

糖皮质激素诱导的亮氨酸拉链蛋白(GILZ)对小鼠骨髓间充质干细胞成骨分化影响研究

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摘要 探讨过表达糖皮质激素诱导的亮氨酸拉链蛋白(glucocorticoid-induced leucine zipper, GILZ)对小鼠骨髓间充质干细胞(bone marrow-derived mesenchymal stem cells, BM-MSCs)成骨分化的影响。以逆转录病毒为介导, 将绿色荧光蛋白(green fluorescent protein, GFP)基因和GILZ基因分别导入小鼠BM-MSCs中, 得到MSC-GFP和MSC-GILZ细胞。以MSC-GFP细胞为对照, 对MSC-GILZ细胞进行成骨分化诱导, 结果显示GILZ过表达显著增加了碱性磷酸酶的表达和矿化骨节的形成。RT-PCR和Western blot结果显示GILZ过表达显著增加了Runx2和Osterix的mRNA和蛋白质表达。结果表明GILZ过表达对小鼠BM-MSCs成骨分化的增强, 可能部分是通过促进成骨分化中两个关键的转录因子Runx2和Osterix的表达来实现的。

关键词 逆转录病毒; 糖皮质激素诱导的亮氨酸拉链蛋白; 骨髓间充质干细胞; 成骨分化

骨质疏松(osteoporosis)是由多种原因引起的一组骨病, 使得骨量减少、骨脆性增加、骨折的风险性增高。糖皮质激素诱导的亮氨酸拉链蛋白(glucocorticoid-induced leucine zipper, GILZ)是亮氨酸拉链蛋白家族的一个新成员, 属于转录因子TSC-22(transforming growth factor β -stimulated clone-22)家族^[1]。国外的研究显示GILZ具有显著增强小鼠骨髓间充质干细胞(bone marrow-derived mesenchymal stem cells, BM-MSCs)成骨分化的能力^[2], 国内尚未见相关报道。成骨细胞起源于BM-MSCs^[3], 本文主要研究了在逆转录病毒介导下, 将GILZ高效稳定导入小鼠骨髓MSC, 并检测GILZ过表达对成骨分化中两个关键的转录因子Runx2和Osterix的mRNA和蛋白质表达的影响, 有助于了解GILZ显著增强小鼠BM-MSCs成骨分化的分子机制, 以期为临床治疗骨质疏松疾病提供思路和实验依据。

1 材料与方法

1.1 材料

1.1.1 试剂 Recombinant Mouse TNF- α (#410-MT)购R&Dsystems公司(美国), Osterix抗体(#ab22552)购自Abcam Inc.公司(美国), Runx2单克隆抗体(#D130-3)

购自MBL International公司(美国), IRDye 800 anti-rabbit IgG (#611-132-122)购自Rockland Immunochemicals, Inc.公司(美国), IRDye 680 anti-mouse IgG (#C00512-04)购自Odyssey® Infrared Imaging System(美国), DMEM购自Hyclone公司(美国), 1-Step NBT/BCIP solution (#34042)购自Pierce Biotechnology公司(美国), TRIzol® 试剂购自Invitrogen公司(美国), Taq-Man反转录试剂和Power SYBR® Green PCR Master Mix (#4367659)购自Applied Biosystems公司(美国), 其它试剂购自Sigma公司(美国)。

1.1.2 仪器 Chromo-4 real-time RT-PCR仪器购自MJ research。

1.1.3 细胞与培养基 所用小鼠BM-MSCs为本实验室分离和保存, 来自6只18个月的C57BL/6雄性小鼠的骨髓, 先经培养富集贴壁细胞, 再分别采用结合有抗体CD45R/B220、CD11b和Sca-1的免疫磁珠筛选纯化, 得到CD45R/B220、CD11b阴性和Sca-1阳性的MSCs。所得小鼠BM-MSCs分别经成骨诱导、成脂

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诱导和成肌诱导培养, 发现其具有成骨分化、成脂分化和成肌分化的三向分化潜能, 详见本实验室已发表文章^[4]。表明用该方法得到的小鼠BM-MSCs是具有多向分化潜能的干细胞。在具体实验操作中发现其传代至25代仍具有三向分化潜能。MSC细胞在含10%胎牛血清(fetal bovine serum, FBS)的DMEM培养基中培养。

1.2 方法

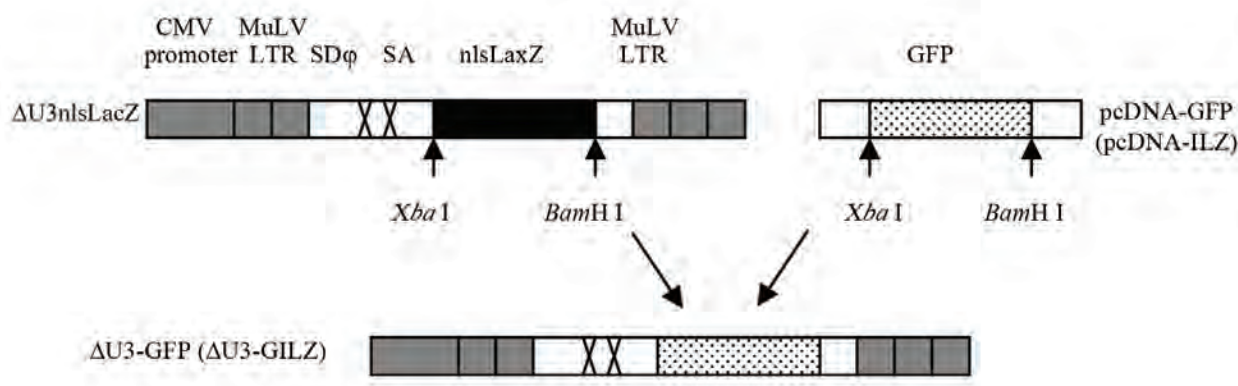
1.2.1 构建表达GFP和GILZ的逆转录病毒载体

GFP和GILZ的逆转录病毒载体(Δ U3-GFP和 Δ U3-GILZ)的构建参照参考文献[2]。简而言之, 将在 Δ U3nlsLacZ载体^[5]中的LacZ序列用限制性内切酶Xba I和BamH I切除, 再将GFP和GILZ cDNA插入。当用PCR扩增GFP cDNA时, 将Xba I和BamH I限制性酶切位点分别合并入PCR产物的5'端和3'端(图1)。

1.2.2 转染293GPG细胞 在60 mm培养皿中种植293GPG细胞, 待细胞生长至大约80%融合度时, 将293GPG培养基换为DMEM, 用Lipofectamine Plus Reagents将表达GFP和GILZ的逆转录病毒载体转染入293GPG细胞, 转染后6 h, 加入等量体积的含20%胎牛血清的DMEM, 分别在24, 48, 72 h收集含有逆转录病毒颗粒Ret-GFP和Ret-GILZ的培养液, 混合。Ret-GFP和Ret-GILZ的滴度分别为 7.9×10^5 cfu/mL和 6.7×10^5 cfu/mL, 均调整至 6.7×10^5 cfu/mL, -80°C 保存备用。

1.2.3 感染MSC细胞 将含有逆转录病毒颗粒Ret-GFP或Ret-GILZ的培养液分别加入培养的小鼠骨髓MSC中, 就可进行GFP或GILZ基因的表达。简而言之, 在60 mm培养皿中种植MSC细胞, 普通培养基中培养, 第2天细胞生长至大约50%融合度时, 除去培养基, 以感染复数(multiplicity of infection, MOI)为10 (感染效率为99%以上)的逆转录病毒颗粒Ret-GFP、Ret-GILZ各1.5 mL感染MSC, 同时培养基中各加入1.5 μL 10 mg/mL polybrene, 6~8 h后除去培养液, 换为普通培养基培养24 h即可出现绿色荧光。

1.2.4 成骨分化 按参考文献[4]的方法进行。简而言之, 将MSC-GFP和MSC-GILZ细胞分别以密度 $1 \times 10^4/\text{cm}^2$ 种植于96孔板, 每一组设置3个复孔, 在5% CO_2 的培养箱中 37°C 培养。第2天将完全培养基换成促成骨分化培养基(osteogenic medium, OS) (由DMEM、2% FBS、5 mmol/L β -磷酸甘油和50 $\mu\text{mol/L}$ 2-磷酸-L-抗坏血酸组成)。对于ALP染色, 用不含钙磷的PBS缓冲液洗涤细胞, 在室温下用4%多聚甲醛固定30 min, 用双蒸水洗涤3次。然后在室温下用一步NBT/BCIP溶液染色15 min, 用双蒸水洗涤, 然后用扫描仪扫描。对于ARS染色, 连续培养21 d, 隔天换液。用不含钙磷的PBS缓冲液洗涤细胞, 用70%冰乙醇固定1 h, 用水洗涤, 再用40 mmol/L Alizarin Red S (ARS) (pH4.2) 在室温下染色10 min。用水洗涤细胞5次, 再用PBS缓冲液室温下震动洗涤细胞15 min



PCR得到GFP和GILZ序列, 在两端加上Xba I和BamH I限制性内切酶位点, 然后克隆入病毒载体 Δ U3nlsLacZ的Xba I和BamH I酶切位点之间。GFP and GILZ sequences were produced by PCR, Xba I and BamH I restriction enzyme sites were added on both ends of the GFP or GILZ sequence, then GFP or GILZ sequence was cloned into the virus vector Δ U3nlsLacZ by replacement of nlsLacZ between the Xba I and BamH I enzymes cut sites.

图1 逆转录病毒质粒 Δ U3-GFP和 Δ U3-GILZ的构建

Fig.1 Construct of retrovirus plasmid Δ U3-GFP and Δ U3-GILZ

以减少非特异性的ARS染色,然后用扫描仪扫描。

1.2.5 免疫荧光细胞染色 培养细胞在无菌盖玻片上,在5% CO₂的培养箱中37 °C培养过夜。用PBS缓冲液洗涤细胞两次,在室温下用4%多聚甲醛固定15 min,用PBS缓冲液洗涤细胞两次,用0.2% Triton X-100透化处理15 min,用PBS缓冲液洗涤细胞两次。室温下用封闭缓冲液(PBS缓冲液中含1%牛血清)封闭1 h。加1:200稀释度的多克隆抗GILZ抗体在盖玻片上,室温下放置1 h,用PBS缓冲液洗涤细胞3次。加1:600稀释度的连接Cy3的羊抗兔IgG,室温下暗处放置1 h,用PBS缓冲液洗涤细胞3次。用300 nm的4',6-二脒基-2-苯吡啶盐酸染色液室温下染细胞核2 min。洗涤,将盖玻片反向放在加了5 μL SlowFade Gold antifade reagent的载玻片上,以透明指甲油封闭盖玻片四周。

1.2.6 RNA的提取和实时RT-PCR RNA的分离、反转录和PCR分析按参考文献[4]的方法进行。简而言之,根据制造商指南使用TRIzol® (Invitrogen公司,美国)试剂分离细胞RNA,用TaqMan Reverse Transcription Reagents (Applied Biosystems)反转录2 μg RNA,特定基因的mRNA水平用Power SYBR® Green PCR Master Mix (Applied Biosystems)和Chromo-4 real-time RT-PCR instrument (MJ research)来分析,设3个复孔。mRNA水平用β-actin作为内标来校正,基因表达以倍数的变化来显示(ΔΔCt方法)^[6]。PCR反应使用的引物序列如表1所示。

1.2.7 Western blot法检测Runx2和Osterix蛋白质的表达 将小鼠MSC-GFP和MSC-GILZ细胞分别在不含和含有1 ng/mL TNF-α的OS中培养7天,收集细胞裂解液,用等量的蛋白质进行10% SDS-PAGE。转至醋酸纤维素膜,5%脱脂奶粉封闭。用1:500稀释的抗鼠Runx2抗体或抗兔Osterix抗体在室温下孵育2 h。PBST洗膜3次,每次10 min。1:20 000稀释的IRDye 680

anti-mouse IgG或IRDye 800 anti-rabbit IgG抗体,室温下孵育1 h。PBST洗膜3次,每次10 min。用Odyssey Infrared Imaging System (LI-COR Biotechnology)扫描。

2 结果

2.1 表达GFP和GILZ的逆转录病毒高效感染MSC且稳定过表达

为了探讨逆转录病毒介导的GFP和GILZ能否在MSC中表达,我们用表达GFP和GILZ的逆转录病毒感染第5代MSC,荧光显微镜下观察GFP在MSC内的表达情况,免疫荧光细胞染色检测GILZ在MSC内的表达情况。结果显示:含有GFP和GILZ的逆转录病毒感染第5代MSC 24 h后,逆转录病毒介导的GFP和GILZ在骨髓MSC中高效表达,达到99% (图2)。证明逆转录病毒感染MSC得到稳定过表达GFP和GILZ的MSC-GFP和MSC-GILZ细胞。

2.2 GILZ过表达显著增强MSC的碱性磷酸酶表达和矿化骨节的形成

MSC细胞具有多向分化潜能,在合适的条件下可分化成为骨细胞、脂肪细胞等^[3]。为探讨MSC过表达GILZ后,是否影响其成骨分化的潜能。我们以MSC-GFP细胞为对照,对MSC-GILZ细胞进行成骨分化诱导,7天后进行碱性磷酸酶染色,21天后进行茜素红钙盐染色。结果显示:MSC-GILZ细胞碱性磷酸酶表达明显增高(图3A)、矿化骨节形成明显增高(图3B),表明GILZ过表达显著增强小鼠BM-MSCs的成骨分化。

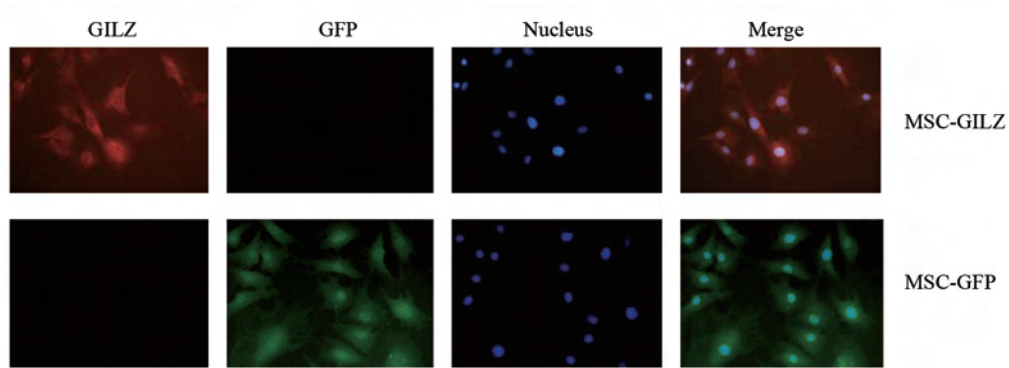
2.3 GILZ过表达显著增强MSC的Runx2和Osterix的mRNA和蛋白质表达

为了进一步探讨GILZ对成骨分化中两个关键的转录因子Runx2和Osterix的mRNA和蛋白质的表达的影响,我们分别采用RT-PCR和Western blot来检测MSC-GFP和MSC-GILZ细胞的Runx2和Osterix

表1 RT-PCR所用的引物序列

Table 1 Primer sequences used in RT-PCR

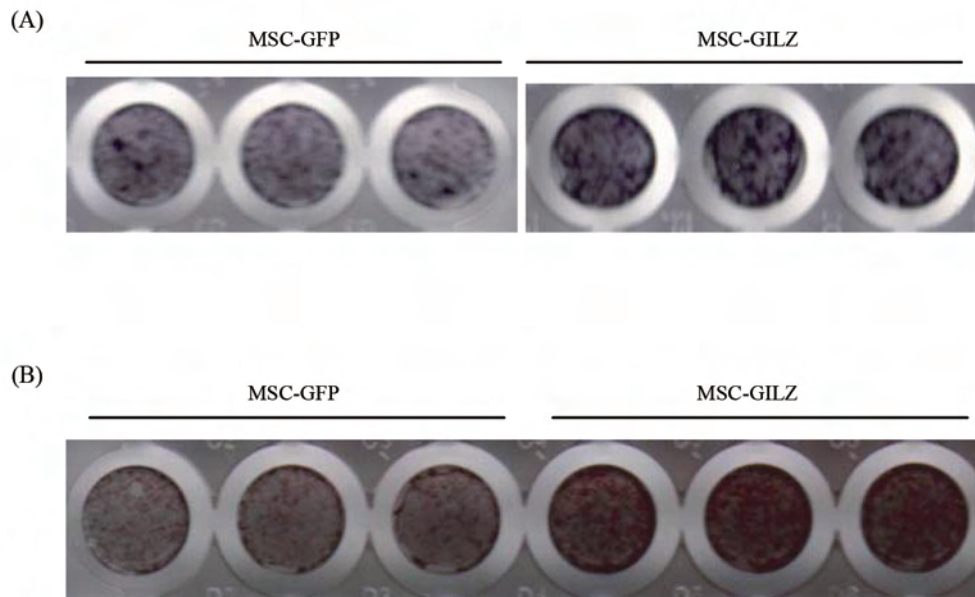
基因	基因库号码	正义链(5'-3')	反义链(5'-3')	产物大小(bp)
Genes	GenBank No.	Forward (5'-3')	Reverse (5'-3')	Size (bp)
Runx2	NM_009820	CCA CCA CTC ACT ACC ACA CG	TCA GCG TCA ACA CCA TCA TT	250
Osterix	NM_130458	ACC AGG TCC AGG CAA CAC	GCA GTC GCA GGT AGA ACG	373
β-actin	NM_007393	CTG GCA CCA CAC CTT CTA CA	GGT ACG ACC AGA GGC ATA CA	190



用表达GFP和GILZ的逆转录病毒(Ret-GFP和Ret-GILZ)感染MSC得到MSC-GFP和MSC-GILZ细胞。GFP的表达用荧光显微镜检测, GILZ的表达用抗GILZ的抗体通过免疫荧光标记来检测。

MSC-GFP and MSC-GILZ cells were prepared by infecting MSC with retroviruses expressing GFP (Ret-GFP) or GILZ (Ret-GILZ). GFP expression was monitored by fluorescence microscopy and GILZ expression was detected by immunofluorescence labeling with anti-GILZ antibody.

图2 MSC-GFP和MSC-GILZ细胞
Fig.2 MSC-GFP and MSC-GILZ cells

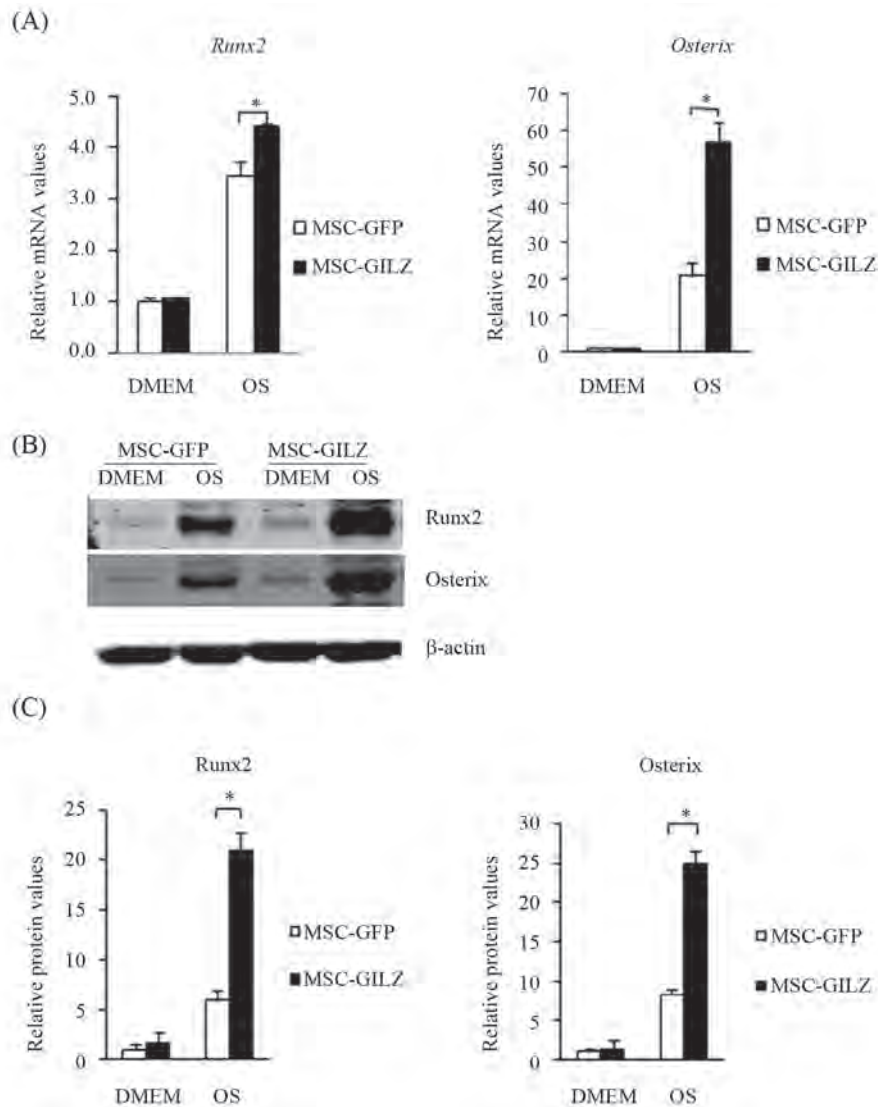


A, B: 如方法中所述培养和处理细胞; A: 第7天用ALP底物对细胞显色, 来检测ALP阳性细胞; B: 第21天用茜素红染色来检测矿化骨结的形成。
A, B: the cells were cultured and treated as the method described; A: cells were incubated with ALP substrate at day 7 to detect ALP positive cells; B: cells were stained with Alizarin Red S at day 21 to visualize mineralized bone matrix.

图3 GILZ增强小鼠骨髓间充质干细胞的成骨分化
Fig.3 GILZ enhances osteogenic differentiation of mouse BM-MSCs

的mRNA和蛋白质的表达。结果显示GILZ过表达显著增强Runx2和Osterix的mRNA (图4A)和蛋白质

(图4B)的表达。表明MSC过表达GILZ增强其成骨分化潜能。



A-C: 实时定量RT-PCR和Western blot分别分析小鼠MSC-GFP和MSC-GILZ细胞*Runx2*和*Osterix*基因的mRNA和蛋白质的表达。将小鼠MSC-GFP和MSC-GILZ细胞分别以密度 $1 \times 10^4/\text{cm}^2$ 种植于完全培养基中, 第二天换成促成骨分化培养基(osteogenic media, OS), 培养7天; A: 提取总RNA, 对等量RNA (2 μg)进行反转录, 分析*Runx2*和*Osterix*基因的mRNA水平, 引物见表1。所得数据用 β -actin来校正, 将在OS中培养的MSC-GFP细胞的mRNA水平设定为1, 以相对于OS中培养细胞的mRNA水平的比值变化来表示($\Delta\Delta\text{Ct}$ 方法)。误差线显示标准偏差, $*P < 0.01$; B: Western blot分析*Runx2*和*Osterix*的蛋白质水平。用针对*Runx2*和*Osterix*的抗体分析每份样品中等量的蛋白质; C: 柱状图对图B进行定量分析。

A-C: mouse MSC-GFP and MSC-GILZ cells were plated at a density of 1×10^4 cells/ cm^2 in regular growth media at the previous day, then switched to osteogenic media (OS) and cultured for 7 days; A: total RNA were extracted, equal amounts of RNA (2 μg) were reverse transcribed and the mRNA levels of the indicated genes were analyzed using SYBR Green master mixture and primers listed in Table 1. Data were normalized to β -actin and expressed as -fold changes relative to the mRNA levels of MSC-GFP cells in OS ($\Delta\Delta\text{Ct}$ method). Error bars indicate standard deviation, $*P < 0.01$; B: *Runx2* and *Osterix* protein levels were analyzed by Western blot. Equal amount of the proteins were detected with anti-*Runx2* and anti-*Osterix* antibodies; C: bar graph showing quantitative results of B.

图4 GILZ增强小鼠BM-MSCs的*Runx2*和*Osterix*的mRNA和蛋白质表达

Fig.4 GILZ enhances *Runx2* and *Osterix* mRNA and protein expression of mouse BM-MSCs

3 讨论

骨质疏松给很多患者的生活带来极大的不便和痛苦, 治疗收效很慢, 一旦骨折又会危及生命, 是一个不容忽视的健康问题。GILZ是1997年意大利科

学家D'Adamio等^[7]在用地塞米松处理的小鼠胸腺淋巴细胞内发现的, 属于转录因子TSC-22家族^[1], 此家族蛋白的成员含有三个截然不同的结构域(图1): N端结构域、中间的亮氨酸拉链结构域、C端的多聚

脯氨酸富集结构域。GILZ具有多种功能: Yang等^[8]研究显示GILZ介导糖皮质激素(glucocorticoid, GC)的抗炎效果, BM-MSC过表达GILZ会抑制炎症因子TNF- α 和IL-1 β 诱导的COX-2的表达, 而且用小发夹RNA (small hairpin RNA, shRNA)降低GILZ会减少GC对COX-2表达的抑制。进一步的研究显示GILZ通过阻止NF- κ B的核转位来抑制COX-2的表达。研究结果表明, GILZ是一种重要的GC抗炎的介导者, 在抗炎治疗中有很大的潜力; Shi等^[9]进一步的研究显示GILZ可结合到编码PPAR- γ 2 (peroxisome-proliferator-activated receptor- γ 2)基因启动子CCAAT增强子结合蛋白(CCAAT/enhancer-binding protein, C/EBP)的串联重复序列上, 抑制其转录, GILZ也抑制编码成脂肪细胞分化的标志基因C/EBP- α 、脂蛋白脂肪酶(lipoprotein lipase)和脂肪细胞蛋白酶(adipsin)的表达; Zhang等^[2]的研究显示GILZ是MSC成骨细胞分化和成脂肪细胞分化的调节者, 过表达GILZ可以使MSC成骨细胞分化和成脂肪细胞分化之间的平衡倾向于成骨分化方向, 结果表明GILZ修饰的干细胞可能用于代谢性骨疾病如骨折修复的治疗。

成骨细胞起源于骨髓间充质干细胞, BM-MSCs是多能性的, 在合适的条件下能分化成为截然不同的细胞系, 如骨细胞、脂肪细胞、肌细胞等^[3]。BM-MSCs向成骨细胞分化需要转录因子的协同作用。*Runx2*和*Osterix*是调节成骨分化和骨形成的两个关键性的转录因子, Otto等^[10]研究表明, *Runx2*纯合子突变小鼠出生后不久会死于呼吸衰竭, 分析其骨骼, 发现此种小鼠没有成骨细胞和骨骼; 杂合子突变小鼠显示出特有的骨骼异常, 表明*Runx2*基因对于成骨分化和骨形成是必需的; Nakashima等^[11]的研究发现*Osterix*基因敲除老鼠没有骨形成, 表明*Osterix*基因对于成骨分化和骨形成是必需的。Zhang等^[2]的研究表明过表达GILZ可显著增强小鼠BM-MSCs的*Runx2*的mRNA表达。本文研究显示过表达GILZ可显著增强小鼠BM-MSCs的*Runx2*和*Osterix*的mRNA和蛋白质的表达。

总之, 实验以逆转录病毒为介导成功地构建过表达GILZ的MSC细胞(MSC-GILZ), 并研究了GILZ对小鼠BM-MSCs成骨分化的影响, 阐明GILZ对小

鼠BM-MSCs成骨分化的增强可能部分是通过促进成骨分化中两个关键的转录因子*Runx2*和*Osterix*的表达来实现的, 为GILZ治疗骨质疏松进一步研究提供了思路和实验依据。

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Effects of Glucocorticoid-induced Leucine Zipper (GILZ) on the Osteogenic Differentiation of Mouse Bone Marrow-derived Mesenchymal Stem Cells

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Abstract To investigate the effect of glucocorticoid-induced leucine zipper (GILZ) overexpression on mouse bone marrow-derived mesenchymal stem cells (BM-MSCs) osteogenic differentiation, green fluorescent protein (GFP) and *GILZ* genes were delivered into BM-MSCs mediated by retrovirus and MSC-GFP and MSC-GILZ cells were prepared. MSC-GILZ cells were cultured in osteogenic medium (OS) using MSC-GFP cells as a control group. The results showed that GILZ significantly increased ALP expression and bone nodules formation. Real-time RT-PCR and Western blot results showed that GILZ significantly increased *Runx2* and *Osterix* mRNA and protein expression. The results indicated that GILZ enhanced mouse BM-MSCs osteogenic differentiation partly through the increasing expression of *Runx2* and *Osterix* which are the two key transcription factors of osteogenic differentiation.

Key words retrovirus; glucocorticoid-induced leucine zipper (GILZ); bone marrow-derived mesenchymal stem cells (BM-MSCs); osteogenic differentiation

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