – 224

[5] Eiges R, Schuldiner M, Drukker M. Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. *Current Biology*, 2001, **11**:514 – 518

[6] Zhao WN(赵文宁), Meng GL(孟国良), Xue YF(薛友纺) *et al*. Labeling of three different mouse ES cell lines with the green fluorescence protein. *Chinese Journal of Genetics* (遗传学报), 2003, **30** (8):743 – 749

[7] Teng L(滕路), Meng GL(孟国良), Xing Y(邢阳) *et al*. Labeling embryonic stem cells with enhance green fluorescent protein on the hypoxanthineguanine phosphoribosyl transferase locus. *Chinese Medical Journal*(中华医学杂志), 2003, **116** (2):267 – 272

[8] Shen G(沈干), Cong XQ(丛笑倩), Wu Z(吴铮) *et al*. Establishment of cell line of mouse embryonic stem cells and its label with GFP. *The Journal of Southeast University* (*Medical Science Edtion*)[东南大学学报(医学版)], 2003, **22** (2):71 – 74

[9] Meng GL(孟国良), Shang KG(尚克刚). Improvement of preparing method of primary mouse embryonic fibroblast cells. *Biotechnology*(生物技术), 1997, **7**(2):38 – 39

[10] Sambrook J, Russell DW *et al*. Molecular Cloning: A Laboratory Manual. 3rd ed, New York: Cold Spring Harbor Laboratory Press, 2001

[11] Mathur A, Hong Y, Kemp BK *et al*. Evaluation of fluorescent dyes for the detection of mitochondrial membrane potential changes in cultured cardiomyocytes. *Cardiovascular Research*, 2000, **46**: 126 – 138

[12] Mummery CD, Oostwaard W, Doevendans P *et al*. Differentiation of human embryonic stem cells to cardiomyocytes role of coculture with visceral endoderm-like cells. *Circulation*, 2003, **107**:2733 – 2740

[13] Thomson JA, Itskovitz-Eldor J, Shapiro SS *et al*. Embryonic stem cell lines derived from human blastocysts. *Science*, 1998, **282**: 1145 – 1147

[14] Reubinoff BE, Pera MF, Fong CY *et al*. Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nature Biotechnology*, 2000, **18**: 399 – 404

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