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山东大学
SHANDONG UNIVERSITY

博士学位论文

Dissertation for Doctoral Degree

(专业学位)

论文题目: 神经肽 Y 在骨折愈合中的作用
及成骨机制研究

Effect of neuropeptide Y on Bone Formation in Fracture
Healing and the Underlying Biological Mechanism

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目 录

中文摘要	1
英文摘要	10
符号说明	20
研究背景	23
第一部分 神经肽Y在小鼠骨折愈合中的作用及相关机制研究	28
前 言	28
材料与方法	30
结 果	38
讨 论	43
结 论	47
附 图	48
附 表	61
参考文献	62
第二部分 神经肽Y在小鼠前成骨细胞MC3T3-E1中的表达和生物学功能	71
前言	71
材料与方法	72
结 果	84
讨 论	87
结 论	89
附 图	90
附 表	97
参考文献	98
第三部分 神经肽Y上调Runx2、Osterix表达促进小鼠前成骨细胞MC3T3-E1成骨的机制研究	102
前 言	102
材料与方法	103
结 果	107
讨 论	109

结 论.....	112
附 图.....	113
附 表.....	115
参考文献.....	116
综述：神经肽Y在骨与能量合成中的生物学功能及研究进展.....	119
致 谢.....	131
攻读学位期间发表的学术论文	132
学位论文评阅及答辩情况表	133
英文文章一	134
英文文章二	154

CATALOG

Abstract in Chinese	1
Abstract in English.....	10
Symbolic decription	20
Background	23
Part I Effect and mechanism of neuropeptide Y on fracture healing of mice	28
Introduction	28
Materials and Methods.....	30
Results	38
Discussion	43
Conclusions	47
Figures	48
Tables	61
References	62
Part II Expression and Biological function of NPY in the Mouse Pre-osteoblast Cell Line MC3T3-E1	71
Introduction	71
Materials and Methods.....	72
Results	84
Discussion	87
Conclusions	89
Figures	90
Tables	97
References	98
Part III NPY upregulates Runx2 and Osterix and enhances osteogenesis in the Mouse Pre-osteoblast Cell Line MC3T3-E1	102
Introduction	102
Materials and Methods.....	103
Results	107

Discussion	109
Conclusions	112
Figures	113
Tables	115
References	116
Reviews: Biological function and research progress of neuropeptide Y in bone and energy metabolism	119
Acknowledgements	131
Published papers.....	132
Paper evaluation table	133
English paper I	134
English paper II	154

神经肽 Y 在骨折愈合中的作用及成骨机制研究

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中文摘要

研究背景

随着全球老龄化进程的加快，骨质疏松带来的骨折及社会、家庭负担逐渐加重。此外，严重的创伤性骨折、肿瘤切除、先天性疾病会导致巨大的节段性骨缺损，给临床工作带来很多困难。生长因子（growth factors）、多肽（peptides）及小分子（small molecules）联合人造骨移植材料（Synthetic bone graft substitutes）可以明显诱导成骨细胞的转移、分化和成熟。研究表明超过 2cm 的骨折后缺损就需要生物活性因子的介入，否则将难以愈合。因此，生长因子、多肽及小分子正成为国内外研究骨折缺损修复的热点。

神经肽 Y (neuropeptide Y, NPY) 是位于大脑和周围神经系统的重要神经递质。NPY 具有促进骨折愈合、改善膝关节骨性关节炎、治疗骨质疏松脆性骨折的多重作用，是一种重要的骨骼神经营养因子。NPY 在周围组织骨细胞、成骨细胞、破骨细胞中可通过直接和间接激活多个通路来促进骨合成及代谢。NPY 可能会参与增强成骨细胞介导的骨合成、抑制破骨细胞介导的骨吸收，是骨折愈合的关键因子。

此外，NPY 对关节软骨修复、维护骨代谢均衡也具有关键的作用。研究表明，关节滑膜、关节软骨、半月板和软骨下骨等关节组织都含有丰富的神经肽。NPY 是重要的交感神经肽，其对髋、膝等关节的神经滋养作用正受到越来越多的关注，并被认为是改善骨性关节炎（osteoarthritis, OA）的潜在治疗靶点。

同时，NPY 是治疗绝经后骨质疏松症（Postmenopausal osteoporosis, PMO）的重要神经营养因子。在人体及啮齿类动物模型中，NPY 通过 NPY Y1 受体（NPY receptor Y1, NPY1R）和 NPY Y2 受体（NPY receptor Y2, NPY2R）调节体内骨量平衡。NPY 沉默的小鼠在面对冷刺激时表现为体内皮质酮水平升高，骨量丢失，其骨丢失速度是野生型小鼠的 3 倍；其分子生物学机制是沉默 NPY 的小鼠

体内中枢和外周组织缺乏 NPY, 不能通过其相应的 NPY2R 受体抑制皮质酮的产生, 继而对抗应激反应造成的骨量丢失。换言之, NPY 具有在应激状态下促进体内骨合成的作用。然而, NPY 通过什么信号通路调控骨折后骨代谢及骨形成, 仍有待于进一步研究。

综上所述, 探究 NPY 与机体成骨的相应分子生物学机制, 对改善骨折后巨大骨缺损的预后具有重要意义及研究价值。本研究体内实验中, 通过建立 C57BL/6 小鼠胫骨骨折模型, 探究了 NPY 及成骨相关基因在骨折初期的细胞分布及表达情况, 揭示出 NPY 可参与骨折早期炎症反应, 可能起到骨诱导的作用; 在体外实验中, 我们合成了 NPY 小干扰 RNA (siRNA) 及 NPY 过表达质粒, 并将其转染入小鼠前成骨 MC3T3-E1 细胞, 进一步检测了 MC3T3-E1 细胞成骨相关基因和蛋白的表达情况, 揭示了 NPY 基因通过上调 Runx2、Osterix 基因表达促进细胞成骨的分子生物学机制。

第一部分 神经肽 Y 在小鼠骨折愈合中的作用及相关机制研究

研究目的:

1. 检测小鼠胫骨闭合骨折愈合过程中 NPY 在小鼠血清中的表达情况。
2. 检测小鼠骨折组织愈合过程中成骨相关因子 NPY, NPY1R, Runx2, Osterix mRNA 及蛋白表达情况。
3. 分析 NPY 在小鼠骨折愈合中的生物学效应。

研究方法:

1. 建立 C57BL/6 小鼠的闭合胫骨骨折模型

以 18 只 SPF 级 C57BL/6 雄性小鼠为实验对象, 在异氟醚诱导麻醉下人工掰断一侧胫骨建立小鼠胫骨闭合骨折模型, 密切监测小鼠饮食及饮水情况。

2. 动态观察小鼠骨折过程中骨痂形成、重塑

分别于建模后第 0、1、7、14 天, 随机取 3 只小鼠, 进行 IVIS®SpectrumCT 检测, 观察骨折部位骨痂形成情况。

3. 检测小鼠骨折愈合过程中血清 NPY 的表达情况

分别于建模后第 1、7、14 天, 随机取出 3 只小鼠, 取血后, 采用酶联免疫吸附测定法 (enzyme linked immunosorbent assay, ELISA) 检验 C57BL/6 小鼠血清中 NPY 的表达水平。

4. 检测小鼠骨折愈合过程中成骨相关基因及蛋白的表达

分别于建模后第 1、7、14 天, 随机取出 3 只小鼠处死, 并取骨折处组织提取 RNA, 应用 RT-PCR 技术检测组织样本中 NPY, NPY1R, Runx2, Osterix mRNA 的表达。

取上述小鼠骨折处组织, 切片石蜡包埋, 行苏木精/伊红染色 (hematoxylin-eosin staining, HE) 或固定在 70%乙醇中分别行 NPY, NPY1R, Runx2, Osterix 的免疫组织化学染色 (Immunohistochemical staining, IHC)。利用 AI 图像分析系统 (Aipathwell) 自动读取石蜡切片免疫染色区域图像, 分析测定阳性染色的灰度值。

研究结果:

1. 成功建立了 C57BL/6 小鼠胫骨闭合骨折模型

通过大体观察小鼠生活状态, 术后第 1 天, 小鼠运动较少, 可能小鼠患处有

轻微疼痛,但不影响进食和饮水。术后第2天,小鼠可逐渐活动。术后2周,小鼠患肢即可负重行走,有时可见双侧肢体同时站立的情况。提示患肢术后2周有足够强度及硬度支撑体重。

2. 宏观及微观角度观察动物模型中骨折愈合良好

从宏观角度,我们应用 IVIS[®]SpectrumCT 三维成像系统动态观测小鼠骨折愈合情况。CT 显示术后14天骨折线模糊不清,骨折端骨性骨痂形成,提示建模成功。从微观角度我们应用 HE 染色,观察建模第14天骨折处出现初级骨化中心,并可见新生骨小梁结构,骨性骨痂形成提示建模成功。

3. 动物模型中血清NPY表达情况

应用ELISA法检测了小鼠骨折后14天细胞外血清中NPY的表达情况。NPY不同时间点(建模后第1、7、14天)浓度范围为925.35pg/ml-1055.2pg/ml。NPY浓度在检测的14天的时间段内有一过性下降后逐渐增高趋势,但整体差异无明显统计学意义($P=0.152$)。

4. 小鼠骨折愈合过程中成骨相关基因及蛋白表达情况

应用 RT-PCR 法检测了小鼠骨折愈合过程中成骨相关基因 mRNA 表达情况,结果显示: NPY mRNA 在骨折早期纤维性骨痂组织中高表达,在软骨痂中中等表达,在骨痂中低表达。建模后第7、14天 NPY mRNA 的表达量分别是第1天的27%和9%,差异具有统计学意义($P<0.001$)。NPY1R mRNA 表达总体差异性不显著($P=0.133$)。建模后第7、14天 NPY1R mRNA 的表达量分别是第1天 NPY1R mRNA 的1.6倍和3.8倍。建模后第7、14天 Runx2 mRNA 的表达量分别是第1天 Runx2 mRNA 的7.9倍和65.6倍。建模后第7、14天 Osterix mRNA 的表达量分别是第1天 Osterix mRNA 的124.0倍和775.1倍。

应用蛋白免疫组化染色和 AI 图像分析系统做了小鼠骨折愈合过程中成骨相关蛋白分析,结果表明: NPY 蛋白在骨折早期纤维性骨痂组织中高表达,建模后第7、14天 NPY 蛋白表达量分别是第1天 NPY 蛋白的37%和55%,差异有统计学意义($P<0.05$)。NPY1R 蛋白在小鼠骨折早期组织中高表达。建模后第7、14天 NPY1R 蛋白表达量分别是第1天 NPY1R 蛋白的8.2%和21%,差异有统计学意义($P<0.001$)。建模后第7、14天 Runx2 蛋白表达量分别是第1天 Runx2 蛋白的15%和19%,差异具有统计学意义($P<0.001$)。建模后第7、14天的 Osterix 蛋白表达量分别是第1天 Osterix 蛋白的12%和10%,差异有统计学意义。

义 ($P < 0.001$)。

研究结论:

1. NPY 基因及蛋白共同参与骨折早期炎症反应, 可能起到骨诱导的作用。
2. 骨折愈合相应成骨因子 NPY、NPY1R、Runx2 和 Osterix 的表达存在时间及空间特异性。

第二部分 神经肽 Y 在小鼠前成骨细胞 MC3T3-E1 中的 表达和生物学功能

研究目的:

1. 检测过表达或干扰 NPY 后在 MC3T3-E1 细胞中 NPY mRNA 和蛋白的表达情况。
2. 观测 NPY 对 MC3T3-E1 细胞成骨分化的影响。

研究方法:

1. 构建 3 种 NPY siRNA 序列

构建针对 NPY 基因的 siRNA1、siRNA2、siRNA3 序列。应用 RFect siRNA 转染试剂法转染后, 应用反转录定量 (RT-qPCR 法) 检测转染 3 种 siRNA 48 小时后细胞 NPY mRNA 的表达情况, 筛选出干扰效率最高的 NPY siRNA 序列。

2. 构建 NPY 过表达质粒并测序验证

构建 NPY 过表达质粒, 测序验证后应用 RFect DNA 转染试剂将 NPY 过表达质粒及对照质粒载体转染入 MC3T3-E1 细胞, 验证 NPY 细胞内表达情况。

3. 检测不同干预组 MC3T3-E1 细胞中 NPY mRNA 的表达情况

将 NPY siRNA、scrambled siRNA、NPY 过表达质粒、空白质粒分别转染入 MC3T3-E1 细胞中, 应用 RT-qPCR 法于第 4、7 天检测 NPY mRNA 的表达情况。

4. 检测过表达或干扰 NPY 后 MC3T3-E1 细胞中 NPY 蛋白的表达情况

按照分组分别于 MC3T3-E1 细胞中加入对应的干扰或过表达质粒, 应用半定量蛋白检测 (Western blot) 法于第 4、7 天检测各组中 NPY 的蛋白表达情况。

5. 观察过表达或干扰 NPY 后 MC3T3-E1 细胞成骨变化

复苏 MC3T3-E1 细胞，加入成骨诱导培养液培养，按照分组分别加入对应的干扰或过表达质粒，14 天后，分别行茜素红、碱性磷酸酶（ALP）、 β -catenin 免疫荧光染色后镜下观察细胞成骨情况。

研究结果：

1. 成功筛选出干扰效率最佳的 NPY siRNA

成功制备三种包含目的基因 NPY 的 siRNA1、siRNA2、siRNA3，验证 NPY siRNA3 在胞内干扰效率最高，为 75%。因此，选用 siRNA3 做后续实验。

2. 成功制备 NPY 过表达质粒

NPY 过表达质粒构建成功后，测序验证符合 NPY 序列。

3. 过表达及干扰 NPY 后检测细胞 NPY 基因表达的影响

过表达 NPY 可显著促进细胞 NPY mRNA 的表达，第 4 天增加了 3.9 倍，第 7 天增加了 4.3 倍 ($P < 0.001$)。应用 siRNA 干扰 NPY 后，可显著减低 NPY mRNA 的表达，在第 4 天下降了 75% ($P < 0.05$)，第 7 天下降了 55%。

4. 过表达及干扰 NPY 后检测细胞 NPY 蛋白表达的影响

过表达 NPY 可显著促进细胞 NPY 蛋白的表达，第 4、7 天，NPY 蛋白分别增加了 1.2 倍和 1.5 倍 ($P < 0.05$)。应用 siRNA 干扰 NPY 后，可显著降低细胞 NPY 蛋白的表达，第 4、7 天，NPY 蛋白分别降低了 45% 和 35% ($P < 0.05$)。

5. 过表达及干扰 NPY 后检测细胞成骨分化的影响

茜素红染色显示过表达 NPY 后 MC3T3-E1 细胞数目增多，体积较大，核染色较深，胞内粒子增多，可见较多的紫红色钙结节；应用 siRNA 干扰 NPY 后，细胞数目较少，核染色较浅，可见较少的紫红色钙结节。ALP 染色显示过表达 NPY 后细胞数目增多，体积较大，核染色较深，胞内黄色钙盐沉积较多，可见较多蓝色颗粒或块状沉淀。应用 siRNA 干扰 NPY 后，细胞数目较少，核染色较浅，蓝色颗粒较少。 β -catenin 免疫荧光细胞染色显示过表达 NPY 后细胞核数目增多，细胞质内绿色荧光较多，应用 siRNA 干扰 NPY 后，细胞核数目较少，胞质内绿色荧光较少。

研究结论：

1. 干扰 NPY 可抑制 MC3T3-E1 细胞成骨分化。
2. 过表达 NPY 可促进 MC3T3-E1 细胞成骨分化。

第三部分 神经肽 Y 上调 Runx2、Osterix 表达促进小鼠 前成骨细胞 MC3T3-E1 成骨的机制研究

研究目的:

揭示 NPY 上调 MC3T3-E1 细胞成骨特异性转录因子 Runx2、Osterix 表达, 促进 MC3T3-E1 细胞成骨分化的机制。

研究方法:

1. 过表达及干扰 NPY 后检测 MC3T3-E1 细胞中 Runx2、Osterix、ALP、OCN mRNA 的表达情况

将 NPY siRNA 和 NPY 过表达质粒分别转染入 MC3T3-E1 细胞中, 应用 RT-qPCR 法于第 4、7 天观察 Runx2、Osterix、ALP、OCN mRNA 的表达情况。

2. 过表达及干扰 NPY 后检测 MC3T3-E1 细胞中 Runx2、Osterix、ALP、OCN 蛋白的表达情况

将 NPY siRNA 和 NPY 过表达质粒分别转染入 MC3T3-E1 细胞中应用 Western blot 法于第 4、7 天检测各组中 Runx2、Osterix、ALP、OCN 的表达情况。

研究结果:

1. NPY 上调 MC3T3-E1 细胞 Runx2、Osterix 基因的表达

1.1 过表达或干扰 NPY 后检测细胞 Runx2 和 Osterix 基因表达的影响

过表达 NPY 可明显上调 MC3T3-E1 细胞 Runx2 和 Osterix 基因的表达, Runx2 mRNA 较对照组在第 4、7 天分别增加了 5.4 倍和 6 倍。Osterix mRNA 在第 4、7 天分别增加了 2.7 倍和 3 倍。应用 siRNA 干扰 NPY 后, Runx2 mRNA 在第 4、7 天分别下降了 50% 和 35%。Osterix mRNA 在第 4、7 天分别下降了 80% ($P<0.001$) 和 55%。

1.2 过表达或干扰 NPY 后检测细胞 Runx2 和 Osterix 蛋白表达的影响

过表达 NPY 可显著上调 MC3T3-E1 细胞 Runx2 和 Osterix 蛋白的表达, Runx2 蛋白较对照组第 4、7 天分别增长了 1.6 和 1.2 倍; Osterix 蛋白表达第 4、7 天分别增长了 1.6 和 2.1 倍 ($P<0.05$)。应用 siRNA 干扰 NPY 后, Runx2 蛋白表达第 4、7 天分别下降了 40% 和 55%; Osterix 蛋白表达第 4、7 天分别下降了 60% 和 55% ($P<0.05$)。

2. NPY 促进 MC3T3-E1 细胞成骨分化

2.1 过表达或干扰 NPY 后检测细胞 ALP 和 OCN 基因表达的影响

过表达 NPY 显著上调 MC3T3-E1 细胞 ALP 和 OCN 基因的表达, ALP mRNA 较对照组第 4、7 天分别增加了 3.7 倍和 4.9 倍, OCN mRNA 在第 4 天和第 7 天分别增加了 4.0 倍和 4.3 倍($P<0.001$)。应用 siRNA 干扰 NPY 后, ALP mRNA 在第 4、7 天分别降低了 56% ($P<0.05$)和 26%, OCN mRNA 第 4 天和第 7 天分别降低了 60%($P<0.05$)和 35%。

2.2 过表达或干扰 NPY 后检测细胞 ALP 和 OCN 蛋白表达的影响

过表达 NPY 明显上调 MC3T3-E1 细胞 ALP 和 OCN 蛋白的表达, ALP 蛋白较对照组第 4、7 天均增加了 1.6 倍; OCN 蛋白第 4、7 天分别增加了 1.4 和 1.6 倍($P<0.05$)。应用 siRNA 干扰 NPY 后, ALP 蛋白第 4 天和第 7 天分别减少了 60% 和 60%; OCN 蛋白第 4、7 天分别减少了 64%和 78%($P<0.05$)。

研究结论:

1. NPY 通过上调 Runx2、Osterix 基因的表达促进 MC3T3-E1 细胞成骨。
2. NPY 有望成为创伤后促进骨折愈合的靶点。

关键词: 骨折, 骨合成, 神经肽 Y, Runx2, Osterix

课题的创新性和局限性

课题的创新性:

(1) 本课题系统阐述了小鼠骨折愈合早、中期不同细胞 NPY、NPY1R、Runx2 和 Osterix 的 mRNA 及蛋白分布表达情况, 并部分揭示了 NPY 可参与骨折早期炎症反应, 可能起到骨诱导的作用。

(2) 本课题成功筛选出干扰效率最高 NPY siRNA 并制备了 NPY 过表达质粒, 验证了 NPY 在小鼠前成骨细胞 MC3T3-E1 中的表达和生物学功能, 从而为深入研究 NPY 的分子生物学机制创造了条件。

(3) 本课题揭示了 NPY 可上调 Runx2 和 Osterix 表达促进小鼠前成骨细胞 MC3T3-E1 的生长、分化, 阐明了 NPY 调控前成骨细胞成骨分化的潜在生物学机制。

课题的局限性:

(1) 体外细胞学实验研究了单一成骨细胞系, 后续可增加人体原代培养成骨细胞系, 来进一步阐述 NPY 的生物学效应。

(2) 本研究仅建立了创伤性骨折模型, 而未建立骨质疏松脆性骨折模型, 今后可行进一步的研究来阐述 NPY 在不同类型骨折中的作用及相关机制。

Effect of neuropeptide Y on Bone Formation in Fracture Healing and the Underlying Biological Mechanism

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Abstract

Background:

With the coming of aging population, the brittle fracture caused by osteoporosis is increasing year by year, which has caused a heavy economic burden to the family and society. Furthermore, tumor resection, cancer, severe trauma or congenital diseases can lead to huge segmental bone defects, it has caused a lot of difficulties for clinical work. Synthetic bone graft substitutes combined with bioactive molecules, such as peptides, growth factors and small molecules, can improve the delivery of bone precursor cells, as well as subsequent bone formation and metabolism. Bioactive molecules are required in patients with larger bone defects (>2 cm). For this purpose, growth factors, peptides and small molecules are currently being evaluated at the pre-clinical and clinical levels. Based on previous findings, combining Neuropeptide Y (NPY) with the appropriate corresponding bone scaffold may bring about effective clinical translations and novel insights into the future of bone fracture.

Neuropeptide Y(NPY) is a 36-amino acid peptide that is a neurotransmitter located in the brain and peripheral nervous system. NPY is an essential skeletal neurotrophic factor and plays a principal role in promoting fracture healing, improving knee osteoarthritis, and treating osteoporotic brittle fracture. Furthermore, NPY can promote bone metabolism through direct and indirect activation of multiple pathways in osteocytes, osteoblasts and osteoclasts. Additionally, NPY enhances osteoblast mediated extracellular osteogenesis and inhibits osteoclast mediated bone resorption and remodeling, which are key factors of fracture healing.

Furthermore, NPY plays an important role in articular cartilage repair and bone metabolism. Studies have found that there are abundant neuropeptides in synovium, articular cartilage, meniscus and subchondral bone. NPY is an important sympathetic

neuropeptide, and its neurotrophic effect on hip and knee joints is attracting more and more attention. NPY is considered as a potential therapeutic target for osteoarthritis (OA).

Additionally, NPY is an important neurotrophic factor in the treatment of postmenopausal osteoporosis (PMO). In human and rodent models, NPY regulates bone mass balance through NPY receptor Y1 (NPY1R) and NPY receptor Y2 (NPY2R). Mice lacking the anxiolytic factor NPY showed more anxious behavior and elevated corticosterone levels. Furthermore, due to the inhibition of osteoblast activity, the bone loss of NPY silenced mice was 3-fold greater than that of wild-type mice under 6-week restraint and cold stress. The stress-protective NPY pathway acts specifically through NPY2R. In other words, NPY can promote bone formation under stress. However, the molecular mechanism by which NPY regulates the osteogenesis of MC3T3-E1 remains unclear, which needs further study.

In conclusion, it is of great significance to investigate the osteogenic molecular biological mechanism of NPY in promoting bone defect healing after fracture. In this study, we established a C57BL/6 mouse tibial fracture model to investigate the distribution and expression of NPY and osteogenic related genes in the early stage of fracture in vivo. It was suggested that NPY was involved in the early inflammatory reaction of fracture and may play a role in bone induction. In vitro, we synthesized NPY small interfering RNA (siRNA) and NPY overexpression plasmid, and transfected them into mouse pre-osteoblast MC3T3-E1 cells. We further detected the expression of osteogenic related genes and proteins, and revealed the molecular biological mechanism of NPY gene promoting osteogenesis by up regulating Runx2 and osterix gene expression.

PART I

Effect and mechanism of neuropeptide Y on fracture healing of mice

Objective:

1. To investigate the serum expression of NPY gene in a C57BL/6 mouse model of tibial closed fracture.
2. To detect the gene and protein expression of osteogenic related factors neuropeptide Y(NPY), neuropeptide Y1 receptor (NPY1R), Runx2, Osterix at different time points of fracture healing in mice.
3. To analyze the biological effect of NPY on fracture healing in mice.

Methods:

1. Model establishment

Eighteen SPF C57BL/6 male mice were selected as experimental subjects. The unilateral tibial fracture was artificially broken without fixation under isoflurane induced anesthesia. The mice were kept and monitored in containers with free access to distilled water and food.

2. Dynamic observation of callus formation and remodeling in the process of fracture in mice

Three mice were randomly taken out under isoflurane induced anesthesia and tested by IVIS[®]SpectrumCT at 0, 1, 7 and 14 days after surgery, respectively, to observe the callus growth at the fracture site.

3. The expression of serum NPY in the fracture healing

Blood was randomly collected from three inner canthus of mice at 1, 7, and 14 days, and the concentration of NPY in serum was detected by ELISA.

4. The expression of osteogenic genes in the fracture healing

Three mice were randomly killed at 1, 7, and 14 days after modeling. At 1, 7 and 14 days, the fracture tissue were taken to extract RNA. The mRNA expressions of NPY, NPY1R, Runx2 and osterix were detected by RT-PCR.

The fracture tissue was extracted and paraffin-embedded sections were performed for hematoxylin eosin staining (HE) or fixed in 70% ethanol for immunohistochemical staining (IHC) of NPY, NPY1R, Runx2 and osterix, respectively. The relative protein was detected by AI image analysis system (alpathwell) after immunohistochemical staining (IHC).

Results:

1. Successfully established the model of closed tibial fracture

Through gross observation, we found that on the 1st day after the operation, the mice did not move a great amount and may have shown a little pain, but it did not affect their eating and water intake. On the 2nd day after operation, the mice could move gradually. The walking function of mice could be initially established at 14 days, and sometimes both limbs of mice could stand at the same time. It is suggested that there is bony connection in fracture.

2. Macroscopic and microscopic observation showed that the tibial fracture healed well in the animal model

From a macroscopic point of view, we used IVIS[®]SpectrumCT to monitor the situation in 14 days after operation. CT showed that the fracture line was indistinct and callus formed at the fracture end, indicating that the modeling was successful. From the microscopic point of view, HE staining showed that primary ossification center and new bone trabecular structure were found at the healing site at 14 days.

3. Changing trend of serum NPY concentration in C57BL/6 mice after operation

ELISA was intended to explore the expression of NPY in the serum of mice. NPY concentrations ranged from 925.35pg/ml-1055.2pg/ml at different time points (1, 7 and 14 days after modeling). The concentration of NPY had a transient decrease and then gradually increased in the 14 days, but the overall difference was not statistically significant ($P=0.152$).

4. Expression levels of osteogenic related genes and proteins during fracture healing in mice

RT-PCR was intended to explore the mRNA expression level of osteogenesis related genes in the process of fracture healing in mice. NPY mRNA was highly expressed in blood callus, moderately expressed in cartilage callus, and lowly expressed in bone callus. The expression of NPY mRNA was decreased by ~0.27-fold at day 7 and 0.09-fold at day 14, in comparison with the day 1 ($P<0.001$). There was no significant difference in NPY1R mRNA expression ($P=0.133$). The expression of NPY1R mRNA was increased by ~1.6-fold at day 7 and 3.8-fold at day 14 ($P=0.059$). The expression of Runx2 mRNA was elevated by ~7.9-fold at day 7 and ~65.6-fold at day 14, in comparison with the day 1. The expression of Osterix mRNA was elevated by ~124.0-fold at 7 days and ~775.1-fold at day 14, in comparison with the day 1.

Immunohistochemical staining and AI image analysis system were used to analyze osteogenic related proteins in the process of fracture healing in mice. NPY protein was highly expressed in the early stage of fracture. The expression of NPY protein was decreased by ~0.37-fold at day 7 and 0.55-fold at day 14, in comparison with the day 1 ($P<0.05$). The expression of NPY1R protein was decreased by ~0.08-fold at 7 days and ~0.21-fold at day 14, in comparison with the day 1 ($P<0.001$). The expression of Runx2 protein was decreased by ~0.15-fold at day 7 and ~0.19-fold at day 14, in comparison with the day 1 ($P<0.001$). The expression of osterix protein was decreased by ~0.12-fold at day 7 and ~0.10-fold at day 14, in comparison with the day 1.

Conclusions:

1. NPY gene and protein are involved in the early inflammatory response of fracture and may play a role in osteoinduction.
2. The expression of osteogenic factors NPY, NPY1R, Runx2 and osterix is specific in time and space.

PART II

Expression and Biological function of NPY in the Mouse

Pre-osteoblast Cell Line MC3T3-E1

Objective:

1. To investigate the mRNA and protein expression after overexpression or interference of NPY in mouse pre-osteoblast cell line MC3T3-E1 in vitro.
2. To detect the effect of NPY in osteogenic differentiation of MC3T3-E1 cells in vitro.

Methods:

1. Construction of three NPY siRNA sequences.

The siRNA1, siRNA2 and siRNA3 sequences targeting NPY gene were constructed. They were transfected into pre-osteoblast cell line MC3T3-E1 by rfect siRNA transfection reagent respectively. Reverse transcription quantitative (RT-q) PCR was intended to explore the expression of NPY mRNA in pre-osteoblast cell line MC3T3-E1. To verify and screen out the NPY siRNA with the best interference efficiency.

2. Construction of NPY overexpression plasmid and sequencing verification

Overexpression plasmid targeting NPY gene were constructed. After sequencing verification, rfect DNA transfection reagent was used to transfect NPY overexpression plasmid and control plasmid vector into MC3T3-E1 cells to verify NPY expression.

3. The expression of NPY mRNA in MC3T3-E1 cells of different intervention groups

NPY siRNA, scrambled siRNA, NPY overexpression plasmid and empty vector plasmid were transfected into MC3T3-E1 cells respectively. Reverse transcription quantitative (RT-q) PCR method was performed to observe the NPY mRNA expression of MC3T3-E1 cells in the groups at 4, 7 days.

4. The expression of NPY protein in MC3T3-E1 cells of different intervention groups

NPY siRNA and NPY overexpression plasmid were transfected into MC3T3-E1 cells. Western blot method was performed to evaluate NPY protein expression in the groups at 4, 7 days.

5. Observe the changes of MC3T3-E1 cells staining after overexpression or interference of NPY.

MC3T3-E1 cells were resuscitated and cultured in osteogenic induction medium. NPY siRNA and NPY overexpression plasmid were added respectively. At 14 days, alizarin red staining, ALP staining and β -catenin immunofluorescence staining were performed respectively for microscopic observation.

Results:

1. Screening of NPY-siRNA sequences with the highest interference efficiency

The siRNA1, siRNA2, siRNA3 containing the target gene NPY were successfully constructed. NPY siRNA3 possessed the highest interference efficiency, up to 75%. As a result, siRNA3 was used for subsequent experimentation.

2. Construction of NPY overexpression plasmids

After the construction of NPY overexpression plasmid, the sequence of NPY was confirmed by sequencing.

3. Effects of NPY overexpression or interference on NPY gene expression

Overexpression of NPY dramatically elevated the expression of NPY mRNA in pre-osteoblast cell line MC3T3-E1 by ~3.9-fold and ~4.3-fold at 4, 7 days, respectively, compared with the control group ($P<0.001$). NPY siRNA decreased the expression of NPY mRNA by ~0.75-fold at 4 days ($P<0.05$) and ~0.55-fold at 7 days, compared with the control group.

4. Effects of NPY overexpression or interference on NPY protein expression

Overexpression of NPY significantly increased the expression of NPY protein by ~1.2-fold and 1.5-fold at 4, 7 days, respectively, compared with the control group ($P < 0.05$). NPY siRNA decreased the expression of NPY protein by ~0.45-fold and 0.35-fold at 4, 7 days, respectively, compared with the control group ($P < 0.05$).

5. Effects of overexpression or interference of NPY on osteoblast differentiation

Alizarin red staining showed that there were more purplish red calcium nodules in MC3T3-E1 cells after overexpression of NPY, and less purplish red calcium nodules after interference of NPY. ALP staining showed that there were more yellow calcium deposits and more blue granules in the cells after overexpression of NPY, and less blue granules after interference of NPY. Immunofluorescence staining of β -catenin showed that there was more green fluorescence in the cytoplasm after overexpression of NPY, and less green fluorescence in the cytoplasm after interference of NPY.

Conclusions:

1. The interference of NPY can inhibit the osteogenic differentiation of MC3T3-E1 cells.
2. Overexpression of NPY promotes osteogenic differentiation of MC3T3-E1 cells.

PART III

NPY upregulates Runx2 and Osterix and enhances osteogenesis in the Mouse Pre-osteoblast Cell Line MC3T3-E1

Objective:

To explore the biological mechanism of NPY upregulating the expression of osteoblast specific transcription factors Runx2 and osterix to promote the osteogenesis of MC3T3-E1 cells in vitro.

Methods:

1. The expression of Runx2, osterix, ALP and OCN mRNA after overexpression or interference of NPY in MC3T3-E1 cells

NPY siRNA and NPY overexpression plasmid were transfected into MC3T3-E1 cells. The mRNA expressions of Runx2, osterix, ALP and OCN were observed by reverse transcriptional quantitative (RT-q) PCR at 4, 7 days.

2. The expression of Runx2, osterix, ALP and OCN proteins after overexpression or interference of NPY in MC3T3-E1 cells

NPY siRNA and NPY overexpression plasmid were transfected into MC3T3-E1 cells. The expression levels of Runx2, osterix, ALP and OCN proteins in the groups were investigated by Western blot at 4, 7 days.

Results:

1. NPY upregulated Runx2 and osterix expression in MC3T3-E1 cells.

1.1 Effects of overexpression or interference of NPY on Runx2 and osterix mRNA expression

Overexpression of NPY significantly increased the expression of Runx2 and Osterix mRNA in MC3T3-E1 cells. Runx2 mRNA was increased by ~5.4-fold and 6-fold at 4, 7 days, respectively, compared with the control group. Osterix mRNA was increased ~2.7-fold and ~3-fold on at 4, 7 days, respectively, compared with the control group.

NPY siRNA decreased the expression of Runx2 and osterix mRNA. Runx2 mRNA was decreased by ~0.50-fold and ~0.35-fold at 4, 7 days, respectively, compared with the control group. Osterix mRNA was decreased by 0.80-fold ($P < 0.001$) and 0.55-fold at 4, 7 days, respectively, compared with the control group.

1.2 Effects of overexpression or interference of NPY on Runx2 and osterix proteins expression

Overexpression of NPY significantly increased the expression of Runx2 and Osterix proteins in MC3T3-E1 cells. Runx2 protein expression was increased by ~1.6-fold and ~1.2-fold at 4, 7 days, respectively, compared with the control group. Osterix protein expression increased by ~1.6-fold and ~2.1-fold at 4, 7 days, respectively, compared with the control group ($P < 0.05$).

NPY siRNA decreased the expression of Runx2 and osterix proteins. Runx2 protein expression was decreased by ~0.40-fold and ~0.55-fold at 4, 7 days, respectively, compared with the control group. Osterix protein expression was decreased by ~0.6-fold and ~0.55-fold at 4, 7 days, respectively, compared with the control group ($P < 0.05$).

2. NPY is critical for osteoblastic differentiation.

2.1 Effects of overexpression or interference of NPY on ALP and OCN mRNA expression

Overexpression of NPY significantly promoted the expression of ALP and OCN mRNA. At 4, 7 days, ALP mRNA was increased by ~3.7-fold and 4.9-fold, and OCN mRNA was increased ~4.0-fold and ~4.3-fold, respectively, compared with the control group ($P < 0.001$).

NPY siRNA decreased the expression of ALP and OCN mRNA. At 4, 7 days, ALP mRNA was decreased by ~0.56-fold and ~0.26-fold, and OCN mRNA was decreased by ~0.60-fold and ~0.35-fold, respectively, compared with the control group. The differences were statistically significant at 4 days ($P<0.05$).

2.2 Effects of overexpression or interference of NPY on ALP and OCN proteins expression

Overexpression of NPY significantly promoted the expression of ALP and OCN proteins. At 4, 7 days, OCN protein expression was increased by ~1.4-fold and ~1.6-fold, and ALP protein expression was increased by ~1.6-fold, respectively, compared with the control group ($P<0.05$).

NPY siRNA decreased the expression of ALP and OCN proteins. At 4, 7 days, ALP protein expression was decreased by ~0.60-fold at 4, 7 days, respectively. OCN protein expression was decreased by ~0.64-fold and ~0.78-fold at 4, 7 days, respectively ($P<0.05$).

Conclusions:

1. NPY may promote the osteogenesis of MC3T3-E1 cells by upregulating the expression of Runx2 and osterix genes.
2. NPY is expected to be a target for the treatment of huge bone defects in traumatic fractures.

Key words: fracture, bone formation, Neuropeptide Y, Runx2, Osterix.

Innovations and Limitations

Innovations:

- (1) The study systematically described the mRNA and protein expression of NPY, NPY1R, Runx2 and osterix in different cells at the early and middle stages of fracture healing in mice, and partially revealed that NPY participated in the early inflammatory reaction of fracture, which may play the role of bone induction.
- (2) In the study, we successfully screened out the most efficient NPY siRNA and prepared the NPY overexpression plasmid, which verified the expression and biological function of NPY in the mouse pre-osteoblast cell Line MC3T3-E1.
- (3) The study revealed that NPY may promote the osteogenesis of MC3T3-E1 cells by upregulating the expression of Runx2 and osterix genes.

Limitations:

- (1) In vitro cytological experiments, we only studied a single osteoblast line, and then we can add human primary osteoblast to further elaborate the biological effects of NPY.
- (2) In vivo animal studies we only established the traumatic fracture model, but we did not establish the osteoporotic brittle fracture model. In the future, we can further study the role of NPY in different fractures and elaborate the related mechanism.

符号说明

缩略语	英文名称	中文名称
NPY	neuropeptide Y	神经肽 Y
siRNA	small interference RNA	小干扰 RNA
Runx2	runt-related transcription factor 2	runt 相关转录因子 2
RIPA	Radio Immunoprecipitation Assay	RIPA 裂解液
PMSF	Phenylmethylsulfonyl fluoride	苯甲基磺胺氟
ALP	Alkaline phosphatase	碱性磷酸酶
OC	osteocalcin	骨钙素
OPN	Osteopontin	骨桥蛋白
NGF	nerve growth factor	神经生长因子
SP	substance P	P 物质
CGRP	calcitonin gene-related peptide	降钙素基因相关肽
VIP	vasoactive intestinal peptide	血管活性肠肽
OVX	ovariectomized	卵巢切除
BDNF	brain-derived neurotrophic factor	脑源性神经营养因子
eNOS	endothelial NO synthase	内皮型一氧化氮合酶
VEGF	vascular endothelial growth factor	血管内皮生长因子
DMSO	dimethyl sulfoxide	二甲基亚砷
PTH	parathyroid hormone	甲状旁腺激素
HRP	horseradish peroxidase	辣根过氧化物酶
BMSCs	bone marrow mesenchymal stem cells	骨髓间充质干细胞
FBS	fetal bovine serum	胎牛血清

ISC	intestinal stem cells	肠干细胞
NPY1R	NPY receptor Y1	NPY Y1 受体
NPY2R	NPY receptor Y2	NPY Y2 受体
NPY4R	NPY receptor Y4	NPY Y4 受体
NPY5R	NPY receptor Y5	NPY Y5 受体
KD	kilodalton	千道尔顿
NICD	notch1 intracellular domain	notch1 胞内结构域
BMSCs	bone marrow-derived stromal cells	骨髓间充质干细胞
BMP	bone morphogenetic protein	骨形态发生蛋白
APA	alkaline phosphatase activity	碱性磷酸酶活性
12×SBE-Oc-pGL3	BMP/Smad-responsive construct	BMP/Smad 响应结构的转活化
LSD	Least Significant Difference	最小显著性差异法
ANOVA	Analysis of Variance	方差分析
OD	optical density	光密度
ELISA	Enzyme linked immunosorbent assay	酶联免疫吸附测定
Pi	pixel	像素
IOD	Integral Optical Density	累积光密度
PBS	phosphate buffer solution	磷酸盐缓冲液
Rpm	revolution per minute	每分钟转数
H-score	Histochemistry score	组织化学评分
RPKM	Reads Per Kilobase per Million mapped reads	基因每千碱基长度的表达数
GDNF	Glial-derived neurotrophic factor	胶质细胞源性神经营养因子
WT	wild-type	野生型
HT	hormone therapy	激素疗法

IHC	immunohistochemistry	免疫组化
ACTH	adrenocorticotropin	促肾上腺皮质激素
α -MSH	α -melanocyte-stimulating hormone	α -黑色素细胞刺激激素
β -ED	β -endorphin	β -内啡肽
TTR	transthyretin	甲状腺素运载蛋白
AR	androgen receptor	雄激素受体
PKN1	Protein kinase N1	蛋白激酶 N1
KLK2	Kallikrein 2	激肽释放酶 2
KLK3	Kallikrein 3	激肽释放酶 3
GPCR	G Protein-Coupled Receptors	G 蛋白偶联受体
cAMP	cyclic 3',5'-adenosinemonophosphate	环磷酸腺苷
BSP	bone sialoprotein	骨涎蛋白
DMP1	dentin matrix protein 1	牙本质基质蛋白 1
MEPE	Matrix extracellular phosphoglycoprotein	细胞外基质磷酸化糖蛋白
OA	osteoarthritis	骨性关节炎
POMC	proopiomelanocortin	原阿片黑皮素
PACAP	pituitary adenylate cyclase-activating peptide	垂体腺苷环化酶激活肽
MLT	Melatonin	褪黑素
NSAID	Nonsteroidal Antiinflammatory Drugs	非甾体抗炎药
OVX	ovariectomized	卵巢切除
PMO	Postmenopausal osteoporosis	绝经后骨质疏松

神经肽 Y 在骨折愈合中作用及成骨机制研究

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研究背景

随着全球人口趋于老龄化，50 岁后 33%的女性和 20%的男性深受骨质疏松的困扰^[1]。骨质疏松带来的脆性骨折比如髌关节、脊柱和手腕等部位的骨折正逐年增加^[2]。早在 1990 年，统计分析发现全世界下肢髌部骨折的直接和间接年费用就已经达到了 348 亿美元，预计在今后 50 年中将大幅增加，给社会及个人带来了沉重的经济负担^[2]。近年来，神经肽类物质在骨折愈合、骨再生、骨重建中的作用得到国内外研究者的重视，并已成为一个研究热点。

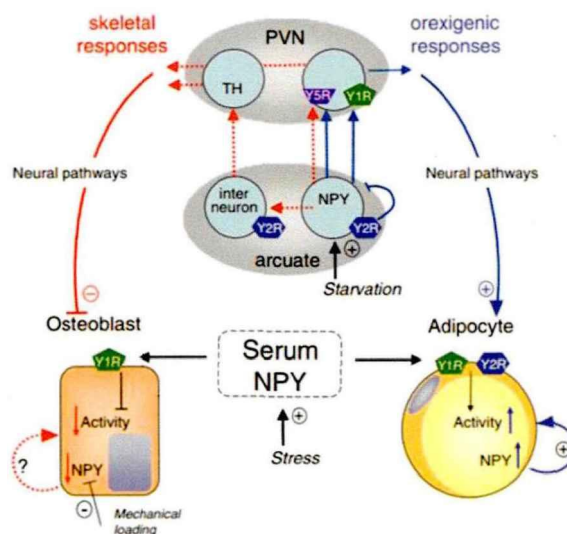
神经肽是人体多种组织形成的，拥有神经生物活性的肽类物质。交感神经纤维含有丰富的神经肽 Y(Neuropeptide Y, NPY)和血管活性肠肽(vasoactive intestinal peptide, VIP)，其对髌、膝等关节的神经滋养作用正受到越来越多的关注^[3]。NPY 被认为是改善骨性关节炎(osteoarthritis, OA)的潜在治疗靶点^[3]。在骨折区域内，降钙素基因相关肽(calcitonin gene-related peptide, CGRP)、P 物质(substance P, SP)和 NPY 含量显著增高，揭示出神经肽类物质在骨折修复、愈合、重建中发挥了重要作用。此外，NPY 在骨折局部骨细胞、成骨细胞、破骨细胞中可通过直接和间接激活多个通路来促进骨合成代谢。NPY 通过抑制破骨细胞增生并增强成骨细胞的活性，来达到维持体内骨量稳定的作用^[4]。研究表明，NPY 是重要的骨骼神经营养因子^[5]，具有促进骨折愈合、治疗髌、膝关节骨性关节炎、改善骨质疏松症的预后等多重作用^[6-10]。

1. 国内外研究现状

临床工作中，我们经常碰到严重创伤性骨折，如伴有 2cm 以上的骨缺损将难以自我愈合。一般需要骨移植替代物和生物活性分子比如生长因子(growth factors)、肽类(peptides)、小分子(small molecules)共同作用来诱导间充质干细胞聚集，从而促进骨折愈合^[11]。NPY 作为重要的神经营养因子，对于严重创伤性骨折的预后具有重要的临床意义^[6, 12]。

NPY 在中枢和外周神经系统中普遍表达，在调节体内骨量和能量合成等多

种生理进程中起到重要的作用^[8,9]。研究表明，在中枢神经系统中，NPY 经下丘脑上的 NPY Y2 受体（NPY receptor Y2, NPY2R）对骨代谢调节发挥作用，是一种重要的神经递质；而在周围骨组织内，NPY 主要是通过 NPY Y1 受体（NPY receptor Y1, NPY1R）受体来发挥其效应^[13]。Baldock PA 教授^[14]等研究指出 NPY 系统主要通过 G-蛋白双受体（Y1 and Y2）介入调节骨和脂肪组织相互作用，这种作用通过下丘脑的特定核和目的组织来实现，如下图所示。该图反应了 NPY 及其受体在骨和脂肪组织中枢和外周调节机制，中枢传导路径起源于纹状核的 NPY 能神经元。通过上调 NPY 的表达，作用于室旁核神经元的 NPY Y1 受体（NPY receptor Y1, NPY1R）和 NPY Y5 受体（NPY receptor Y5, NPY5R），从而传导动物摄食反射（蓝色箭头）。同时，室旁核神经元的 NPY Y1 受体和 NPY5R 受体介

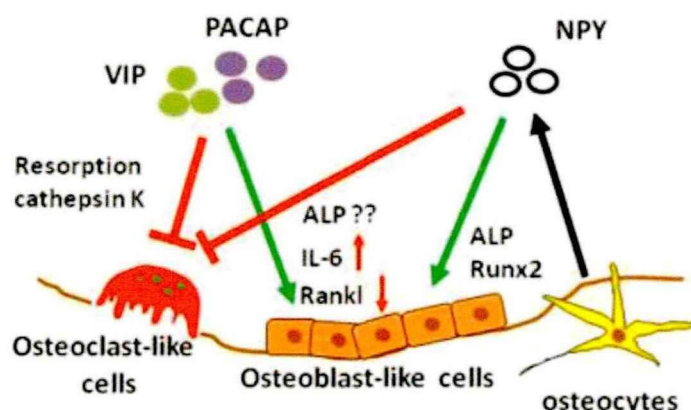


导中枢神经元诱导的成骨细胞的应答（红色箭头）。然而，NPY 在成骨细胞内自身基因调控机制尚有待于进一步阐明（红色虚线），有待于我们进一步的研究。

Baldock PA 教授在 NPY 沉默鼠的研究中指出，在持续冷刺激下，NPY 沉默的小鼠表现为体内皮质酮水平升高，骨量丢失速度加快，其骨丢失速度是野生型小鼠的 3 倍；其分子生物学机制是沉默 NPY 的小鼠体内中枢和外周系统的 NPY 严重不足，不能通过其 NPY2R 受体抑制皮质酮的产生，也不能对抗应激反应造成的骨量丢失，更深层的研究是体内的成骨细胞骨合成代谢受到了严重的影响^[15]。由此可见，NPY 在应激状态下具有上调成骨细胞骨代谢合成的生物学效应。然而，在 NPY1R 沉默鼠中，NPY 的成骨作用却得到了质疑^[12]。Baldock PA 教授等研究指出 NPY1R 基因沉默的小鼠成骨能力提高，提示了阻断 NPY1R 通路可诱导体内骨合成代谢^[16]。Daniela M Sousa 等研究了外源性阻断 NPY1R 受体，也可造成

小鼠骨量增加,具体的机制可能是反馈性促使成骨细胞内源性上调成骨转录因子 Runx2、Osterix 的表达来实现的^[12、13]。由此可见, NPY 及其受体在体内外骨代谢领域的生物学效应是复杂多变的,有待于系统的研究。

NPY 不光参与中枢性调控骨代谢,在周围局部骨组织中的骨髓间充质干细胞、成骨细胞、破骨细胞、骨细胞中同样展现了重要的调控效力^[8、17、18]。研究表明, NPY 和 NPY1R 可诱导骨髓间充质干细胞分化为成骨细胞,并促进其骨合成^[19-21]。骨骼中的骨细胞、破骨细胞和成骨细胞均表达 NPY 及其受体,其中成骨



细胞表面以 NPY 和 NPY1R 受体居多,可能是细胞成骨的重要靶点^[22]。

上图展示了 NPY 在局部组织骨代谢的作用,提示 NPY 在骨组织内可通过上调白介素-6(interleukin-6, IL-6)和下调核因 κ B 受体活化因子配体(receptor activator of nuclear factor kappa-B ligand, RANKL)诱导成骨细胞骨合成,同时可下调破骨细胞介导的骨吸收。从而, NPY 在周围骨代谢中具有抑制破骨并增强成骨的双重生物效应(红箭头-抑制,绿箭头-激活)。

NPY 及其受体信号传导通路在促进骨折愈合、修复 OA,调节骨代谢方面发挥了非常重要作用^[3、5、16]。Hua Long 等人证明在成角骨折愈合的早期和晚期 NPY 明显增加,与凸侧相比,凹侧 NPY 纤维在术后 1 周增加了 5 倍,而凸侧 NPY 纤维在第 3 和 8 周则增加了 6 倍,提示 NPY 在骨折愈合早期和晚期具有重要的生物学功能^[23]。此外, Eitner 教授等研究也证实,正常大鼠和绵羊膝关节髌旁区滑膜内含有大量感觉纤维和酪氨酸羟化酶阳性的交感神经(NPY 和 VIP)纤维,能促进大鼠和绵羊膝关节成骨细胞的增生、分化^[5]。而在失交感神经支配的 OA 模型中,其滑膜组织不能够对损伤产生炎症反应,不能修复 OA 软骨缺损,从而更容易发生膝关节骨性关节炎^[5]。

2. 神经肽 Y 及其受体的分布及生物学功能

NPY 的分子生物学效应主要通过 4 个 G 蛋白偶联受体(G protein-coupled receptors, GPCRs)来完成^[24]。GPCRs 具有 7 个 α 螺旋跨膜片段, 分别为 NPY1R、NPY2R, NPY4R、NPY5R 和 NPY6R^[18]。各种受体的分布不相同, 在与 NPY 的亲和力方面, $\text{NPY2R} > \text{NPY1R} > \text{NPY5R}$, $\text{NPY4R} = \text{NPY6R}$ ^[18]。神经生长因子(nerve-growth factor, NGF)和脑源性神经营养因子(brain-derived neurotrophic factor, BDNF)等多种特异性生长因子可调节 NPY 的表达^[25-28]。NPY 在机体中枢神经系统中下丘脑的重要核团弓状核中表达最为丰富^[18]。

NPY 可通过中枢 μ 和 κ 阿片受体来诱导动物摄食行为^[29]。与阿片肽(opioid peptides)、强啡肽 A(dynorphin A)和 β -内啡肽(β -endorphin, β -ED)可通过 μ 和 κ 阿片受体来诱导动物摄食行为类似^[29]。应用 κ 阿片受体拮抗剂或 κ 反义探针的预处理, NPY 诱导的摄食反射呈剂量依赖性减少^[29]。此外, NPY 还可通过多种 NPY 受体参与动物摄食行为, 其中 NPY1R 和 NPY5R 受体与中枢神经系统正向调节摄食行为相关^[30-33]。与之相反, 激活 NPY2R 或 NPY4R 受体会抑制进食行为^[34, 35]。

NPY 及其受体不光存在于中枢系统, 也广泛存在于外周多种组织。例如肠粘膜下和肠肌丛神经节细胞内发现了 NPY 及其 NPY1R 受体^[36]。NPY 被检测到参与调控胃肠粘膜肠内神经元, 1 $\mu\text{mol/L}$ 浓度的 NPY 即能显著促进胃肠粘膜肠内神经元增殖, 减少细胞凋亡^[37]。在 NPY-siRNA、NPY-Y1 受体拮抗剂和 NPY (-/-) 小鼠体内培养的肠神经细胞中, 胶质细胞源性神经营养因子(Glia-derived neurotrophic factor, GDNF)介导的神经细胞增殖和活性明显降低^[37]。与野生型小鼠相比, NPY (-/-) 小鼠胃排空加快, 肠道转运延迟。此外, 脂肪细胞中同样发现有 NPY 及其 NPY1R 和 NPY2R 受体, NPY 在外周交感神经元/肾上腺兴奋期间可直接作用于其 NPY1R 和 NPY2R 受体, 刺激体内脂肪累积^[14]。除此之外, NPY 还可通过 NPY2R 受体激活一氧化氮(Nitric oxide, NO)释放来参与血管生成, 机制是 NPY 可刺激机体血管组织直接释放内皮型一氧化氮合酶(endothelial NO synthase, eNOS)或间接激活成纤维细胞生长因子(fibroblast growth factor)和血管内皮生长因子(vascular endothelial growth factor, VEGF)^[38]。

3. 神经肽 Y 主要相关信号通路

NPY 是一种具有调节能量消耗和骨量平衡双重效能的神经递质^[39]。目前已

知关于 NPY 的神经通路有以下几种：1.抗精神病药途径（代谢副作用），Antipsychotics Pathway (Metabolic Side Effects), 20 个相关基因, 相关度分值 0.893; 2.交感神经通路(神经效应器连接), Sympathetic Nerve Pathway(Neuroeffector Junction), 23 个相关基因, 相关度分值 0.765; 3.内皮素通路, Endothelin Pathways, 34 个相关基因, 相关度分值 0.638; 4.脂肪细胞脂降解调节通路, Regulation of lipolysis in adipocytes, 56 个相关基因, 相关度分值 0.510; 5.脂肪细胞因子信号通路, Adipocytokine signaling pathway, 69 个相关基因, 相关度分值 0.446; 6.环磷酸腺苷信号通路, cAMP signaling pathway, 216 个相关基因, 相关度分值 0.255; 7.神经生物学信号通路, Neuroscience signaling pathway, 341 个相关基因, 相关度分值 0.191; 8.活化的蛋白激酶 N1(PKN1)刺激 AR（雄激素受体）调节基因激肽释放酶 2 和激肽释放酶 3（KLK2 和 KLK3）的转录信号通路, Activated PKN1 stimulates transcription of AR (androgen receptor) regulated genes KLK2 and KLK3, 588 个相关基因, 相关度分值 0.159; 9.肽配体结合受体信号通路, Peptide ligand-binding receptors, 676 个相关基因, 相关度分值 0.128; 10.G 蛋白偶联受体信号通路, Signaling by GPCR, 2598 个相关基因, 相关度分值 0.080。

近年来, NPY 在骨合成代谢领域和相关神经通路的研究正逐渐兴起^[4, 40-43]。本课题第 1 部分通过 RT-PCR 和组织免疫组化染色的方法, 检测了小鼠骨折愈合过程中不同细胞中 NPY、NPY1R、Runx2 和 Osterix 的表达, 提示 NPY 基因及蛋白共同参与骨折早期炎症反应, 可能起到骨诱导的作用。第 2、3 部分从分子生物学角度阐明, NPY 过表达质粒转染 MC3T3-E1 细胞后, 可通过上调其 Osterix 和 Runx2 表达来促进细胞成骨分化, 揭示了 NPY 在体外小鼠前成骨细胞系的潜在分子生物学机制。

4. 展望

生物活性分子如生长因子、多肽和小分子与骨移植支架相结合, 可诱导间充质干细胞增生、分化, 有利于骨折后骨缺损的修复^[11]。这对未来合并巨大骨缺损的骨折预后产生积极的影响, 具有重要的临床应用价值。

第一部分 神经肽 Y 在小鼠骨折愈合中作用及相关机制研究

前言

随着人口老龄化的发展,全球 50 岁后 1/3 的女性和 1/5 的男性遭受着骨质疏松带来一系列并发症的威胁^[1]。骨质疏松导致的髋关节、脊柱和手腕等部位的脆性骨折病人数目正逐年增加^[2]。在美国,每年 790 万骨折患者中大约有 10% 的患者出现骨不愈合和/或延迟愈合,进而导致病人严重残疾^[44]。据统计,1990 年全世界髋部骨折的直接和间接年费用已高达 348 亿美元,预计在今后 50 年中将大幅度增加^[2]。所以,我们有必要对骨折后的愈合及相关机制做进一步深入的研究。

众所周知,创伤后骨折愈合修复是一个复杂的骨诱导、骨合成及骨重塑过程,涉及了多种成骨相关细胞,细胞因子 (cytokines), 生长因子 (growth factors) 和细胞外环境 (extracellular environment) ^[45-47]。骨折愈合的三个重要的基础要素包括骨折处细胞再生、骨和软组织的血管再生和稳定的力学环境^[48]。长骨骨折的修复一般会经历最初的血肿形成,纤维性骨痂形成,软骨骨痂形成,骨性骨痂形成,骨痂重塑的过程^[49]。骨折后不久,间充质干细胞被招募到损伤部位并分化为纤维母细胞,而后纤维母细胞渐渐转化为软骨母细胞和骨母细胞,并逐渐纤维化后构成纤维性骨痂。7 天左右,纤维性骨痂渐渐被软骨基质构成的软骨痂桥接爬行替代,形成透明软骨。随后,骨母细胞被招募到该部位,骨折处软骨痂钙化。最终,钙化的软骨被重塑,骨性骨痂被成熟的板层骨所取代,恢复伤前骨组织形态^[45, 48]。创伤骨折后如合并巨大骨缺损,将造成骨折端不稳定,进而影响骨折愈合,而生长因子、多肽和小分子等的介入有望解决这一临床难题。

NPY 是一种由 36 个氨基酸构成的多肽,普遍参与调控体内中轴及外周骨量平衡^[14, 18]。NPY 在周围局部骨组织骨髓间充质干细胞、破骨细胞、骨细胞、成骨细胞中广泛表达,参与了相应的骨代谢^[8, 17, 18]。在人体及啮齿类动物模型中,NPY 主要通过其 NPY1R 和 NPY2R 受体调节体内骨量平衡。Hua Long 等人证明在成角骨折愈合的早期和晚期 NPY 明显增加,与凸侧相比,凹侧 NPY 纤维在第 7 天增加了 5 倍,而凸侧 NPY 纤维在第 21 和 56 天则增加了 6 倍,提示 NPY 在骨折愈合早期和晚期参与了骨折愈合^[23]。而 NPY 沉默的小鼠在应对冷刺激时体内皮质酮水平升高,骨量丢失速度是野生型小鼠的 3 倍;其分子生物学机制是沉默 NPY 的小鼠不能通过 NPY2R 受体抑制皮质酮的产生,也不能对抗应激反应

造成的骨量丢失^[15]。进一步的研究发现, NPY 具有在应激状态下促进成骨细胞再生、加快骨组织代谢的作用^[15]。NPY1R 作为 NPY 在骨骼中的主要受体, 在人体及啮齿动物中含量较高, 可能在骨折愈合过程中具有比较重要的作用^[13]。然而, 目前尚不清楚 NPY 及其 NPY1R 与成骨特异性转录因子如 Runx2、Osterix 在骨折愈合中各类细胞中的分布及表达情况, 有必要进行系统详细的研究。

本部分的研究建立了 C57BL/6 小鼠的闭合胫骨骨折模型, 分别从宏观和微观角度观察了骨折愈合及重塑的过程。在 IVIS[®]SpectrumCT 三维成像动态观察下, 展现了小鼠骨折愈合的进程。我们通过骨折愈合过程中不同细胞的 HE 染色, 揭示了细胞水平的成骨过程。我们应用 ELISA 方法检测了小鼠骨折愈合中不同时间点的 NPY 血清蛋白表达情况, 揭示了 NPY 在细胞外浓度的变化情况。此外, 通过应用 RT-PCR 技术检测了小鼠骨折愈合不同时间点标本中 NPY、NPY1R、Runx2、Osterix 基因的表达情况, 在基因水平揭示了不同成骨相关基因在骨折愈合中的作用。通过免疫组化染色及 AI 图像分析系统检测了小鼠骨折愈合不同时间点标本中 NPY、NPY1R、Runx2、Osterix 蛋白表达情况, 在蛋白水平揭示了不同成骨相关基因在骨折愈合中的作用。

本部分研究探讨了在动物体内骨折愈合与成骨相关基因之间的关系, 为我们后续体外研究 NPY 上调 Runx2 和 Osterix 表达促进前成骨细胞成骨提供了研究基础, 并逐步揭示 NPY 在骨折愈合中的潜在分子生物学机制。

材料与amp;方法

1. 材料

1.1 实验动物

18 只雄性 C57BL/6 小鼠购于山东大学动物实验中心，14 周龄，体重为 20~26g，平均(22.1 ± 3.8)g，饲养于 SPF(Specific Pathogen Free)级实验动物屏障环境中，温度(22±2℃)、湿度(55±5%)，室内紫外线定期照射，定期进行蒸馏水、饲料、垫料和鼠笼等高压灭菌及更换。所有动物相关实验程序均按照山东大学第二医院实验动物伦理委员会批准的方案执行[批号:KYLL-2021(LW)019]。

1.2 主要试剂

二甲苯	国药集团化学试剂有限公司 (Sinopharm chemical Co.Ltd.)
EDTA (PH8.0) 抗原修复液	武汉谷歌生物有限公司 (Google Bio.)
柠檬酸 (PH6.0) 抗原修复液	Google Bio.
EDTA (PH9.0) 抗原修复液	Google Bio.
PBS 缓冲液	Google Bio.
双氧水	Sinopharm chemical Co.Ltd.
BSA	Google Bio.
正常兔血清	Boster
盐酸	Sinopharm chemical Co.Ltd.
氨水	Sinopharm chemical Co.Ltd.
中性树胶	Google Bio.
组化试剂盒 DAB 显色剂	DAKO
苏木素染液套装	Google Bio.
神经肽 Y (NPY) 检测试剂盒	武汉云克隆科技股份有限公司 (Cloud-Clone Corp.)
FastStart Universal SYBR Green Master (Rox)	Roche
RevertAid First Strand cDNA Synthesis Kit	Thermo
三氯甲烷	美国 Affinity Biosciences 公司

HyPure TMMolecular Biology Grade Water	HyClone
脱钙液	武汉赛维尔生物科技有限公司 (Servicebio)
异丙醇	Sinopharm chemical Co.Ltd.
无水乙醇	Sinopharm chemical Co.Ltd.
RNA 提取液	Sinopharm chemical Co.Ltd.
Osterix 抗体 (cat. no. bs-1110R)	北京博奥森生物技术有限公司 (Bioss Bio.)
NPY1R 抗体 (cat. no. bs-1070R)	Bioss Bio.
Runx2 抗体(cat. no. PAB011Mu01)	Cloud-Clone Corp.
NPY 抗体 (cat. no. DF6431)	美国 Affinity Biosciences 公司
二抗(horseradish peroxidase-linked guinea pig anti-rabbit IgG polyclonal)(cat. no. SAA544Rb59)	Cloud-Clone Corp.

1.3 主要设备及耗材

脱水机	武汉俊杰电子有限公司
包埋机	武汉俊杰电子有限公司
病理切片机	上海徕卡仪器有限公司
冻台	武汉俊杰电子有限公司
组织摊片机	浙江省金华市科迪仪器设备有限公司
烤箱	天津市莱玻璃仪器设备有限公司
载玻片及盖玻片	江苏世泰实验器材有限公司
正置光学显微镜	日本尼康
成像系统	日本尼康
AI 图像分析系统	Servicebio
高速低温组织研磨仪	Servicebio
微波炉	格兰仕电器有限公司
酶标仪	Thermo Fisher

37℃孵育箱	黄骅菲斯福实验仪器有限公司
脱色摇床	Google Bio.
涡旋混合器	Google Bio.
移液枪	Dragon
掌上离心机	Google Bio.
组化笔	Gene tech
显微镜	CIC
台式高速冷冻型微量离心机	DragonLab
荧光定量 PCR 仪	Roche
超微量分光光度计	Thermo Fisher
标准试剂型纯水仪	青岛 FLOM 科技有限公司
离心管	Axygen Bioscience
超声波清洗器 CX-250	北京医疗设备二厂
电磁搅拌仪 ETS-D4	德国 IKA 公司
微量注射泵 KDS100	美国 KDS 公司

1.4 主要试剂配制

(1) 10×PBS 缓冲液

将 90g NaCl、60g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 和 4g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 溶于双蒸水中，充分搅动溶化后定容至 1000ml。用时与水按体积比 1:9 稀释成 1×PBS。

(2) 1×转膜缓冲液

取 Gly 14.4g、Tris 3.0g、200ml methanol，双蒸水定容至 1000ml。

(3) 10×TBS 缓冲液

将 80g NaCl 和 24.4g Tris 溶于双蒸水中，充分搅动溶化后双蒸水定容到 1000ml，室温下储存。

(4) TBST 洗脱液

用双蒸水将 10×TBS 缓冲液溶解稀释 10 倍，将 1/1000 体积的 Tweens 加入后混匀，室温下储存。

(5) 5%脱脂奶粉

用 1×TBST 溶液将脱脂奶粉 5.0g 稀释后，定容至 100ml。

(6) 4%多聚甲醛溶液

将 2.0g 多聚甲醛用 1×PBS 50mL 溶液, 充分搅动溶化, 调整 PH 值为 7.4, 4℃ 冰箱存储。

(7) EDTA 修复液

将 30.27g Tris-NaCl 和 1.46g EDTA 溶解于双蒸水中, 搅动溶化后双蒸水扩容至 500mL, 室温存储。应用时, 用双蒸水将其稀释 10 倍。

2. 实验方法

2.1 建立 C57BL/6 小鼠闭合胫骨骨折模型

(1) 将小鼠放入 3%异氟醚和 60ml/min O₂ 的容器中, 持续 5-10min。在姿势反射消失后, 从麻醉诱导透明容器中取出小鼠, 并在 3%异氟醚和 60ml/min O₂ 的吸入面罩中维持麻醉。在确保没有后爪反射后人工随机掰断一侧胫骨中段, 建立闭合骨折模型。

(2) 分别于建模 0、1、7、14 天, 在异氟醚诱导麻醉下随机取出 3 只小鼠, 通过 IVIS®SpectrumCT 检测观察骨痂形成的情况, 观测完送回鼠笼。

(3) 从 1、7、14 天小鼠随机取 3 只在异氟醚诱导麻醉下眼内眦取血, 血清 ELISA 法检测血清学 NPY 浓度指标。

(4) 小鼠骨折愈合过程中成骨相关基因的表达: 分别于建模后第 1、7、14 天随机取 3 只小鼠在异氟醚诱导麻醉下脱颈处死, 并取胫骨骨折处组织提取 RNA; 应用 RT-PCR 技术检测组织样本中 NPY, NPY1R, Runx2, Osterix mRNA 的表达。

(5) 小鼠骨折愈合过程中成骨相关蛋白的表达: 取上述小鼠骨折处样本, 石蜡包埋切片, 行苏木精/伊红染色 (hematoxylin-eosin staining, HE) 或固定在 70%乙醇中分别行 NPY, NPY1R, Runx2, Osterix 的免疫组织化学染色 (Immunohistochemical staining, IHC)。利用 AI 图像分析系统 (Aipathwell) 自动读取石蜡切片免疫染色 3 处区域图像, 分析测定阳性染色的灰度值。

2.2 组织病理学检查

2.2.1 组织切片的制作

(1) 取材: 新鲜 C57BL/6 小鼠胫骨骨折处组织用 4%多聚甲醛固定液固定 24h。将待测部位组织从固定液取出, 用眼科剪修整。

(2) 脱钙: 流水冲去多聚甲醛后, 标本放在脱钙液中震荡, 事软化程度 (以细针较易穿进组织) 为脱钙终点。

(3) 脱水: 将组织置于脱水盒内, 在脱水机器内按顺序梯度酒精脱水。75%乙醇 4.0h-85%乙醇 2.0h-90%乙醇 2.0h-95%乙醇 1.0h-100%乙醇 I 0.5h-100%乙醇 II 0.5h-醇苯 10min-二甲苯 I 10min-二甲苯 II 10min-蜡 I 1.0h-蜡 II 1.0h-蜡 III 1.0h。

(4) 包埋: 包埋机启动加热至 65℃, 石蜡融解后, 将标本浸入包埋。蜡块标本于-20℃冻台下凝结后修整。

(5) 切片: 将蜡块在石蜡切片机内切片, 厚约 3μm。40℃ 温水中, 将切片在摊片机上悬浮展平, 60℃ 烘箱内烤干。烤干蜡块后取出常温保存备用。

2.2.2 切片脱蜡

(1) 融蜡: 将已干燥的切片置于 60℃烤箱中 20min。

(2) 脱蜡: 将标本切片依次放入: 二甲苯 (dimethylbenzene) I 15min-二甲苯II 15min-二甲苯 III 15min。

(3) 复水: 将切片依次放入 100%乙醇I 5min-100%乙醇II 5min-85%乙醇 5min-75%乙醇 5min-蒸馏水洗。

2.2.3 苏木素/伊红染色观察骨折处细胞形态

(1) 苏木素将上述步骤脱蜡后的组织切片染色 5-10min, 双蒸水冲洗 10min。

(2) 将切片置入 1%盐酸溶液分化片刻, 双蒸水冲洗 1min。

(3) 将切片置入 1%氨水溶液返蓝约 10sec, 双蒸水冲洗 1min。

(4) 伊红染色: 将组织切片轻轻甩干后加入伊红染色液, 室温孵育 3min 后水洗 3min。

(5) 脱水封片: 切片依次置入 100%乙醇 I 5min-100%乙醇 II 5min-100%乙醇III 5min-二甲苯I 5min-二甲苯II 5min 中脱水透明, 中性树胶封片。

(6) 每张切片镜下随机取 3 处视野在 200 倍和 400 倍下拍照留存。

2.3 石蜡切片免疫组化染色

(1) 石蜡切片脱蜡至水同 2.2.2。

(2) 抗原修复: 在 PH 6.0 的柠檬酸抗原修复缓冲液中放置组织切片, 修复抗原, 中火 8min 到沸腾, 关火 8min 后再行中低火 7min (注意避免干片)。冷却后将玻片放在 PBS 中摇匀, 5min/次×3 次。

(3) 阻断内源性过氧化物酶: 切片置于 3%H₂O₂ 液体中, 避光室温孵育 25 min, 将玻片放在 PBS (PH7.4) 中在摇床上清洗, 5min/次×3 次。

(4) 血清封闭: 在组化圈内滴入 10%兔血清, 将标本完全覆盖, 室温封闭 0.5h。

(5) 封闭一抗：将封闭液甩干，滴入按一定比例配好的一抗（NPY、NPY1R、Runx2、Osterix），切片展平放置在湿盒内，4℃ 孵育过夜。

(6) 封闭二抗：在摇床上摇晃置于 PBS (PH7.4) 中的玻片冲洗 3 次，5min/次。甩干切片后在圈内滴加（1:2000）二抗（horseradish peroxidase-linked guinea pig anti-rabbit IgG polyclonal）覆盖组织，室温培育 50min。

(7) DAB 显色：在摇床上晃动置于 PBS 中的玻片，5min/次×3 次。甩干切片后将 DAB 显色液滴入圈内，镜下棕黄色显色后，蒸馏水冲洗终结显色。

(8) 复染细胞核：苏木素复染 3min，蒸馏水洗，1%的盐酸酒精分化几秒，蒸馏水洗，氨水返蓝，流水洗涤。

(9) 脱水封片：将切片依次放入 75%乙醇 5min-85%乙醇 5min-100%乙醇I 5min-100%乙醇II 5min -二甲苯I 5min 中脱水透明，甩干后中性树胶封片。

(10) 免疫组化显微镜镜检，图像采集分析

使用 AI 图像分析系统 (Alpathwell) 自动读取石蜡切片免疫染色 3 处区域图像，计算像素面积。阴性无色，计 0 分；弱阳性淡黄色，计 1 分；中度阳性棕黄色，计 2 分；强阳性棕褐色，计 3 分。

分析指标如下，阳性面积比：阳性面积/组织面积。反映阳性面积的多少[50]；组织化学评分（Histochemistry score, H-score），将每张切片内阳性数量及染色强度转变为数值，从而达到对组织染色半定量的目的。 $H\text{-Score} = \sum (pi \times i) = (\text{percentage of weak intensity area} \times 1) + (\text{percentage of moderate intensity area} \times 2) + (\text{percentage of strong intensity area} \times 3)$, pi 表示阳性信号像素面积占比；i 代表着色强度。鉴于 H-score 统计的相对准确性^[51, 52]，我们实验中主要采用 H-score 评分来反应细胞蛋白表达强度。

2.4 ELISA 法检测 C57BL/6 小鼠骨折模型中不同时间点血清中 NPY 浓度

(1) 取样：分别于建模后 1、7、14 天，随机取出 3 只小鼠行眼内眦取血，取血后以消毒纱布按压眼部 1-2min，可轮流穿刺双眼数次，一次采血 0.1ml。将全血标本 1000rpm×20min，取上清液放在-20℃存储，避免反复冻融。

(2) 按照小鼠神经肽 Y(NPY)检测试剂盒操作说明，于临用前 15min 内进行相关试剂准备。

(3) 取 40μl 血清加入 160μl PBS，使得血清标本稀释 5 倍。

(4) 加样: 空白孔、标准孔、待测标本孔。空白孔加标本稀释液 100 μ l, 其余在酶标板孔底各加标准品或待测标本 100 μ l, 避免触及孔壁和产生气泡, 慢慢晃匀, 酶标板覆膜, 37 $^{\circ}$ C 反应 2h。

(5) 将加样孔内液体移去, 每孔用洗涤液 0.35ml 冲洗, 浸泡 2min, 采用吸水纸移干孔内液体。重复 3 次。最后, 将残余的洗涤缓冲液全部吸除干净。

(6) 将检测溶液 B 0.10ml 分别加入各孔, 覆膜后 37 $^{\circ}$ C 孵育 30 min。

(7) 将孔内液体丢弃, 甩干, 洗板 5 次, 方法同步骤 5。

(8) 将底物溶液 90 μ l 加入各孔, 酶标板覆膜, 37 $^{\circ}$ C 避光显色约 15min。

(9) 将 50 μ l 终止溶液加入各孔, 待蓝色立转黄色时终止反应。

(10) 待见酶标板底无气泡及水滴后, 随即用酶标仪在 450nm 波长检测各孔的光密度 (O.D.值), 进而求出 NPY 的血清浓度。

2.5 荧光定量 PCR 法

2.5.1 总 RNA 抽提

(1) 将新鲜组织剪碎, 高速低温组织研磨仪内高速研磨 10min。

(2) 加入 1ml 的 Trizol Reagent, 室温裂解 8min。

(3) 将 Trizol Reagen 吸取到 2ml 离心管中, 吸打混匀, 静置 5min。

(4) 12000rpm $\times$ 3min 取上清。

(5) 取三氯甲烷 0.20ml 滴入, 剧烈摇晃使混合均匀, 液体呈乳状, 室温放置 10min。

(6) 4 $^{\circ}$ C 下 12000rpm $\times$ 15min。

(7) 移上清液到新离心管中, 并加入等体积的异丙醇 (isopropanol), 颠倒混匀。

(8) -20 $^{\circ}$ C 放置 10min。

(9) 4 $^{\circ}$ C 下 12000rpm $\times$ 10min, 离心管底的白色沉淀物就是 RNA。

(10) 吸干上清液, 移入 75%酒精 1ml, 颠倒混匀, 使白色沉淀物悬浮。

(11) 4 $^{\circ}$ C 下 12000rpm $\times$ 5min。

(12) 弃上清, 吸净管底乙醇, 在超净台上将离心管吹 3min, 晾干沉淀。

(13) 移入无 RNA 酶的水 30 μ l 溶化 RNA。

(14) 应用 Nanodrop 2000 仪器测定 RNA 纯度及浓度: 仪器调 0 后取 2 μ l 待测 RNA 溶液进行吸光值检测。

(15) 如 RNA 溶液浓度过高可恰当比例的稀释, 使之最终浓度达到 100ng/ μ l。

2.5.2 反转录

- (1) 取 1 个 PCR 管，加入含 100ng RNA 以上的溶液，作为反转录的模板。
- (2) 加入 1 μ l 特异性 miRNA 反转录引物。
- (3) 移入不含核糖核酸酶的去离子水，补充容量至 12 μ l。
- (4) 于 PCR 仪上 65 $^{\circ}$ C 保温 5min 后，立即放冰上冷却。
- (5) 依次加入 4 μ l 5 $\times$ ES Reaction Mix，1 μ l RNA inhibitor，2 μ l 10mM dNTPs 和 1 μ l 反转录酶，抽吸混合均匀。
- (6) 于 PCR 仪上 42 $^{\circ}$ C保温 1h，然后 80 $^{\circ}$ C保温 5min 将反转录酶灭活。

2.5.3 定量 PCR（逆转录定量 PCR 的引物序列如附表 1）

- (1)取 200 μ l PCR 管，按下列反应体系配制，每个反转录产物配制 3 管（表 1-1）。

表 1-1 PCR 体系中各反应液组成

名称	体积
2 $\times$ qPCR Mix	12.5 μ l
基因引物（7.5 μ M）	2.0 μ l
反转录产物	2.5 μ l
ddH2O	8.0 μ l

表 1-2 PCR 反应条件

Cycle	Cycle Point	
预变性	95 $^{\circ}$ C	5min
循环（44 次）	Step 1 @ 95 $^{\circ}$ C	15 sec
	Step 2 @56 $^{\circ}$ C	30 sec
	Step 3 @72 $^{\circ}$ C	20 sec
熔解曲线	75 $^{\circ}$ C $\rightarrow$ 95 $^{\circ}$ C，每 20 sec 升温 1 $^{\circ}$ C	

2.5.4. 结果处理

$\Delta\Delta$ CT 法：A=CT(目的基因，待测标本)- CT(内标基因，待测标本); B=CT(目的基因，对照标本)- CT(内标基因，对照标本); $\Delta\Delta$ CT=A-B;表达倍数= $2^{-\Delta\Delta$ CT}。

3. 统计学分析

数据结果以均数 $\pm$ 标准差(means $\pm$ SD)表示。数据结果分析使用 SPSS18.0 统计分析软件。ELISA 法检测血清 NPY 浓度；各组基因的表达量采用 $2^{-\Delta\Delta$ CT；NPY 蛋白表达量采用 AI 免疫组化自动分析软件 H-score 分值来估算 NPY 阳性强度。使用 SPSS 18.0 软件中 One-way ANOVA 方法进行各组总体差异检验，各组间两两比较采用 Uncorrected Fisher's LSD 方法， $P<0.05$ 为差异有统计学意义。使用 GraphPad Prism v.6.0 软件柱状图绘图。

实验结果

1. 构建 C57BL/6 小鼠胫骨闭合骨折模型成功

为了最大程度地模拟人体胫骨骨折愈合的过程,我们借助小鼠构建了胫骨骨折模型。18 只小鼠中 17 只构建闭合胫骨骨折模型成功,1 只小鼠麻醉后呼吸抑制最终死亡。术后其余 17 只小鼠均可自主进食,进水,无运动功能、感觉功能、社交行为等异常表现。

通过大体观察小鼠生活状态,术后第 1 天,小鼠运动较少,但不影响进食和饮水。术后第 2 天,小鼠可逐渐活动。术后 2 周,小鼠患肢即可负重行走,有时可见双侧肢体同时站立的情况(图 1)。提示患肢术后 2 周有足够强度及硬度支撑体重。

2. 宏观及微观角度观察动物模型中骨折愈合良好

从宏观角度,我们应用 IVIS[®]SpectrumCT 三维成像观察小鼠胫骨骨折愈合情况。CT 三维重建显示:建模即时,胫骨横行骨折,骨皮质不连续,骨折线清晰(图 2);建模后第 1 天,胫骨骨折断端略有移位,对位可(图 3);建模后第 7 天,胫骨骨折线模糊不清,对位可,曲度略微成角(图 4)。建模后第 14 天,骨折线模糊不清,对位对线可,骨折端骨痂形成(图 5)。CT 三维重建的影像学检查再现了小鼠骨折骨性骨痂逐渐形成的过程。同时,宏观分析小鼠闭合骨折具有较人类骨折愈合时间更短,骨痂形成更快的特点,为下一步研究骨痂中的成骨相关因子的表达创造了必要条件。

通过宏观观察确认建模成功后,我们又从微观的角度进一步了解了小鼠骨折的后成骨的历程。HE 染色显示:建模后第 1 天:骨折处可见水肿及炎细胞浸润,纤维蛋白、胶原纤维、网状原纤维等形成松散的网络纤维性骨痂结构(图 6.A、B)。建模后第 7 天:纤维结缔组织增生,局部见纤维组织转化为软骨组织,形成软骨痂,并可见少量骨样基质形成(图 6.C、D)。建模后第 14 天:软骨细胞被骨的骨膜外壳包绕,开始肥大和肿胀,出现初级骨化中心,并可见新生骨小梁结构,构成骨性骨痂(图 6.E、F)。从微观 HE 染色揭示了小鼠骨折愈合过程中组织成骨相关细胞逐渐聚集、分化、成熟的过程,为下一步研究不同细胞中成骨相关因子的表达创造了研究基础。

3. 建模 14 天内血清 NPY 浓度变化趋势

为了进一步研究 NPY 是否通过细胞外途径参与骨折术后骨合成,我们应用

ELISA 法检测了小鼠骨折后 14 天细胞外血清中 NPY 蛋白表达情况。NPY 不同时间点（建模后第 1、7、14 天）浓度范围为 925.35 pg/ml-1055.2 pg/ml（图 7）。NPY 浓度在检测的 14 天的时间段内有一过性下降随后增高的趋势，但整体差异无明显统计学意义（ $P=0.152$ ）。唐鹏等研究发现，SD 大鼠骨折后血清中的 NPY 浓度在骨折后 4 周才能明显增高，8 周后达到峰值，具有统计学意义^[53]。与唐鹏等研究一致，我们也发现血清中的 NPY 在小鼠骨折后有一定增长的趋势，但短期内血清中 NPY 变化趋势并不大。

4. 小鼠骨折愈合过程中成骨相关基因及蛋白表达情况

4.1 建模骨折处组织 NPY 基因的表达

4.1.1 建模后第 1、7、14 天骨折处 NPY mRNA 的表达

PA Baldock 在 2014 年研究表明 NPY 在应急反应下可以刺激成骨细胞增生^[15]。Grässel S 等研究发现骨组织含有丰富的 NPY，是关节软骨重要的神经营养因子^[3]。为了进一步验证 NPY 在骨折这种应激反应中体内不同细胞中的定位及表达情况，我们采用 RT-PCR 检测了 NPY mRNA 在骨折愈合过程中的表达，结果显示：NPY mRNA 在骨折后第 1 天纤维性骨痂组织中高表达，结合 HE 及免疫组化染色，考虑与局部的炎症细胞、前成骨细胞、血管内皮细胞中 NPY mRNA 高表达有关。建模后第 7、14 天骨折组织中 NPY mRNA 的表达量分别是第 1 天的 27%和 9%，差异具有统计学意义（ $P<0.001$ ，图 12. A），提示在软骨痂及骨痂中 NPY mRNA 有下降的趋势。由此可见，NPY mRNA 在骨折炎症期可能参与诱导前成骨细胞、炎症细胞、血管内皮细胞聚集，促进骨折愈合。为了进一步揭示 NPY 在骨折愈合中的作用，我们做了组织中 NPY 蛋白的染色及分析。

4.1.2 建模后第 1、7、14 天骨折处不同细胞 NPY 蛋白分布

为了进一步了解 NPY 蛋白在体内不同细胞的分布情况，我们应用 AI 图像分析系统检测了 NPY 免疫组化蛋白在骨折愈合过程中的表达分布情况，结果显示：建模后第 1 天，早期骨折纤维性骨痂处的炎症细胞、前成骨细胞、血管内皮细胞及脂肪细胞等 NPY 染色胞浆阳性表达，呈棕色表现，整体 NPY 阳性面积比率均值为 28.0%（图 8.A、B）。建模后第 7 天，骨折软骨痂中软骨细胞及纤维细胞等 NPY 染色胞浆阳性表达，整体 NPY 阳性面积比率均值为 11.2%（图 8.C、D）。建模后第 14 天，骨性骨痂中成骨细胞、软骨细胞、破骨细胞及周围纤维结缔组织细胞 NPY 免疫组化染色散在阳性表达，新生骨表面的破骨细胞和成骨细胞 NPY 染

色弥漫阳性表达，强度较强，骨小梁中骨细胞散在的阳性，强度较弱，整体 NPY 阳性面积比率均值为 23.2%（图 8.E、F）。

4.1.3 建模后第 1、7、14 天骨折处 NPY 蛋白表达分析

为了进一步了解 NPY 蛋白在骨折愈合中的表达，我们应用 AI 图像分析系统做了 NPY 阳性面积强度（H-score 评分）统计分析，结果显示：小鼠骨折组织中 NPY 蛋白在骨折早期纤维性骨痂高表达，提示 NPY 蛋白可能主要来源于骨折初期的炎症细胞、前成骨细胞、血管内皮细胞等组织。建模后第 7、14 天软骨痂及骨性骨痂中 NPY 蛋白的表达量分别是第 1 天纤维性骨痂的 37%和 55%，差异有统计学意义（ $P<0.05$ ，图 12. B）。提示 NPY 蛋白与 NPY mRNA 有相似的生物学效应，在骨折早期即介入炎症反应，可能在骨折愈合诱导成骨方面具有比较重要的作用。

4.2 建模后第 1、7、14 天骨折处 NPY1R 受体的表达

4.2.1 建模后第 1、7、14 天骨折处 NPY1R mRNA 的表达

Daniela M Sousa 等研究表明：外源性口服 8 周选择性 NPY1R 受体拮抗剂 BIBO3304 后，小鼠骨量呈剂量依赖性增高，提示外源性阻断 Y1 受体信号可能反馈性使成骨细胞内源性上调成骨转录因子 Osterix、Runx2 的表达来增强成骨^[12, 13]。为了更好验证 NPY1R 与细胞成骨的相关性，我们在小鼠骨折愈合不同组织不同时期，应用 RT-PCR 检测 NPY1R mRNA 表达，结果显示：NPY1R mRNA 表达并没有明显统计学意义（ $P=0.133$ ，图 12. C）。虽然 NPY1R 可能在基因水平未参加细胞的成骨，但是蛋白表达决定细胞行为，为此我们做了进一步的 NPY1R 蛋白染色及分析，来求证 NPY1R 在骨折愈合中的作用。

4.2.2 建模后第 1、7、14 天骨折处 NPY1R 蛋白分布

为了进一步了解 NPY1R 蛋白在体内不同细胞的分布情况，我们采用 AI 图像分析系统检测了 NPY1R 免疫组化蛋白表达分布情况，结果显示：建模后第 1 天，骨折早期纤维性骨痂中的炎症细胞、前成骨细胞、脂肪细胞及血管内皮细胞等呈阳性表达，整体 NPY1R 阳性面积比率均值为 29.6%（图 9.A、B）。建模后第 7 天，骨折软骨痂中纤维细胞及生成的软骨细胞等 NPY1R 免疫组化染色散在阳性表达，整体 NPY1R 阳性面积比率均值为 4.1%（图 9.C、D）。建模后第 14 天，骨折骨痂中软骨细胞、破骨细胞、成骨细胞及周围纤维结缔组织细胞 NPY1R 免疫组化染色散在阳性表达，整体 NPY1R 阳性面积比率均值为 10.0%（图 9.E、F）。

4.2.3 建模后第 1、7、14 天骨折处 NPY1R 蛋白表达分析

为了进一步理解 NPY1R 在体内的生物学作用，我们又研究了 NPY1R 受体蛋白的表达情况。免疫组化阳性面积强度（H-score 评分）统计分析显示：小鼠早期骨折纤维性骨痂中 NPY1R 蛋白在炎症细胞、前成骨细胞、血管内皮细胞及脂肪细胞高表达。建模后第 7、14 天软骨痂及骨性骨痂中 NPY1R 蛋白的表达量分别是第 1 天纤维性骨痂的 8.2% 和 21%，差异有统计学意义（ $P < 0.001$ ，图 12.D）。

4.3 建模后第 1、7、14 天骨折处成骨特异性转录因子 Runx2 的表达

4.3.1 建模后第 1、7、14 天骨折处组织中 Runx2 mRNA 的表达

研究表明，Runx2 是一个关键的早期成骨转录因子，介导间充质干细胞转化为成骨细胞以及成骨细胞特异性基因的表达^[54]。此外，Runx2 在成骨细胞终末分化中起不可替代作用^[55]。为此我们在小鼠骨折愈合期间不同时期，采用 RT-PCR 法检测了 Runx2 mRNA 的表达情况，结果显示：小鼠骨折初期纤维性骨痂中 Runx2 mRNA 低表达，随骨折愈合，软骨痂或骨痂中的 Runx2 mRNA 高表达。建模后第 7、14 天在软骨痂中中等表达，在骨痂中高表达。Runx2 mRNA 的表达量分别是第 1 天的 7.9 倍和 65.6 倍，第 14 天 Runx2 mRNA 的量约是第 7 天的 8.3 倍（图 13.A）。

Runx2 mRNA 水平变化体现了和文献一致的结果，Runx2 mRNA 可能与成骨细胞终末分化有一定的联系。Runx2 mRNA 表达的增高提示成骨细胞终末分化成熟。但是我们也了解到 Runx2 早期可能也介导间充质干细胞的表达及分化，为了更好理解 Runx2 在骨折成骨中的作用，我们又进行了其相关蛋白的检测。

4.3.2 建模后第 1、7、14 天骨折处组织中 Runx2 蛋白分布

为了进一步了解 Runx2 蛋白在体内不同细胞的分布情况，我们应用 AI 图像分析系统检测了 Runx2 免疫组化蛋白表达分布情况，结果表明：建模后第 1 天，早期骨折纤维性骨痂中的炎症细胞、前成骨细胞、血管内皮细胞及脂肪细胞等 Runx2 呈棕色阳性表达，整体 Runx2 阳性面积比率均值为 30.2%（图 10.A、B）。建模后第 7 天，骨折软骨痂中纤维细胞及生成的软骨细胞等 Runx2 免疫组化染色散在阳性表达，整体 Runx2 阳性面积比率均值为 7.5%（图 10.C、D）。建模后 14 天，骨折骨痂中软骨细胞、成骨细胞、破骨细胞及周围纤维结缔组织细胞 Runx2 免疫组化染色散在阳性表达，整体 Runx2 阳性面积比率均值为 12.1%（图 10.E、F）。

4.3.3 建模后第 1、7、14 天骨折处组织中 Runx2 蛋白表达分析

为了进一步验证 Runx2 蛋白在骨折愈合不同时期的作用,我们对 Runx2 蛋白免疫组化阳性面积强度(H-score 评分)统计分析,结果显示:小鼠骨折组织中 Runx2 蛋白的表达在骨折早期纤维性骨痂中高表达。建模后第 7、14 天 Runx2 蛋白的表达量分别是第 1 天的 15%和 19%,差异有统计学意义($P<0.001$,图 13 B)。结果提示纤维性骨痂中 Runx2 蛋白可能在骨折初期起比较重要的作用,而随着骨折的进行,软骨痂及骨痂中的 Runx2 蛋白略有下降。

4.4 建模后第 1、7、14 天骨折处组织中成骨特异性转录因子 Osterix 的表达

4.4.1 建模后第 1、7、14 天骨折处组织中 Osterix mRNA 的表达

研究表明,特异性转录因子 Osterix 在机体发育中的骨骼中特异表达。在 Osterix 敲除的小鼠中,无法形成骨骼。为了进一步探究 Osterix 在体内骨折愈合中的作用,我们应用 RT-PCR 方法检测了 Osterix mRNA 的表达,结果显示:小鼠骨折初期纤维性骨痂中 Osterix mRNA 低表达,随骨折愈合,软骨痂或骨痂中的 Osterix mRNA 高表达。建模后第 7、14 天 Osterix mRNA 的表达量分别是第 1 天的 124.0 倍和 775.1 倍,第 14 天 Osterix mRNA 的量约是第 7 天的 6.3 倍(图 13. C)。从而进一步验证了 Osterix 基因可能是成骨分化的重要转录基因,其在骨折愈合中可能具有重要生物学作用。

4.4.2 建模后第 1、7、14 天骨折处组织中 Osterix 蛋白分布

为了进一步了解 Osterix 蛋白在体内不同细胞分布情况,我们采用 AI 图像分析系统检测了 Osterix 免疫组化蛋白表达分布情况,结果表明:建模后第 1 天,骨折纤维性骨痂中的炎症细胞、血管内皮细胞、前成骨细胞等 Osterix 呈棕色阳性表达,整体 Osterix 阳性面积比率均值为 21.2%(图 11.A、B)。建模后第 7 天,骨折软骨痂中软骨细胞及纤维细胞等 Osterix 免疫组化染色散在阳性表达,整体 Osterix 阳性面积比率均值为 3.8%(图 11.C、D)。建模后第 14 天,骨折骨痂中的成骨细胞、软骨细胞、破骨细胞及周围纤维结缔组织细胞 Osterix 免疫组化染色散在阳性表达,整体 Osterix 阳性面积比率均值为 3.3%(图 11.E、F)。

4.4.3 建模后第 1、7、14 天骨折处组织中 Osterix 蛋白表达

为了进一步验证 Osterix 蛋白在骨折成骨不同时期的作用,我们对 Osterix 蛋白 H-score 评分统计分析,结果表明:Osterix 蛋白在骨折早期纤维性骨痂中高表达。建模后第 7、14 天 Osterix 蛋白的表达量分别是第 1 天 Osterix 蛋白的 12%和 10%,差异有统计学意义($P<0.001$,图 13. D)。

讨 论

神经肽 Y (NPY)是一种由 36 个氨基酸构成的多肽,是一种位于大脑和自主神经系统中的重要神经递质^[56]。NPY 是一种维持食欲和调节能量平衡的典型生物因子,在中枢神经系统中发挥着关键作用^[57],以及在慢性骨丢失引起的应激反应中起到保护作用^[15]。相关文献指出,NPY 在骨髓间充质干细胞、前成骨细胞、脂肪细胞及成骨细胞等多种细胞类型中表达,并在调节细胞骨代谢起到重要的作用^[16, 58, 59]。关节滑膜、关节软骨、半月板和软骨下骨等髌、膝等关节的组织都含有丰富的神经肽 Y,是骨骼重要的神经营养因子^[3]。NPY 在中枢及周围组织中均会参与骨代谢的平衡,并与一系列的相关转录因子调节骨合成和骨重塑^[18, 60]。

神经肽 Y (NPY)受体是 G 蛋白偶联受体超家族的成员,目前在人类中有五个亚型(Y1、2、4、5 和 6)^[39]。然而,在啮齿类动物中,骨量主要是由 NPY 的 Y1 和 Y2 受体调节的^[9]。小鼠骨折愈合需要多种细胞介质及多种细胞通路参与^[61, 62]。本部分研究初步探讨了 NPY、NPY1R、Runx2、Osterix 参与小鼠骨折愈合的相应机制,为下一步体外研究 NPY 调控小鼠前成骨细胞成骨分化的潜在生物学机制打下了基础。

1. NPY 与骨折愈合不同细胞的相关性研究

据文献报道,NPY 能显著增强骨折早期血痂中骨髓间充质干细胞(BMSC)成骨分化,并显著上调 β -catenin mRNA 表达水平以及 p-GSK-3 β 、 β -catenin 等蛋白表达水平^[63]。此外,NPY 能上调骨髓间充质干细胞(BMSC)血管内皮生长因子的表达,进而增强其自身细胞的迁移能力,促进成骨^[63]。在本部分研究中,NPY 基因在骨折早期纤维性骨痂炎症细胞、间充质干细胞等细胞中 mRNA 及蛋白水平均高表达,提示骨髓间充质干细胞等相关细胞可能通过 NPY 途径促进骨折愈合,NPY 可能起到诱导成骨的作用。

早在 1985 年, M Walter 等在骨缺损早期植入成纤维细胞和纤维细胞浸渍过的植入物,形态学和荧光组织学研究后发现即可发展成软骨细胞、软骨细胞、成骨细胞和骨细胞^[64]。成纤维细胞多在骨折后第 3 天起即可出现于骨折局部血肿中,术后 5 天在骨折断端血肿中成纤维细胞不断增生,并分泌许多 I 型胶原,形成松散的纤维结缔组织及纤维骨痂。7 天左右,纤维骨痂逐渐转变为软骨痂,第 2 周即可在软骨细胞中地神经纤维中发现有 NPY 表达^[65]。在本部分研究中,术后第 7 天,成纤维细胞、纤维细胞及生成的软骨细胞等 NPY 蛋白表达。提示 NPY 可能也参

与并调控成纤维细胞的增生及表达, 促进骨折愈合。

骨折发生后,成骨细胞^[62, 66]和破骨细胞^[67, 68]被激活,骨折愈合过程发挥重要作用。成骨细胞移行到骨折区域分泌新的骨基质^[69, 70]。在新生骨连接骨折的骨端后,破骨细胞诱导骨吸收和成骨细胞诱导的骨形成交替进行, 协同完成骨重塑过程。本部分研究中, 新生骨表面的成骨细胞和破骨细胞 NPY 蛋白散在阳性表达, 提示 NPY 可能参与调节成骨、破骨细胞的增殖及表达, 利于骨折愈合。

由此可见, NPY 在周围局部骨组织骨髓间充质干细胞、成骨细胞、成纤维细胞、破骨细胞、骨细胞中发挥了比较重要的调节作用^[8, 17, 18]。

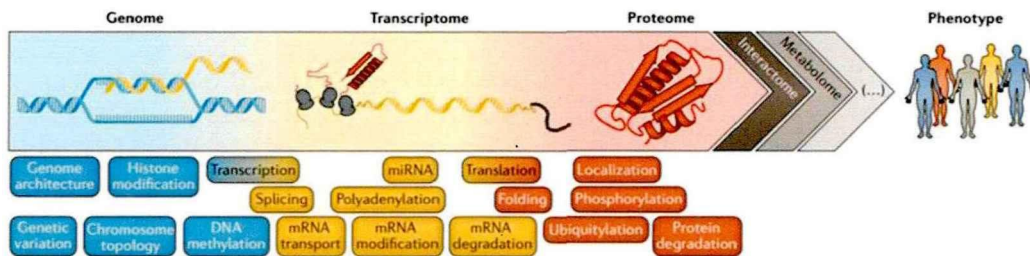
2. NPY、NPY1R、Runx2、Osterix 在骨折愈合不同时期作用及表达

统计分析发现, NPY mRNA 和蛋白在骨折术后第 1 天纤维骨痂期均高表达, 提示 NPY 可能参与诱导间充质干细胞、前成骨细胞、炎症细胞、血管内皮细胞聚集, 促进骨折愈合。然而, 我们也观测到 NPY mRNA 和蛋白水平在建模术后第 14 天不同步的情况, NPY 蛋白表达呈现增高, 而 mRNA 显示降低。我们追踪标本及查阅文献后发现, 这可能与骨折取样地点有关, 骨折凸起侧与凹陷侧的 NPY 蛋白表达可能是不一致的。骨折早期凹陷侧的 NPY 纤维蛋白表达是高的, 而凸起侧 NPY 纤维蛋白是低的, 随着骨折的进行这种趋势会逆转回来。Hua Long 等人研究表明, 成角骨折早期和晚期 NPY 明显增加, 与凸侧相比, 凹侧 NPY 纤维在第 7 天增加了 5 倍, 而凸侧 NPY 纤维在第 21 和 56 天则增加了 6 倍, 提示 NPY 在骨折愈合早期和晚期在凸侧和凹侧的神经纤维可能不一致^[23]。所以, NPY 蛋白可能具有空间特异性, 有必要今后做更多更详细的研究, 阐述 NPY 的相应生物学机制。

NPY1R 蛋白在骨折愈合多种细胞浆中均得到表达, 特别是骨折初期炎症细胞、前成骨细胞、脂肪细胞及血管内皮细胞等胞浆内 NPY1R 蛋白高表达。NPY1R 在第 7 天和第 14 天的软骨痂及骨痂中表达趋势与 NPY 基本相似, NPY1R 蛋白与 NPY 蛋白在骨折愈合中的变化趋势是一致的, 提示 NPY1R 可能与 NPY 协同参与骨折早期成骨诱导, 相关文献^[41, 71, 72]也证实了 NPY 与 NPY1R 之间的作用机制, 这与我们的研究结果基本一致。但是我们也发现 NPY1R 在骨折第 1 天 mRNA 和蛋白水平不一致的情况, 查阅文献, 我们得知一些基因只在特定的环境条件下影响生物体的表型, 并且基因表达是一个复杂的过程, 在许多层面上受到调节。基因的转录受转录因子、表观遗传标记、染色质拓扑等多种因素控制^[73]。如下图所

示, 展示的是基因表达的相关步骤。随着骨折愈合的进行, 软骨痂及骨痂中的 NPY1R 的 mRNA 和蛋白水平是一致的, NPY1R 呈现逐渐增高的过程。

Runx2 基因是非常关键早期成骨转录因子, 介导间充质干细胞分化为成骨细胞以及成骨相关特异性基因的表达^[74-76]。此外, Runx2 基因在成骨细胞终末分化中起不可替代的作用^[77-80]。本部分研究中, Runx2 蛋白在骨折早期高表达, 提示了 Runx2 蛋白可能影响间充质干细胞的早期分化。同时 Runx2 蛋白在骨折愈合过程中持续表达, 提示 Runx2 可能参与了多种细胞介质的成骨活动。本部分研究



中, NPY 基因与 Runx2 基因表达趋势基本一致, 提示 NPY 与 Runx2 可能协同调控细胞成骨, 这也为我们下一步体外研究 NPY 对 Runx2 基因的调控作用提供了一定研究基础。不过, 我们也发现了 Runx2 基因在骨折第 1 天 mRNA 和蛋白表达不同步的情况, 除了上面提到的可能因素, 还可能与 Runx2 与体内多种调节机制有关, 这与 Hecht J et al 和 Moon JS et al 等^[81, 82]发现 Runx2 基因在同一时间点的 mRNA 和蛋白存在差异表达的研究结果是一致的, 需要今后做更多的研究来揭示其原因。

Nakashima 教授早在 2002 年便首先发现了 Osterix (Osx) 基因, Osterix 也被称为 SP7, 一种锌指转录因子, 通常在成骨细胞中表达, 调节成骨细胞的分化和成熟^[83]。Osterix 的亚细胞结构主要位于细胞核内^[83]。但是最近的研究表明, 不光在成骨细胞及软骨细胞中表达 Osterix 基因, 在转移性乳腺癌细胞中及多种癌细胞中也存在过表达 Osterix 基因的情况^[84]。根据 Xiao, Z. S.等的研究发现, Runx2 是 Osterix 基因的上游调控基因。Runx2-I 还参与维持 Runx2-II 缺陷(Runx2-II^{-/-})小鼠 1/2 的 Osterix 基因表达; 因此, 1/2 的 Osterix mRNA 受到上游 Runx2 基因的调控^[85]。本部分研究中, 通过对照 Osterix 与 Runx2 基因及蛋白表达情况, 进一步揭示了 Osterix 可能受 Runx2 调控的相关机制, 这与上述文献研究基本类似。同时, 这也为我们下一步体外研究 NPY 对 Runx2、Osterix 基因的调控作用提供了一定的研究基础。我们的研究同样提示 Osterix 蛋白在骨折愈合早期可能起重要的作用, 参与了骨折愈合进程。但是, 我们也观测到 Osterix 基因在骨折早期存在 mRNA

与蛋白表达不同步的情况，可能与基因的表达受多种因素调节有关，并且一些基因只在特定的环境条件下影响生物体的表型^[86]。综上所述，Runx2 与 Osterix 的蛋白表达的相对一致性，可能 Runx2 与 Osterix 有共同的传导通路，有待于进一步的研究。

通过本部分的研究揭示了 NPY 基因及蛋白共同参与骨折早期炎症反应，可能起到骨诱导的作用；骨折愈合相应成骨因子 NPY、NPY1R、Runx2 和 Osterix 的表达存在时间及空间特异性。然而，NPY 在骨折愈合中的时空特异性，有待于更深入的研究，以进一步明确 NPY 在整个骨折愈合中的作用机制；建立更多的骨折模型和对照研究，以进一步探索 NPY 与骨折周围神经、血浆的关系。为了更好的揭示 NPY 在骨折、骨质疏松、骨性关节炎等疾病患者的作用机理，在伦理委员会审批通过的前提下，可进一步检测机体不同组织中的 NPY 及其受体的表达情况，分析后对临床将有更多的指导意义。

结 论

1. NPY 基因及蛋白共同参与骨折早期炎症反应，可能起到骨诱导的作用。
2. 骨折愈合相应成骨因子 NPY、NPY1R、Runx2 和 Osterix 的表达存在时间及空间特异性。

附图

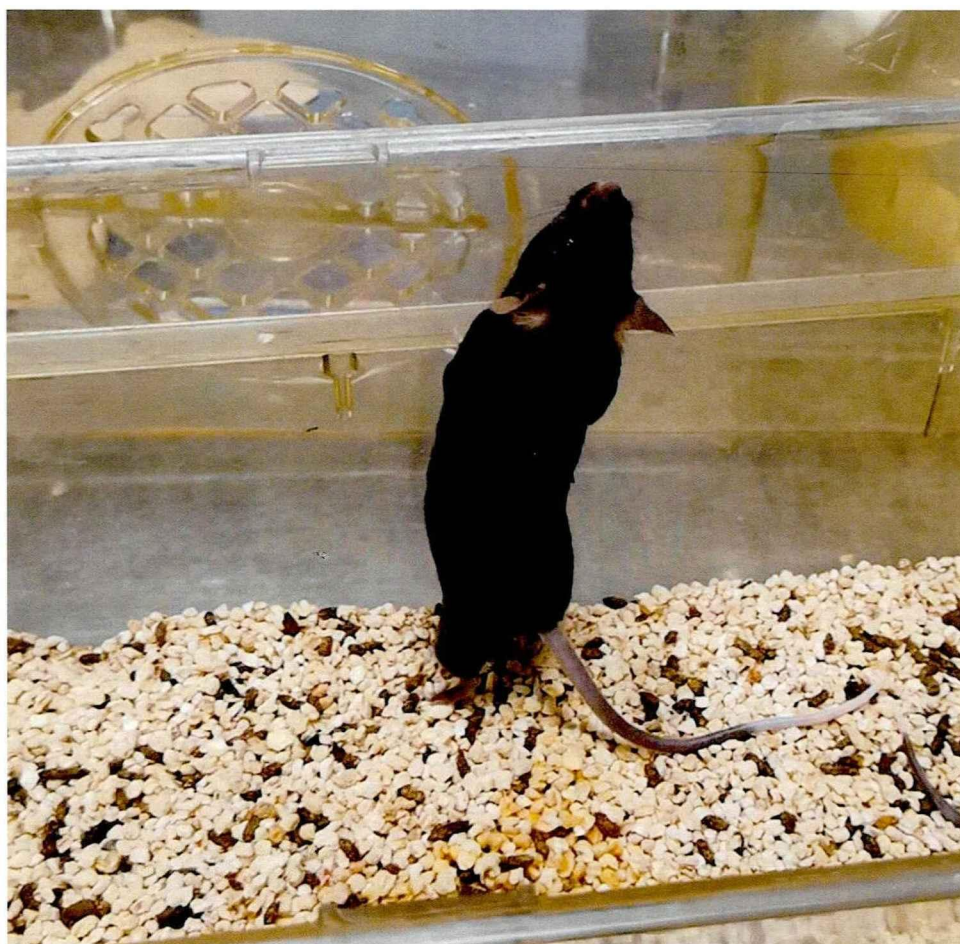


图 1 小鼠骨折模型左侧胫骨骨折术后 14 天大体图像

小鼠左侧胫骨闭合骨折术后 2 周观察，患肢可负重，有时可见双侧肢体同时站立的情况。



图 2. 建模成功即时行 CT 三维重建图像

建模成功即时行 CT 三维重建显示，胫骨横行骨折，骨皮质不连续，骨折线清晰。



图 3 建模后第 1 天 CT 三维重建图像

建模后第 1 天 CT 三维重建显示，胫骨骨折断端略有移位，对位可。

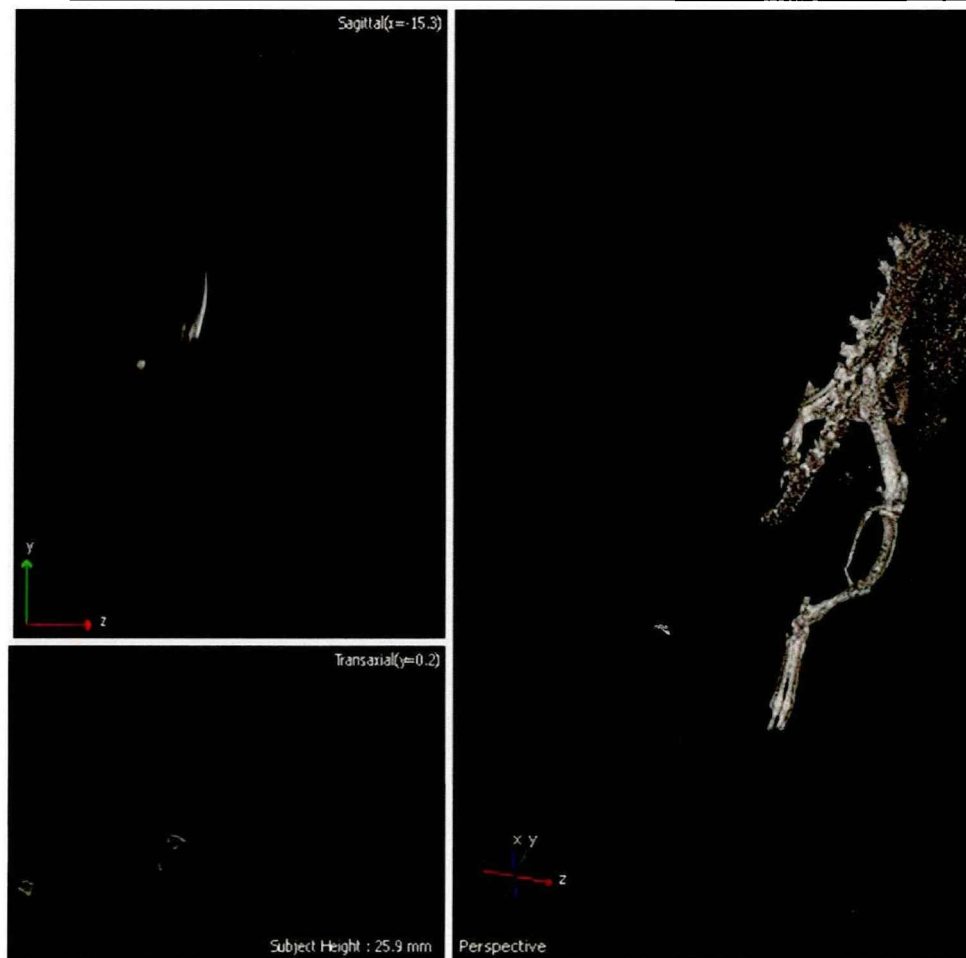


图 4 建模后第 7 天 CT 三维重建图像

建模后第 7 天 CT 三维重建显示，胫骨骨折线模糊不清，对位可，曲度略微成角。

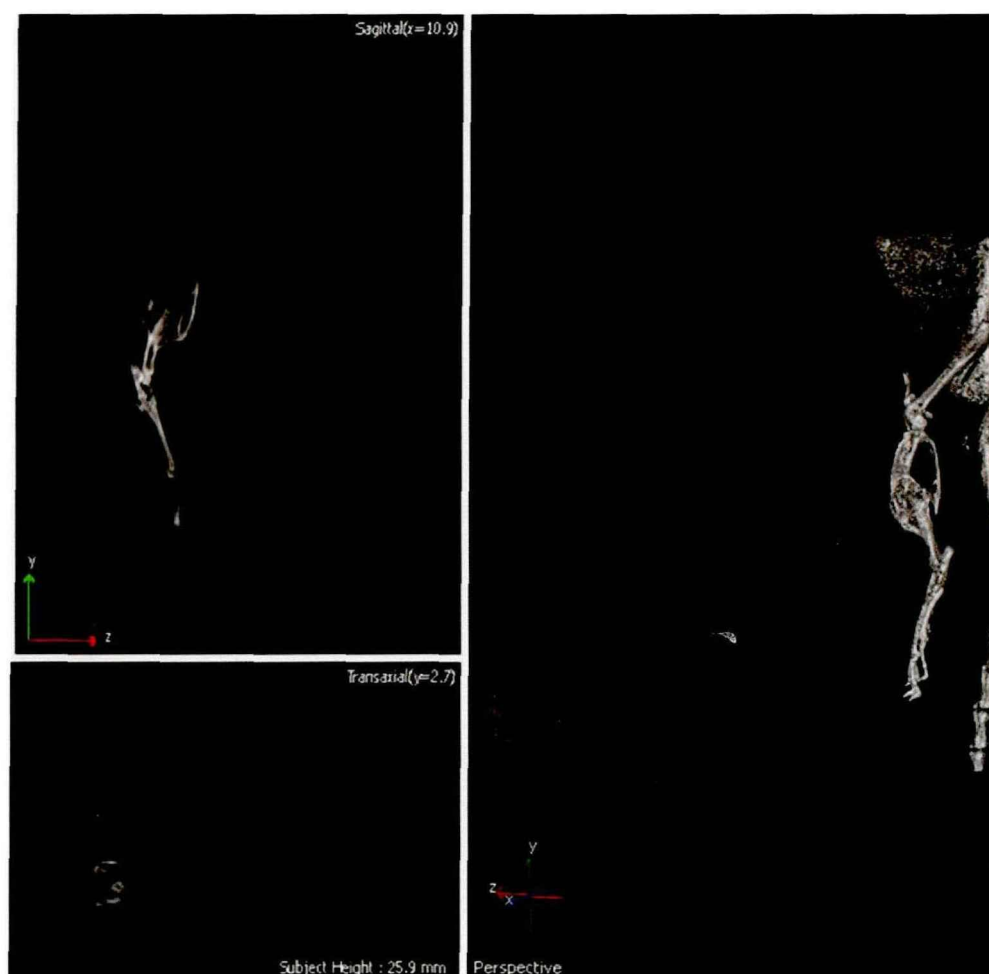


图 5 建模后第 14 天 CT 三维重建显示

建模后第 14 天 CT 三维重建显示，骨折线模糊不清，对位对线可，骨折端骨性骨痂形成。

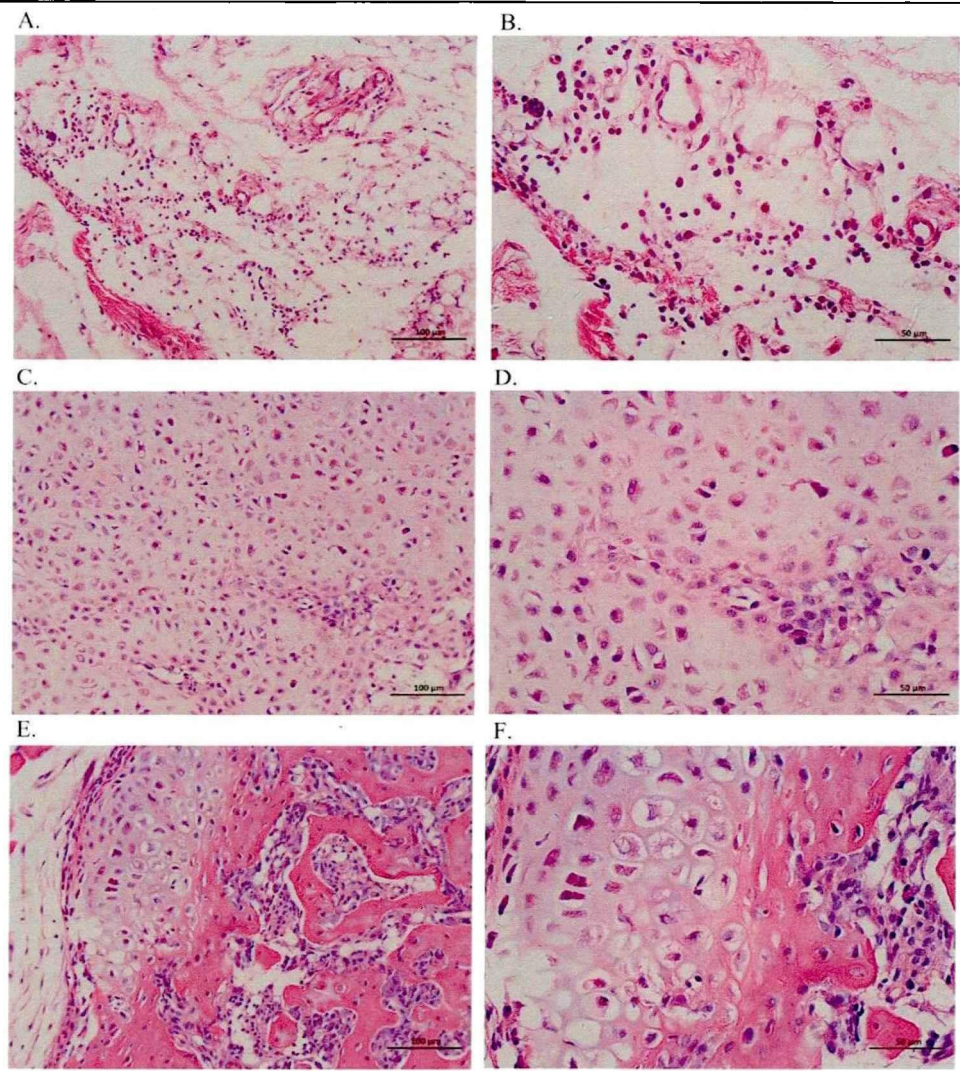


图 6 小鼠骨折模型术后 HE 染色

建模后第 1 天：骨折处可见水肿及炎细胞浸润，纤维蛋白、胶原纤维、网状纤维等构成疏松的纤维性骨痂结构（图 A、B）。建模后第 7 天：纤维结缔组织增生，局部见纤维组织转化为软骨组织，构成软骨痂，并可见少量骨样基质形成（图 C、D）。建模后第 14 天：软骨细胞被骨的骨膜外壳包绕，开始肥大和肿胀，出现初级骨化中心，并可见新生骨小梁结构，构成骨性骨痂（图 E、F）。（图 A、C、E: 200×；图 B、D、F: 400×）。

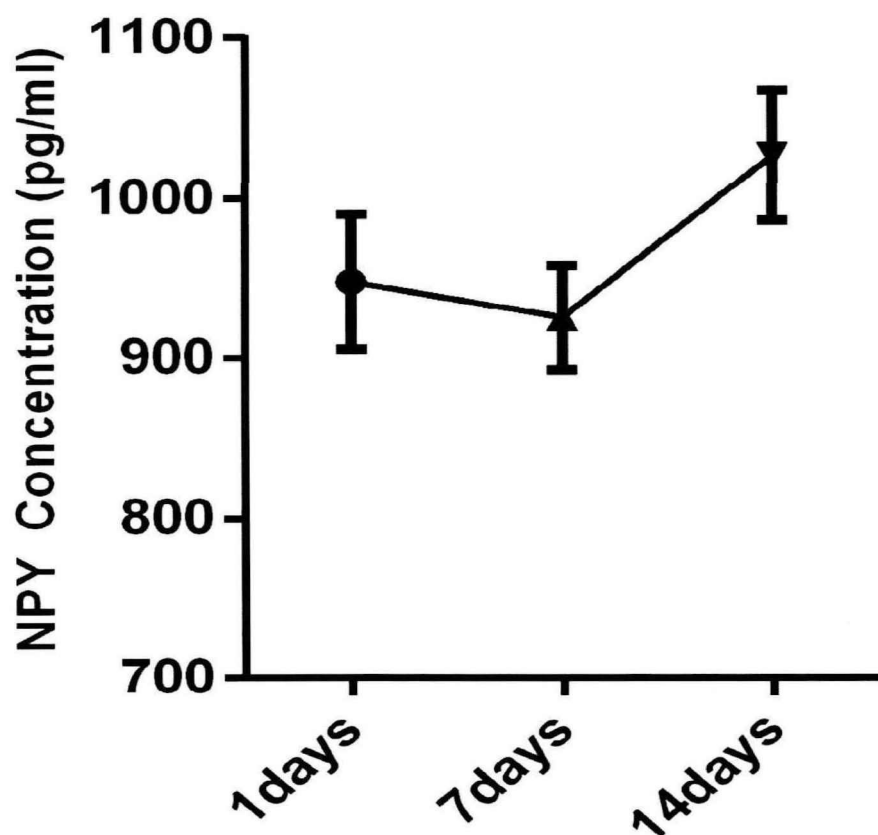


图 7 小鼠骨折模型血清中 NPY 浓度检测结果

NPY 不同时间点(建模后第 1、7、14 天)浓度范围为 925.35 pg/ml-1055.2 pg/ml。NPY 浓度在检测的 14 天的时间段内浓度有一过性下降后逐渐增高趋势,但整体差异无明显统计学意义 ($P=0.152$)。

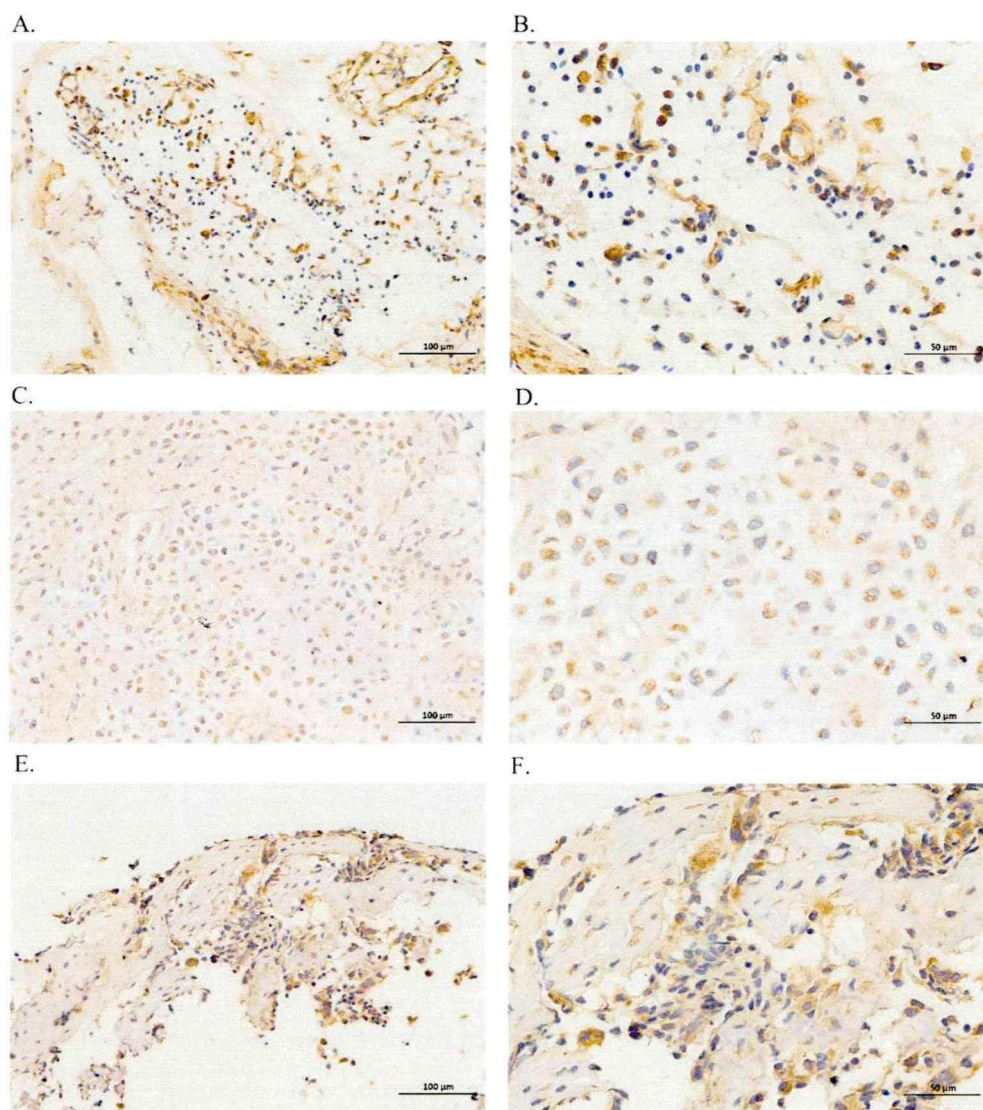


图 8 小鼠骨折模型中 NPY 蛋白免疫组化染色

建模后第 1 天，骨折早期纤维性骨痂处的炎症细胞、前成骨细胞、脂肪细胞及血管内皮细胞等 NPY 染色胞浆阳性表达，呈棕色表现，整体 NPY 阳性面积比率均值为 28.0%（图 A、B）。建模后第 7 天，骨折软骨痂中软骨细胞及纤维细胞等 NPY 染色胞浆阳性表达，整体 NPY 阳性面积比率均值为 11.2%（图 C、D）。建模后第 14 天，骨性骨痂中成骨细胞、软骨细胞、破骨细胞及周围纤维结缔组织细胞 NPY 染色散在阳性表达，新生骨表面的成骨、破骨细胞 NPY 染色弥漫阳性表达，强度较强，骨小梁中骨细胞散在的阳性，强度较弱，整体 NPY 阳性面积比率均值为 23.2%（图 E、F）。（A、C、E: 200×；B、D、F: 400×）。

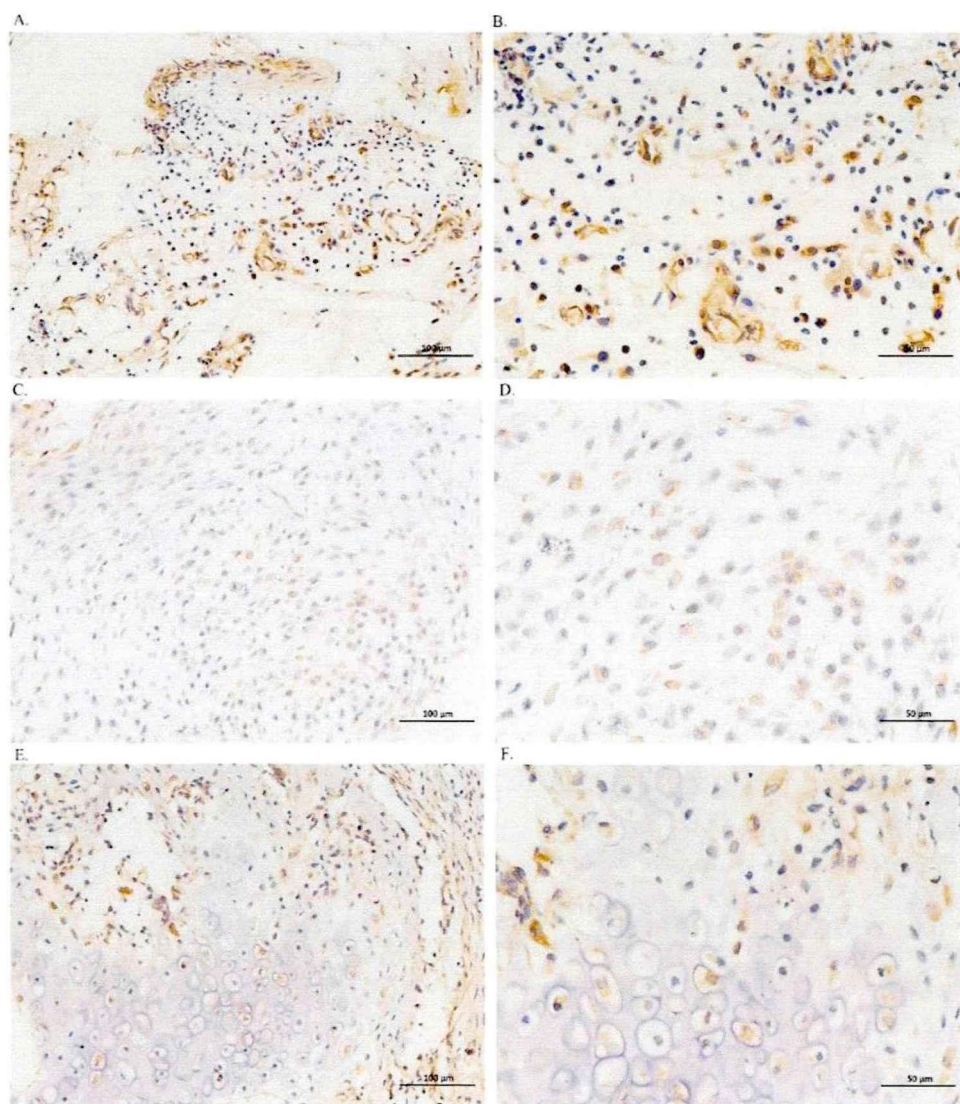


图 9 小鼠骨折模型中 NPY1R 蛋白免疫组化染色

建模后第 1 天，骨折早期纤维性骨痂中的炎症细胞、前成骨细胞、血管内皮细胞及脂肪细胞等呈阳性表达，整体 NPY1R 阳性面积比率均值为 29.6% (图 A、B)。建模后第 7 天，骨折软骨痂中纤维细胞及软骨细胞等 NPY1R 染色散在阳性表达，整体 NPY1R 阳性面积比率均值为 4.1% (图 C、D)。建模后第 14 天，骨折骨痂中软骨细胞、成骨细胞、破骨细胞及周围纤维结缔组织细胞 NPY1R 染色散在阳性表达，整体 NPY1R 阳性面积比率均值为 10.0% (图 E、F)。(图 A、C、E: 200×; 图 B、D、F: 400×)。

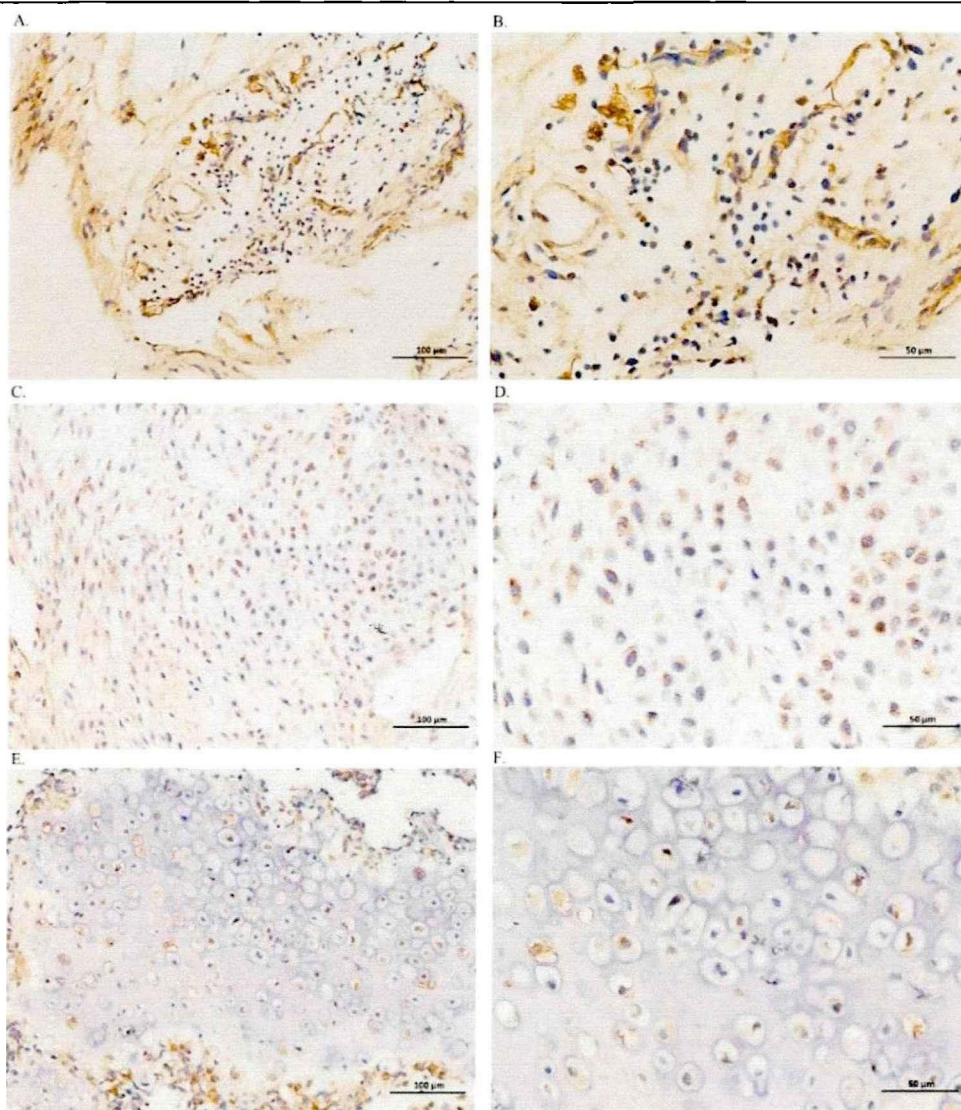


图 10 小鼠骨折模型中 Runx2 蛋白免疫组化染色

建模后第 1 天，骨折早期纤维性骨痂中的炎症细胞、前成骨细胞、血管内皮细胞及脂肪细胞等 Runx2 呈棕色阳性表达，整体 Runx2 阳性面积比率均值为 30.2%(图 A、B)。建模后第 7 天，骨折软骨痂中纤维细胞及软骨细胞等 Runx2 染色散在阳性表达，整体 Runx2 阳性面积比率均值为 7.5% (图 C、D)。建模后 14 天，骨折骨痂中软骨细胞、成骨细胞、破骨细胞及周围纤维结缔组织细胞 Runx2 染色散在阳性表达，整体 Runx2 阳性面积比率均值为 12.1% (图 E、F)。(图 A、C、E: 200×; 图 B、D、F: 400×)。

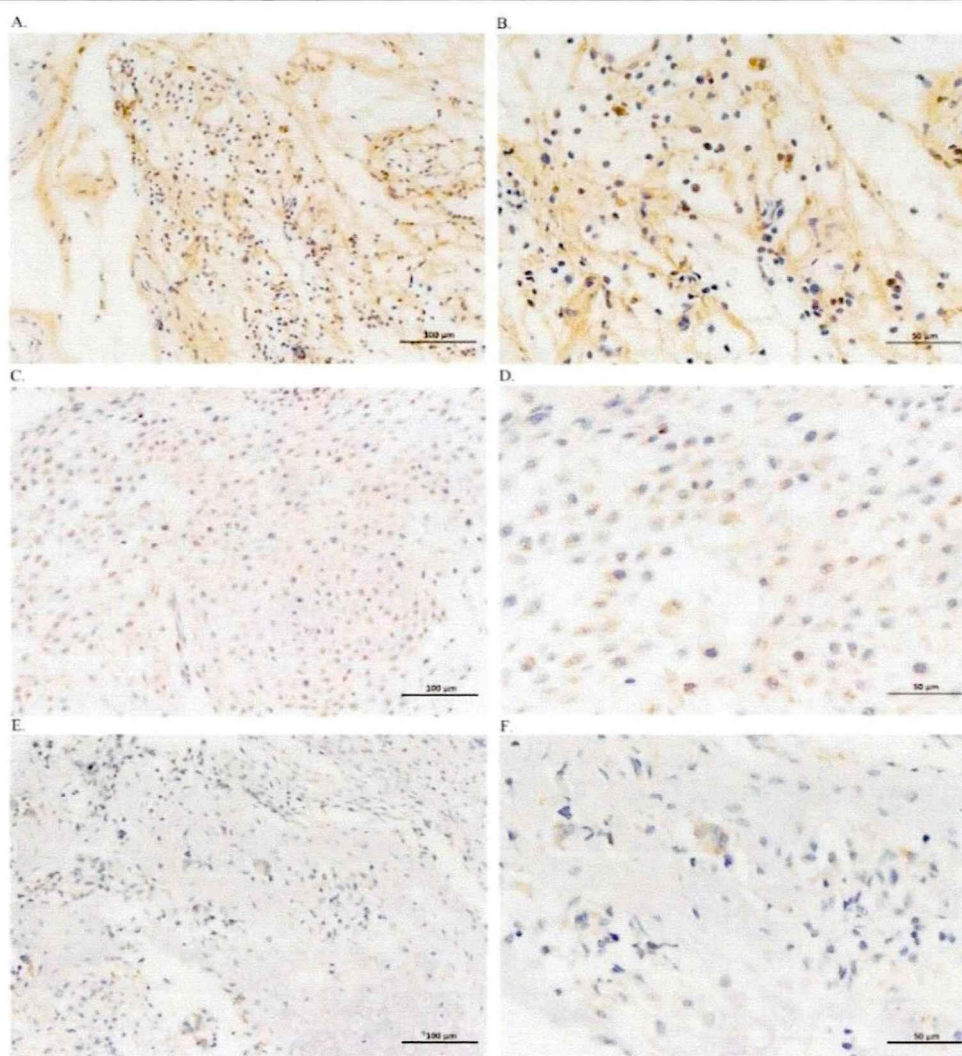


图 11 小鼠骨折模型中 Osterix 蛋白免疫组化染色

建模后第 1 天，骨折早期纤维性骨痂中的炎症细胞、前成骨细胞、血管内皮细胞等 Osterix 呈棕色阳性表达，整体 Osterix 阳性面积比率均值为 21.2%（图 A、B）。建模后第 7 天，骨折软骨痂中软骨细胞及纤维细胞等 Osterix 染色散在阳性表达，整体 Osterix 阳性面积比率均值为 3.8%（图 C、D）。建模后第 14 天，骨折骨痂中的软骨细胞、成骨细胞、破骨细胞及周围纤维结缔组织细胞 Osterix 染色散在阳性表达，整体 Osterix 阳性面积比率均值为 3.3%（图 E、F）。（图 A、C、E: 200×；图 B、D、F: 400×）。

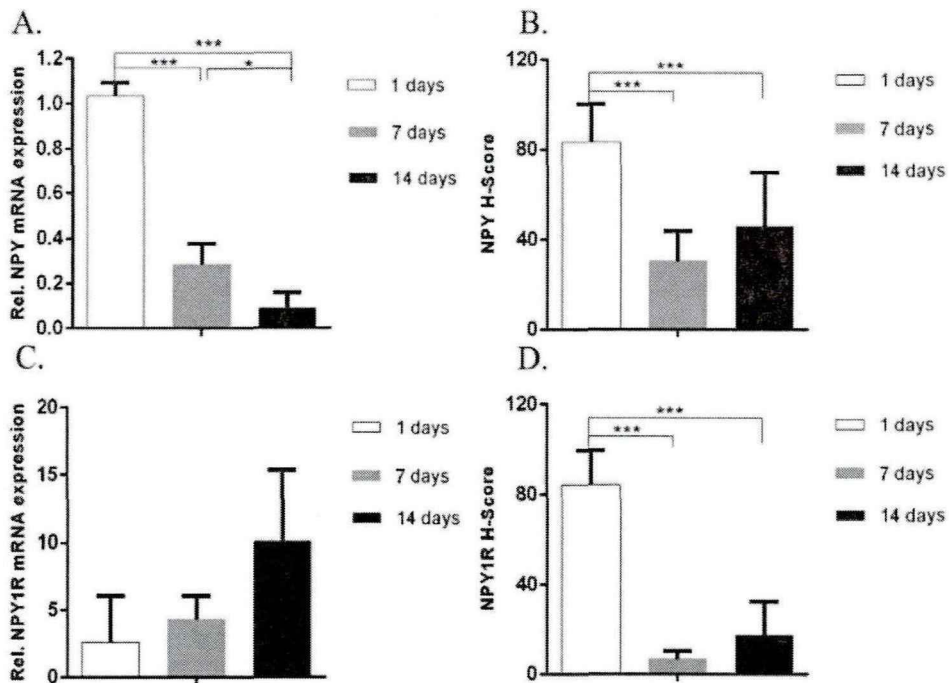


图 12 小鼠骨折模型各个时间点骨折处 NPY 和 NPY1R mRNA 及蛋白表达

建模后第 7、14 天骨折组织中 NPY mRNA 的表达量分别是第 1 天的 27%和 9%，差异有统计学意义 ($P<0.05$ ，图 A)；建模后第 7、14 天软骨痂及骨性骨痂中 NPY 蛋白的表达量分别是第 1 天纤维性骨痂的 37%和 55%，差异有统计学意义 ($P<0.05$ ，图 B)；NPY1R mRNA 表达在骨折愈合不同时期不同组织中并没有明显统计学意义 ($P=0.133$ ，图 C)。建模后第 7、14 天软骨痂及骨性骨痂中 NPY1R 蛋白的表达量分别是第 1 天纤维性骨痂的 8.2%和 21%，差异具有统计学意义 ($P<0.001$ ，图 D)。

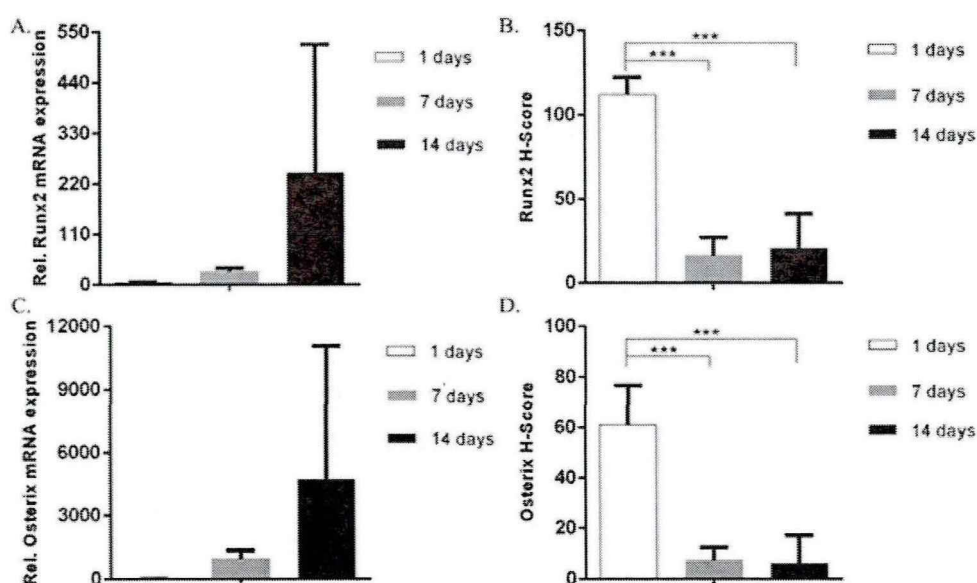


图 13 小鼠骨折模型各个时间点骨折处 Runx2、Osterix mRNA 及蛋白表达

建模后第 7、14 天骨折组织中 Runx2 mRNA 的表达量分别是第 1 天的 7.88 倍和 65.62 倍，第 14 天 Runx2 mRNA 的量约是第 7 天的 8.34 倍，但无显著统计学意义(图 A);建模后第 7、14 天 Runx2 蛋白的表达量分别是第 1 天的 15%和 19%，差异有统计学意义 ($P<0.001$ ，图 B);建模后第 7、14 天 Osterix mRNA 的表达量分别是第 1 天的 123.98 倍和 775.06 倍，第 14 天 Osterix mRNA 的量约是第 7 天的 6.25 倍，但无明显统计学意义 (图 C);建模后第 7、14 天 Osterix 蛋白的表达量分别是第 1 天的 12%和 10%，差异有统计学意义 ($P<0.001$ ，图 D)。

附表

表 1 逆转录定量 PCR 的引物序列

Name	Sequence
NPY	5'-CTCGTGTGTTTGGGCATTC-3'
NM_023456	3'-TAGTGTCGCAGAGCGGAGTA-5'
NPY1R	5'-TATACCACTCTTCTCTTGGTGCTG-3'
NC_000004.12	3'-CTGGAAGTTTTTGTTCAGGAACCCA-5'
Runx2	5'-GACGAGGCAAGAGTTTCACC-3'
NM_009820	3'-GGACCGTCCACTGTCACTTT-5'
Osterix	5'-GATGGCGTCCTCTCTGCTTG-3'
NM_130458.3	3'-TCITTGTGCCTCCTTTCCCC-5'
GAPDH	5'-TCCACCACCCTGTTGCTGTA-3'
NM_002046.3	3'-ACCACAGTCCATGCCATCAC-5'

参考文献

- [1] J.A. Kanis, An update on the diagnosis of osteoporosis, *Current rheumatology reports*, 2 (2000) 62-66.
- [2] N. Harvey, E. Dennison, C. Cooper, Osteoporosis: impact on health and economics, *Nature reviews. Rheumatology*, 6 (2010) 99-105.
- [3] S. Grässel, D. Muschter, Do Neuroendocrine Peptides and Their Receptors Qualify as Novel Therapeutic Targets in Osteoarthritis?, *International journal of molecular sciences*, 19 (2018).
- [4] F. Coluzzi, M.S. Scerpa, M. Centanni, The Effect of Opiates on Bone Formation and Bone Healing, *Curr Osteoporos Rep*, 18 (2020) 325-335.
- [5] A. Eitner, J. Pester, S. Nietzsche, G.O. Hofmann, H.G. Schaible, The innervation of synovium of human osteoarthritic joints in comparison with normal rat and sheep synovium, *Osteoarthritis and cartilage*, 21 (2013) 1383-1391.
- [6] C. Yamada, Y. Yamada, K. Tsukiyama, K. Yamada, N. Udagawa, N. Takahashi, K. Tanaka, D.J. Drucker, Y. Seino, N. Inagaki, The murine glucagon-like peptide-1 receptor is essential for control of bone resorption, *Endocrinology*, 149 (2008) 574-579.
- [7] J. Kaludjerovic, W.E. Ward, Bone-specific gene expression patterns and whole bone tissue of female mice are programmed by early life exposure to soy isoflavones and folic acid, *The Journal of nutritional biochemistry*, 26 (2015) 1068-1076.
- [8] I. Matic, B.G. Matthews, T. Kizivat, J.C. Igwe, I. Marijanovic, S.T. Ruohonen, E. Savontaus, D.J. Adams, I. Kalajzic, Bone-specific overexpression of NPY modulates osteogenesis, *J Musculoskelet Neuronal Interact*, 12 (2012) 209-218.
- [9] S.J. Allison, P.A. Baldock, H. Herzog, The control of bone remodeling by neuropeptide Y receptors, *Peptides*, 28 (2007) 320-325.
- [10] N.J. Lee, K.L. Doyle, A. Sainsbury, R.F. Enriquez, Y.J. Hort, S.J. Riepler, P.A. Baldock, H. Herzog, Critical role for Y1 receptors in mesenchymal progenitor cell differentiation and osteoblast activity, *J Bone Miner Res*, 25 (2010) 1736-1747.
- [11] A. Ho-Shui-Ling, J. Bolander, L.E. Rustom, A.W. Johnson, F.P. Luyten, C. Picart, Bone regeneration strategies: Engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives, *Biomaterials*, 180 (2018) 143-162.

- [12] P.A. Baldock, N.J. Lee, F. Driessler, S. Lin, S. Allison, B. Stehrer, E.J. Lin, L. Zhang, R.F. Enriquez, I.P. Wong, M.M. McDonald, M. During, D.D. Pierroz, K. Slack, Y.C. Shi, E. Yulyaningsih, A. Aljanova, D.G. Little, S.L. Ferrari, A. Sainsbury, J.A. Eisman, H. Herzog, Neuropeptide Y knockout mice reveal a central role of NPY in the coordination of bone mass to body weight, *PLoS One*, 4 (2009) e8415.
- [13] D.M. Sousa, P.A. Baldock, R.F. Enriquez, L. Zhang, A. Sainsbury, M. Lamghari, H. Herzog, Neuropeptide Y Y1 receptor antagonism increases bone mass in mice, *Bone*, 51 (2012) 8-16.
- [14] Y.C. Shi, P.A. Baldock, Central and peripheral mechanisms of the NPY system in the regulation of bone and adipose tissue, *Bone*, 50 (2012) 430-436.
- [15] P.A. Baldock, S. Lin, L. Zhang, T. Karl, Y. Shi, F. Driessler, A. Zengin, B. Horner, N.J. Lee, I.P. Wong, E.J. Lin, R.F. Enriquez, B. Stehrer, M.J. During, E. Yulyaningsih, S. Zolotukhin, S.T. Ruohonen, E. Savontaus, A. Sainsbury, H. Herzog, Neuropeptide y attenuates stress-induced bone loss through suppression of noradrenaline circuits, *J Bone Miner Res*, 29 (2014) 2238-2249.
- [16] P.A. Baldock, S.J. Allison, P. Lundberg, N.J. Lee, K. Slack, E.J. Lin, R.F. Enriquez, M.M. McDonald, L. Zhang, M.J. During, D.G. Little, J.A. Eisman, E.M. Gardiner, E. Yulyaningsih, S. Lin, A. Sainsbury, H. Herzog, Novel role of Y1 receptors in the coordinated regulation of bone and energy homeostasis, *J Biol Chem*, 282 (2007) 19092-19102.
- [17] K. Tatemoto, Neuropeptide Y: complete amino acid sequence of the brain peptide, *Proc Natl Acad Sci U S A*, 79 (1982) 5485-5489.
- [18] H. Horsnell, P.A. Baldock, Osteoblastic Actions of the Neuropeptide Y System to Regulate Bone and Energy Homeostasis, *Curr Osteoporos Rep*, 14 (2016) 26-31.
- [19] L. Teixeira, D.M. Sousa, A.F. Nunes, M.M. Sousa, H. Herzog, M. Lamghari, NPY revealed as a critical modulator of osteoblast function in vitro: new insights into the role of Y1 and Y2 receptors, *J Cell Biochem*, 107 (2009) 908-916.
- [20] H. Hosaka, A. Nagata, T. Yoshida, T. Shibata, T. Nagao, T. Tanaka, Y. Saito, I. Tatsuno, Pancreatic polypeptide is secreted from and controls differentiation through its specific receptors in osteoblastic MC3T3-E1 cells, *Peptides*, 29 (2008) 1390-1395.
- [21] Z. Wang, D. Jin, Y. Tuo, X. Guo, J. Wen, J. Zhou, L. Xia, Y. Zhang, [Calcitonin gene-related peptide promoting migration of rat bone marrow mesenchymal stem cells

and stimulating expression of vascular endothelial growth factor], *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi*, 25 (2011) 1371-1376.

[22] W. Xie, F. Li, Y. Han, Z. Li, J. Xiao, Neuropeptides are associated with pain threshold and bone microstructure in ovariectomized rats, *Neuropeptides*, 81 (2020) 101995.

[23] H. Long, M. Ahmed, P. Ackermann, A. Stark, J. Li, Neuropeptide Y innervation during fracture healing and remodeling. A study of angulated tibial fractures in the rat, *Acta orthopaedica*, 81 (2010) 639-646.

[24] J.M. Allen, T.E. Adrian, K. Tatemoto, J.M. Polak, J. Hughes, S.R. Bloom, Two novel related peptides, neuropeptide Y (NPY) and peptide YY (PYY) inhibit the contraction of the electrically stimulated mouse vas deferens, *Neuropeptides*, 3 (1982) 71-77.

[25] N. Takei, K. Sasaoka, H. Higuchi, Y. Endo, H. Hatanaka, BDNF increases the expression of neuropeptide Y mRNA and promotes differentiation/maturation of neuropeptide Y-positive cultured cortical neurons from embryonic and postnatal rats, *Brain Res Mol Brain Res*, 37 (1996) 283-289.

[26] A. Barnea, G. Cho, G. Lu, M. Mathis, Brain-derived neurotrophic factor induces functional expression and phenotypic differentiation of cultured fetal neuropeptide Y-producing neurons, *J Neurosci Res*, 42 (1995) 638-647.

[27] A. Barnea, J. Roberts, Induction of functional and morphological expression of neuropeptide Y (NPY) in cortical cultures by brain-derived neurotrophic factor (BDNF): evidence for a requirement for extracellular-regulated kinase (ERK)-dependent and ERK-independent mechanisms, *Brain Res*, 919 (2001) 57-69.

[28] J.L. Barreto-Estrada, W.E. Medina-Ortiz, J.E. Garcia-Arraras, The morphological and biochemical response of avian embryonic sympathoadrenal cells to nerve growth factor is developmentally regulated, *Brain Res Dev Brain Res*, 144 (2003) 1-8.

[29] Y. Israel, Y. Kandov, E. Khaimova, A. Kest, S.R. Lewis, G.W. Pasternak, Y.X. Pan, G.C. Rossi, R.J. Bodnar, NPY-induced feeding: pharmacological characterization using selective opioid antagonists and antisense probes in rats, *Peptides*, 26 (2005) 1167-1175.

[30] H. Higuchi, [Neuropeptide Y (NPY)], *Nihon Yakurigaku Zasshi*, 132 (2008) 310-312.

- [31] H. Higuchi, T. Niki, T. Shiiya, Feeding behavior and gene expression of appetite-related neuropeptides in mice lacking for neuropeptide Y Y5 receptor subclass, *World journal of gastroenterology*, 14 (2008) 6312-6317.
- [32] J.C. Erickson, K.E. Clegg, R.D. Palmiter, Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y, *Nature*, 381 (1996) 415-421.
- [33] A. Kanatani, S. Mashiko, N. Murai, N. Sugimoto, J. Ito, T. Fukuroda, T. Fukami, N. Morin, D.J. MacNeil, L.H. Van der Ploeg, Y. Saga, S. Nishimura, M. Ihara, Role of the Y1 receptor in the regulation of neuropeptide Y-mediated feeding: comparison of wild-type, Y1 receptor-deficient, and Y5 receptor-deficient mice, *Endocrinology*, 141 (2000) 1011-1016.
- [34] S. Lin, Y.C. Shi, E. Yulyaningsih, A. Aljanova, L. Zhang, L. Macia, A.D. Nguyen, E.J. Lin, M.J. During, H. Herzog, A. Sainsbury, Critical role of arcuate Y4 receptors and the melanocortin system in pancreatic polypeptide-induced reduction in food intake in mice, *PLoS One*, 4 (2009) e8488.
- [35] H. Higuchi, Molecular analysis of central feeding regulation by neuropeptide Y (NPY) neurons with NPY receptor small interfering RNAs (siRNAs), *Neurochem Int*, 61 (2012) 936-941.
- [36] A.E. Peaire, A. Krantis, W.A. Staines, Distribution of the NPY receptor subtype Y1 within human colon: evidence for NPY targeting a subpopulation of nitrergic neurons, *J Auton Nerv Syst*, 67 (1997) 168-175.
- [37] M. Anitha, B. Chandrasekharan, J.R. Salgado, E. Grouzmann, S. Mwangi, S.V. Sitaraman, S. Srinivasan, Glial-derived neurotrophic factor modulates enteric neuronal survival and proliferation through neuropeptide Y, *Gastroenterology*, 131 (2006) 1164-1178.
- [38] L.E. Kuo, K. Abe, Z. Zukowska, Stress, NPY and vascular remodeling: Implications for stress-related diseases, *Peptides*, 28 (2007) 435-440.
- [39] S. Lin, D. Boey, H. Herzog, NPY and Y receptors: lessons from transgenic and knockout models, *Neuropeptides*, 38 (2004) 189-200.
- [40] K. Wolf, E. Höcherl, T. Farkas, C. Pfister, [Expression and dynamics of peptidergic nerve fibers in granulation tissue after distance osteosynthesis], *Der Unfallchirurg*, 104 (2001) 948-954.

- [41] P. Dong, X. Gu, G. Zhu, M. Li, B. Ma, Y. Zi, Melatonin Induces Osteoblastic Differentiation of Mesenchymal Stem Cells and Promotes Fracture Healing in a Rat Model of Femoral Fracture via Neuropeptide Y/Neuropeptide Y Receptor Y1 Signaling, *Pharmacology*, 102 (2018) 272-280.
- [42] S. Liu, J.Q. Wu, J.J. Hu, L. Wang, Z. Wang, H. Meng, L.J. Zhuo, J.X. Zheng, [Neuropeptide Y Y1 receptor antagonist PD160170 promotes osteogenic differentiation of rat bone marrow mesenchymal stem cells in vitro and femoral defect repair in rats], *Nan Fang Yi Ke Da Xue Xue Bao*, 38 (2018) 669-676.
- [43] W. Xie, F. Li, Y. Han, Y. Qin, Y. Wang, X. Chi, J. Xiao, Z. Li, Neuropeptide Y1 receptor antagonist promotes osteoporosis and microdamage repair and enhances osteogenic differentiation of bone marrow stem cells via cAMP/PKA/CREB pathway, *Aging (Albany NY)*, 12 (2020) 8120-8136.
- [44] G. Victoria, B. Petrisor, B. Drew, D. Dick, Bone stimulation for fracture healing: What's all the fuss?, *Indian J Orthop*, 43 (2009) 117-120.
- [45] G.S. Baht, D. Silkstone, P. Nadesan, H. Whetstone, B.A. Alman, Activation of hedgehog signaling during fracture repair enhances osteoblastic-dependent matrix formation, *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*, 32 (2014) 581-586.
- [46] L. Vi, G.S. Baht, H. Whetstone, A. Ng, Q. Wei, R. Poon, S. Mylvaganam, M. Gryn timer, B.A. Alman, Macrophages promote osteoblastic differentiation in-vivo: implications in fracture repair and bone homeostasis, *J Bone Miner Res*, 30 (2015) 1090-1102.
- [47] D.M. Sousa, M.M. McDonald, K. Mikulec, L. Peacock, H. Herzog, M. Lamghari, D.G. Little, P.A. Baldock, Neuropeptide Y modulates fracture healing through Y1 receptor signaling, *Journal of orthopaedic research : official publication of the Orthopaedic Research Society*, 31 (2013) 1570-1578.
- [48] T.A. Einhorn, The cell and molecular biology of fracture healing, *Clinical orthopaedics and related research*, (1998) S7-21.
- [49] A. Schindeler, M.M. McDonald, P. Bokko, D.G. Little, Bone remodeling during fracture repair: The cellular picture, *Seminars in cell & developmental biology*, 19 (2008) 459-466.

- [50] F. Yue, Y. Cheng, A. Breschi, J. Vierstra, W. Wu, T. Ryba, R. Sandstrom, Z. Ma, C. Davis, B.D. Pope, Y. Shen, D.D. Pervouchine, S. Djebali, R.E. Thurman, R. Kaul, E. Rynes, R.C. Hardison, M.A. Beer, B. Ren, A comparative encyclopedia of DNA elements in the mouse genome, *Nature*, 515 (2014) 355-364.
- [51] T. Todenhöfer, C. Gratzke, Re: Prostate-specific Membrane Antigen Heterogeneity and DNA Repair Defects in Prostate Cancer, *European urology*, 77 (2020) 559-560.
- [52] R. Guo, L.D. Berry, D.L. Aisner, J. Sheren, T. Boyle, P.A. Bunn, Jr., B.E. Johnson, D.J. Kwiatkowski, A. Drilon, L.M. Sholl, M.G. Kris, MET IHC Is a Poor Screen for MET Amplification or MET Exon 14 Mutations in Lung Adenocarcinomas: Data from a Tri-Institutional Cohort of the Lung Cancer Mutation Consortium, *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer*, 14 (2019) 1666-1671.
- [53] P. Tang, C. Duan, Z. Wang, C. Wang, G. Meng, K. Lin, Q. Yang, Z. Yuan, NPY and CGRP Inhibitor Influence on ERK Pathway and Macrophage Aggregation during Fracture Healing, *Cell Physiol Biochem*, 41 (2017) 1457-1467.
- [54] N.C. Laddha, M. Dwivedi, M.S. Mansuri, M. Singh, H.H. Patel, N. Agarwal, A.M. Shah, R. Begum, Association of neuropeptide Y (NPY), interleukin-1B (IL1B) genetic variants and correlation of IL1B transcript levels with vitiligo susceptibility, *PLoS One*, 9 (2014) e107020.
- [55] L. Li, M. Sapkota, M. Gao, H. Choi, Y. Soh, Macrolactin F inhibits RANKL-mediated osteoclastogenesis by suppressing Akt, MAPK and NFATc1 pathways and promotes osteoblastogenesis through a BMP-2/smad/Akt/Runx2 signaling pathway, *Eur J Pharmacol*, 815 (2017) 202-209.
- [56] S.C. Baraban, Neuropeptide Y and limbic seizures, *Rev Neurosci*, 9 (1998) 117-128.
- [57] A.P. Silva, C. Cavadas, E. Grouzmann, Neuropeptide Y and its receptors as potential therapeutic drug targets, *Clin Chim Acta*, 326 (2002) 3-25.
- [58] L.E. Kuo, J.B. Kitlinska, J.U. Tilan, L. Li, S.B. Baker, M.D. Johnson, E.W. Lee, M.S. Burnett, S.T. Fricke, R. Kvetnansky, H. Herzog, Z. Zukowska, Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome, *Nat Med*, 13 (2007) 803-811.

- [59] K. Yang, H. Guan, E. Arany, D.J. Hill, X. Cao, Neuropeptide Y is produced in visceral adipose tissue and promotes proliferation of adipocyte precursor cells via the Y1 receptor, *FASEB J*, 22 (2008) 2452-2464.
- [60] E.C. Khor, P. Baldock, The NPY system and its neural and neuroendocrine regulation of bone, *Curr Osteoporos Rep*, 10 (2012) 160-168.
- [61] F. Marco, F. Milena, G. Gianluca, O. Vittoria, Peri-implant osteogenesis in health and osteoporosis, *Micron (Oxford, England : 1993)*, 36 (2005) 630-644.
- [62] T.A. Einhorn, L.C. Gerstenfeld, Fracture healing: mechanisms and interventions, *Nature reviews. Rheumatology*, 11 (2015) 45-54.
- [63] S. Liu, D. Jin, J.Q. Wu, Z.Y. Xu, S. Fu, G. Mei, Z.L. Zou, S.H. Ma, Neuropeptide Y stimulates osteoblastic differentiation and VEGF expression of bone marrow mesenchymal stem cells related to canonical Wnt signaling activating in vitro, *Neuropeptides*, 56 (2016) 105-113.
- [64] M. Walter, J.M. Müller, H.W. Keller, U. Brenner, [Ossification of the collagen implant], *Aktuelle Traumatologie*, 15 (1985) 254-259.
- [65] A. Bjurholm, Neuroendocrine peptides in bone, *International orthopaedics*, 15 (1991) 325-329.
- [66] J. Pajarinen, T. Lin, E. Gibon, Y. Kohno, M. Maruyama, K. Nathan, L. Lu, Z. Yao, S.B. Goodman, Mesenchymal stem cell-macrophage crosstalk and bone healing, *Biomaterials*, 196 (2019) 80-89.
- [67] E. Gómez-Barrena, P. Rosset, D. Lozano, J. Stanovici, C. Ermthaller, F. Gerbhard, Bone fracture healing: cell therapy in delayed unions and nonunions, *Bone*, 70 (2015) 93-101.
- [68] K.D. Hankenson, K. Gagne, M. Shaughnessy, Extracellular signaling molecules to promote fracture healing and bone regeneration, *Advanced drug delivery reviews*, 94 (2015) 3-12.
- [69] R. Marsell, T.A. Einhorn, The biology of fracture healing, *Injury*, 42 (2011) 551-555.
- [70] K.D. Hankenson, G. Zimmerman, R. Marcucio, Biological perspectives of delayed fracture healing, *Injury*, 45 Suppl 2 (2014) S8-s15.

- [71] L. Wang, F. Rao, K. Zhang, M. Mahata, J.L. Rodriguez-Flores, M.M. Fung, J. Waalen, M.G. Cockburn, B.A. Hamilton, S.K. Mahata, D.T. O'Connor, Neuropeptide Y(1) Receptor NPY1R discovery of naturally occurring human genetic variants governing gene expression in cells as well as pleiotropic effects on autonomic activity and blood pressure in vivo, *J Am Coll Cardiol*, 54 (2009) 944-954.
- [72] Y. Okahisa, H. Ujike, T. Kotaka, Y. Morita, M. Kodama, T. Inada, M. Yamada, N. Iwata, M. Iyo, I. Sora, N. Ozaki, S. Kuroda, Association between neuropeptide Y gene and its receptor Y1 gene and methamphetamine dependence, *Psychiatry Clin Neurosci*, 63 (2009) 417-422.
- [73] C. Buccitelli, M. Selbach, mRNAs, proteins and the emerging principles of gene expression control, *Nature reviews. Genetics*, 21 (2020) 630-644.
- [74] J. Chen, G. He, Y. Wang, D. Cai, MicroRNA-223 promotes osteoblast differentiation of MC3T3-E1 cells by targeting histone deacetylase 2, *International Journal of Molecular Medicine*, (2018).
- [75] X. Zhang, M. Yang, L. Lin, P. Chen, K.T. Ma, C.Y. Zhou, Y.F. Ao, Runx2 overexpression enhances osteoblastic differentiation and mineralization in adipose--derived stem cells in vitro and in vivo, *Calcif Tissue Int*, 79 (2006) 169-178.
- [76] H. Luo, H. Gao, F. Liu, B. Qiu, Regulation of Runx2 by microRNA-9 and microRNA-10 modulates the osteogenic differentiation of mesenchymal stem cells, *Int J Mol Med*, 39 (2017) 1046-1052.
- [77] J. Han, J. Zuo, D. Zhu, C. Gao, The correlation between SNPs within the gene of adrenergic receptor and neuropeptide Y and risk of cervical vertigo, *J Clin Lab Anal*, 32 (2018) e22366.
- [78] Y. Li, Z. Liu, Y. Tang, W. Feng, C. Zhao, J. Liao, C. Zhang, H. Chen, Y. Ren, S. Dong, Y. Liu, N. Hu, W. Huang, Schnurri-3 regulates BMP9-induced osteogenic differentiation and angiogenesis of human amniotic mesenchymal stem cells through Runx2 and VEGF, *Cell Death Dis*, 11 (2020) 72.
- [79] T. Komori, Molecular Mechanism of Runx2-Dependent Bone Development, *Mol Cells*, 43 (2020) 168-175.
- [80] T. Shimizu, Participation of Runx2 in mandibular condylar cartilage development, *Eur J Med Res*, 11 (2006) 455-461.

- [81] J.S. Moon, S.H. Kim, S.H. Oh, Y.W. Jeong, J.H. Kang, J.C. Park, H.J. Son, S. Bae, B.I. Park, M.S. Kim, J.T. Koh, H.M. Ko, Relaxin augments BMP-2-induced osteoblast differentiation and bone formation, *J Bone Miner Res*, 29 (2014) 1586-1596.
- [82] J. Hecht, V. Seitz, M. Urban, F. Wagner, P.N. Robinson, A. Stiege, C. Dieterich, U. Kornak, U. Wilkening, N. Brieske, C. Zwingman, A. Kidess, S. Stricker, S. Mundlos, Detection of novel skeletogenesis target genes by comprehensive analysis of a *Runx2*(-/-) mouse model, *Gene Expr Patterns*, 7 (2007) 102-112.
- [83] K. Nakashima, X. Zhou, G. Kunkel, Z. Zhang, J.M. Deng, R.R. Behringer, B. de Crombrughe, The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation, *Cell*, 108 (2002) 17-29.
- [84] S. Qu, J. Wu, Q. Bao, B. Yao, R. Duan, X. Chen, L. Li, H. Yuan, Y. Jin, C. Ma, Osterix promotes the migration and angiogenesis of breast cancer by upregulation of S100A4 expression, *Journal of cellular and molecular medicine*, 23 (2019) 1116-1127.
- [85] Z.S. Xiao, A.B. Hjelmeland, L.D. Quarles, Selective deficiency of the "bone-related" *Runx2*-II unexpectedly preserves osteoblast-mediated skeletogenesis, *J Biol Chem*, 279 (2004) 20307-20313.
- [86] A. Gann, Jacob and Monod: from operons to EvoDevo, *Curr Biol*, 20 (2010) R718-723.

第二部分 NPY 在小鼠前成骨细胞 MC3T3-E1 中的表达和生物学功能

前言

严重的创伤性骨折会出现难以修复的节段性骨缺损^[1],进而造成骨折不愈合。研究表明超过 2cm 的骨缺损就需要生物活性因子的介入^[2]。因此,生长因子、多肽及小分子正成为国内外研究的热点^[2]。生长因子、多肽及小分子联合人造骨移植材料可以明显促进成骨细胞的传导,促进骨折愈合^[2]。NPY 作为多肽之一,是维持骨量稳定的重要神经营养因子^[3]。NPY 与多种复合骨支架相结合会对大面积创伤性骨缺损的修复产生积极的影响。因此,探讨 NPY 在 MC3T3-E1 细胞中的成骨机制,对于促进骨折愈合具有重要的临床意义。

NPY 是哺乳动物和人类外周和中枢神经系统中含量较多的肽类激素,对于维持内环境稳定具有重要作用^[4]。NPY 及其受体大量存在于机体神经系统中,并骨稳态的主要调控因子^[5,6]。最新研究表明,NPY 不光存在于神经系统,也可由外周组织中成骨细胞产生,从而促进骨合成代谢^[7]。而 NPY 在小鼠前成骨细胞 MC3T3-E1 中的调控机制则知之甚少,有必要更加系统的研究。

Rfect 质粒 DNA 和 Rfect siRNA 转染试剂法是由常州百代生物技术有限公司专门设计(专利申请号:20100022618),转染 DNA 质粒和 siRNA 到真核细胞的新式方法。Rfect 试剂来自动物源性游离脂质转染试剂,可用于制备 Rfect 载体和质粒 DNA 或 siRNA 的复合物,广泛应用于多种贴壁细胞、原代细胞和悬浮细胞。Rfect 试剂采用动物源性纳米材料制备,具有转染效率高、毒性低的特点。Rfect 载体有以下特性:基因转染效率非常高,接近 80-90%;干扰效率非常明显,接近 80-90%;较小的细胞毒性,明显减少非特异性细胞应激反应^[8,9]。相关研究表明,低浓度的 siRNA 和质粒 DNA 可获得高水平的基因干扰和过表达^[8,9]。目前该转染方法是近年来应用研究的热点之一。

本部分我们将靶向抑制 NPY 的小干扰 RNA (small interfering RNA, siRNA) 和 NPY 过表达质粒应用 Rfect 试剂载体分别转染入前成骨 MC3T3-E1 细胞中,观察其对前成骨 MC3T3-E1 细胞成骨的影响,继而探究 NPY 在小鼠前成骨细胞 MC3T3-E1 中的表达和生物学功能。本部分为下一步 NPY 促进前成骨 MC3T3-E1 细胞成骨分化的机制研究创造基础。

材料与amp;方法

1. 材料

1.1 细胞株

MC3T3-E1 细胞购于协和医学院细胞库。

1.2 主要药品和试剂

DMEM 试剂	美国 Gibco 公司
RFect 小核酸转染试剂盒	常州百代生物科技有限公司
RFect 质粒 DNA 转染试剂盒	常州百代生物科技有限公司
T25 细胞培养板	广州洁特生物有限公司 (Jet Bio)
细胞培养板 (6 孔)	Jet Bio
茜素红 S 染色液	北京索莱宝科技有限公司
ALP 染色液	北京索莱宝科技有限公司
10% Fetal Bovine Serum (FBS)	美国 Gibco 公司
minimum essential medium (MEM)	美国 Gibco 公司
NPY siRNA 序列	武汉擎科创新生物科技有限公司
scrambled siRNA 序列	武汉擎科创新生物科技有限公司
NPY 过表达质粒	武汉擎科创新生物科技有限公司
空白质粒	武汉擎科创新生物科技有限公司
FastStart Universal SYBR Green Master (Rox)	瑞士 Roche 公司
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher
无水乙醇	Sinopharm chemical Co.Ltd.
异丙醇	Sinopharm chemical Co.Ltd.
一抗(NPY) (cat. no. DF6431)	Affinity Biosciences
二抗(horseradish peroxidase-linked guinea pig anti-rabbit IgG polyclonal)(cat. no. SAA544Rb59)	Cloud-Clone Corp
一抗(beta Catenin)(ab22656)	Abcam 公司
二抗(山羊抗小鼠 IgG H&L)(ab150113)	Abcam 公司

三氯甲烷	Sinopharm chemical Co.Ltd.
破膜工作液 (G1204)	Google Bio.
DAPI (G1012)	Google Bio.
抗荧光淬灭封片剂	Google Bio.
RNA 提取液	天根生化科技北京有限公司
胰蛋白酶	中国碧云天生物公司
二甲基亚砜 DMSO	美国 Sigma 公司
甲苯磺酰氟	美国 Sigma 公司
蛋白酶抑制剂	美国 Sigma 公司
HyPure TMMolecular Biology Grade Water	美国 HyClone 公司

1.3 主要仪器设备

高速冷冻型微量离心机	上海 DragonLab 公司
荧光定量 PCR 仪	瑞士 Roche 公司
超微量分光光度计	Thermo Fisher
标准试剂型纯水仪	青岛富勒姆科技有限公司
离心管	美国 Axygen Biosciences 公司
TIP 头	美国 Axygen Biosciences 公司
超声波清洗器	北京医疗设备二厂
电磁搅拌仪	德国 IKA 公司
脱色摇床	谷歌生物
微量注射泵	美国 KDS 公司
精密电子天平	瑞士梅特勒公司
移液器	德国 Eppendorf 公司
旋涡混合器	江苏海门其林贝尔仪器公司
pH 计	瑞士梅特勒公司
电恒温水浴箱	上海精宏实验设备有限公司

超低温冰箱	日本 SANYO 公司
组化笔	Gene tech
荧光分光光度计	日本 Hitachi 公司
正置荧光显微镜	日本尼康
成像系统	日本尼康
普通冰箱	青岛 Haier 公司
二氧化碳培养箱	德国 Heraeus 公司
凝胶化学发光检测系统	Thermo Fisher
多样品研磨珠均质仪	美国 Omni International 公司

1.4 主要试剂的配制

- (1) α -MeM: MeM 和 10%胎牛血清。
- (2) 细胞冻存液: DMSO1.0ml, 胎牛血清 9.0ml, 混匀。现用现配。
- (3) RIPA 细胞裂解液:1mmol/l PMSF, 50mmol/l Tris(pH 7.4), 1%TritonX-100, 150mmol/l NaCl, 0.1% SDS, 1% 去氧胆酸钠, 25mmol/l β -glycerophosphate, 2mmol/l sodium pyrophosphate, 1mmol/l Na_3VO_4 , 1mmol/l EDTA, 0.5 $\mu\text{g/ml}$ leupeptin。
- (4) 无缝克隆试剂盒试剂: LB 液体培养基 LB/Amp(100mg/l) 固体培养皿, 灭菌 0.1mol/l 氯化钙, 灭菌 Ep 管, 灭菌 0.1mmol/l 氯化钙/15%甘油, 去离子水。

2. 实验方法

2.1 MC3T3-E1 细胞成骨诱导培养

2.1.1 细胞复苏

- (1) 迅速将冷冻管从液氮中取出, 移至 37℃水浴融化, 约 1min。
- (2) 用酒精棉球快速消毒冷冻管周围。
- (3) 将细胞移入含有 4ml 新鲜培养基的离心管中, 吹匀。
- (4) 1000rpm $\times$ 5min, 弃去上清液。
- (5) 离心管内加入 5ml 新鲜培养基后吹匀。再将细胞悬液加入培养瓶中, 37℃、5%CO₂ 温箱孵育过夜。

(6) 24h 后, 更换培养基, 细胞汇合度达到 80-90% 传代。

2.1.2 细胞传代

- (1) 弃上清, 用不含 Ca^{2+} 、 Mg^{2+} 的 PBS 清洗细胞 1-2 次。
- (2) 添加 2ml 0.25% Trypsin 于培养皿中, 37℃ 温箱中消化 1min。镜下观察细胞大都脱落并变圆时, 迅速取回, 敲打培养瓶数下后, 5ml 培养基加入终止消化。
- (3) 小心吹打贴壁的 MC3T3-E1 细胞, 待完全脱落后吸出, 800rpm×10min, 弃上清, 补加 2ml 培养液后吹匀。
- (4) 按 7ml/瓶补加培养液, 将细胞悬液按 1:2 的比例移入 6ml 新培养液的瓶中, 放在 37℃, 5%CO₂ 的温箱中孵育。

2.1.3 细胞冻存

- (1) 细胞生长至对数生长期, 形态良好时冻存细胞。冻存前 24h 注意换液。
- (2) 同细胞传代 1-4。
- (3) 弃上清, 离心管中加入冻存液, 吹匀 (MC3T3-E1 细胞悬液的细胞密度约 $2 \times 10^6/\text{ml}$)。
- (4) 将 MC3T3-E1 细胞悬液依次滴进标记的无菌冻存管中, 1.0ml/管。
- (5) 将冻存管顺序放进: 4℃ 冰箱 30min, -20℃ 冰箱 30min, -80℃ 冰箱过夜, 次日移入液氮中存储。

2.2 siRNA 干扰载体的构建及制备

3 组 NPY siRNA1, NPY siRNA2, NPY siRNA3, 对照序列 scrambled NPY siRNA(序列如附表 1)由武汉擎科生物科技有限公司合成。将最终浓度 10nmol/l 的 NPY siRNA 与 10 μl Rfect siRNA 转染试剂室温混合, 制备 NPY siRNA-Rfect 复合物, 转染入生长至 70%-80% 融合的培养孔 MC3T3-E1 细胞中, 用 Trizol 法采集并提取 MC3T3-E1 细胞 NPY RNA, 逆转录成 NPY cDNA, RT-qPCR 检测 NPY mRNA 的表达。观察 NPY siRNA 的干扰效率及对 MC3T3-E1 细胞生物学影响。

2.2.1 NPY siRNA-Rfect 复合物制备步骤

(1) 细胞接种

转染前 1d, 在 6 孔板中接种细胞 (2ml /孔), 每孔加入 2500 μl α -MEM 培养基, 使 MC3T3-E1 细胞转染时密度 80% 左右。

(2) NPY siRNA-Rfect 复合物准备 (按 Rfect siRNA 转染试剂盒说明)

- 1) 25 pmol siRNA 用 250 μl 无血清培养基稀释。

2) 10 μ l Rfect 试剂用 0.25ml 无血清培养基稀释。吹匀, 室温培育 5min。

3) 将 Rfect 稀释液与 NPY siRNA 稀释液混匀(总体积 0.5ml), 室温孵育 20min。

(3) 将 NPY siRNA-Rfect 复合物转染入完全培养基的细胞

1) 将 500 μ l NPY siRNA-Rfect 复合物加入培养孔内, 每孔含 200 μ l 培养的 MC3T3-E1 细胞, 慢慢摇晃培养板, 混匀。

2) 37 $^{\circ}$ C 培养 48h, 检测基因抑制的效果。

(4) 制备 Scrambled siRNA-Rfect 复合物

Scrambled siRNA 作为阴性对照组, 制备及转染方法参照 siRNA 操作步骤进行。

2.3 RT-qPCR 法检测 NPY siRNA-Rfect 复合物对 NPY 48h 的干扰效果

2.3.1 实验分组

Control: Scrambled siRNA 组; NPY siRNA1; NPY siRNA2; NPY siRNA3。

2.3.2 细胞样本总 RNA 抽提

(1) 用剪口枪头, 小心吸弃液体培养基。

(2) 加入 1ml 的 Trizol Reagent, 将 MC3T3-E1 细胞从培养皿底部刷起, 使其悬浮于 Trizol Reagent。

(3) 其余操作步骤同第一部分 2.5.1。

2.3.3 反转录

操作步骤同第一部分 2.5.2。

2.3.4 定量 PCR 检测目的基因表达情况

操作步骤同第一部分 2.5.3。

2.3.5 结果处理

数据处理同第一部分 2.5.4。

2.4 NPY 过表达质粒及空白质粒的构建及制备

2.4.1 实验原理

通过 PCR 或酶切获得 NPY 基因, 通过同源重组/组装或连接构建入 pIRES2-EGFP(H316) 载体, 将重组载体转化 E.coli DH5 α 感受态细胞。用菌落 PCR 对转化子筛选, 阳性克隆送测序。经检验无误的克隆送 NPY 质粒抽提。

2.4.2 目的基因片段的获得

(1) 通过 PCR 进行扩增得到目的基因

从 GenBank 中查询 NPY 基因序列及其上下游序列，用 VectorNTI 软件进行引物设计。

(2) PCR 扩增目的基因

使用高保真的 PrimeSTAR 酶扩增 NPY 基因，反应体系和条件如下：

(3) PCR 体系中各反应液组成（表 2-3）

表 2-3 PCR 体系中各反应液组成

名称	体积
5× PrimeSTAR®Buffer (Mg ²⁺ plus)	5μl
dNTP Mixture(各 2.5mmol/l)	2μl
上游引物 (10μmol/l)	0.5μl
下游引物 (10μmol/l)	0.5μl
质粒 DNA(200ng/μl)	1μl
PrimeSTAR®HS DNA Polymerase(2.5u/μl)	0.25μl
ddH ₂ O	Up to 25μl

(4) PCR 扩增（表 2-4）

表 2-4 PCR 反应条件

Cycle	Cycle Point	
循环（44 次）	Step 1 @ 98°C	10s
	Step 2 @55°C	5 sec 或 15 sec
	Step 3 @72°C	1min/kb
熔解曲线	Step 1 @ 98°C	10 sec
	Step 2 @68°C	1min/kb

(5) 通过酶切得到目的基因片段

PCR 产物行琼脂糖凝胶电泳检验扩增效果，用限制性内切酶 *EcoR* IV 及 *Hind* III 对 PCR 产物酶切，并用 TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.3.0 对 NPY 基因片段进行胶回收。

2.4.3 线性化表达载体的制备

用限制性内切酶 *EcoR* IV 及 *Hind* III 对表达载体 pIRES2-EGFP(H316)进行酶切，酶切反应体系（表 2-5），37°C 水浴中培育 2h。用 TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.3.0 对目的载体 pIRES2-EGFP(H316)进行胶回收。

表 2-5 载体酶切反应体系

名称	体积
10×反应 Buffer	1μl
pIRES2-EGFP(H316)载体(200ng/μl)	2μl
限制性内切酶	2μl
ddH ₂ O	Up to 10μl

2.4.4 目的基因构建入线性化表达载体

采用无缝克隆试剂盒将 pIRES2-EGFP(H316)载体和 NPY 基因片段 (表 2-6)以摩尔比 1:2 加入离心管中重组反应。吹匀后在 37℃孵育 0.5h, 随后放置在冰上 5min, 直接转化或-20℃存储, 待转化时解冻转化。

2-6 酶连接体系

名称	体积
5×反应 Buffer	4μl
pIRES2-EGFP(H316) 载体	1.5μl
NPY 片段	1.5μl
无缝连接酶	2μl
ddH ₂ O	Up to 20μl

2.4.5 感受态细胞 Ecoli DH5α 的制备

- (1) 取 Ecoli DH5α 菌划线, 过夜培养。
- (2) 在 LB 液体培养基 5ml 中用无菌枪头轻挑菌落, 振荡孵育 (200rpm, 37℃) 12h, 直至 Ecoli DH5α 菌对数生长期。
- (3) 在 LB 液体培养基中接种 Ecoli DH5α 菌 (1ml/100ml), 振荡孵育 (200rpm, 37℃) 2h, 至 OD₆₀₀=0.4。
- (4) 分装入 2 个 50ml 离心管中, 4℃, 3kg, 200rpm×10min, 弃上清。
- (5) 10ml 的 0.1mmol/l CaCl₂ 滴入离心管中, 重悬细胞沉淀, 冰上摇晃 10min。
- (6) 4℃, 3000g, 200rpm×10min, 弃上清。
- (7) 10ml 预冷的 0.1mmol/l CaCl₂ 滴入离心管中, 重悬细胞沉淀, 冰上摇晃 30min。
- (8) 4℃, 3000g, 200rpm×10min, 弃上清。
- (9) 2ml 预冷的 0.1mmol/l CaCl₂ 加 15%甘油混合溶液滴入离心管中, 摇匀, 重悬

菌体。

(10) 分装，在灭菌的 Ep 管中装 100μl，-80℃保存。

2.4.6 感受态细胞 Ecoli DH5α 的转化

- (1) 在 42℃的水浴内，涂布棒浸入 75%的乙醇。
- (2) 从-20℃冰箱中取出 NPY 质粒 DNA 和从 4℃冰箱中取出 Ecoli DH5α 细菌的 EP 管各一只，冰盒内放置。
- (3) 在净化工作台内，将 NPY 质粒 1μl 放入 100μl Ecoli DH5α 细菌中，冰上 30 min。
- (4) 置于 42℃热水浴中 90s，不要摇动管，立即放在冰盒中，冰上 2min。
- (5) 加入 1000μl LB 液体培养基中，37℃，200rpm×30min，行细菌活化。
- (6) 取转化的菌液涂平板，涂于 LB 琼脂固体培养基上。
- (7) 倒置铺菌皿，37℃培养 12h，长出单菌落。
- (8) 小量质粒提取，EcoR IV及 Hind III进行酶切。
- (9) 将构建的重组真核质粒命名为 NPY 过表达质粒（CMV-NPY-IRES2-EGFP）(图 4)。

2.4.7 菌落 PCR 鉴定阳性转化子

将平板上生长良好的转化子挑取重植在 10μl LB 溶液中，取 0.5μl 为模板行菌落 PCR 鉴定。PCR 反应体系及循环条件（表 2-7、2-8）

表 2-7 PCR 体系中各反应液组成

名称	体积
Premix Taq (TaKaRa Taq™ Version 2.0 plus dye)	12.5μl
模板	0.5μl
上游引物 (20μmol/l)	0.5μl
下游引物 (20μmol/l)	0.5μl
ddH ₂ O	Up to 25μl

表 2-8 PCR 反应条件

Cycle	Cycle Point	
Cycling (30 次)	Step 1 @ 94℃	30 sec
	Step 2 @55℃ or 60℃	30 sec
	Step 3 @72℃	1min/kb
Cycling (30 次)	Step 1 @ 98℃	10 sec

2.4.8 阳性克隆送测序

菌落鉴定得到的阳性克隆，送基因测序公司进行测序验证。合成的 NPY 序列编号（K99669-G0143325-1_N-5），测序结果如图 5 所示。

2.4.9 NPY 过表达质粒小提

测序通过的阳性克隆，进行 CMV-NPY-IRES2-EGFP 质粒提取。具体步骤见试剂盒说明书。

2.4.10 将构建的 NPY 重组质粒转染入 MC3T3-E1 细胞（按照 Rfect 质粒 DNA 转染试剂盒说明）

按照 Rfect 质粒 DNA 转染试剂盒说明，将制备的 NPY 过表达质粒（CMV-NPY-IRES2-EGFP）与 Rfect 试剂构建复合物，将复合物悬液直接转染入融合度 70%-80% 培养孔细胞中，轻轻晃动培养板，使培养液混合均匀。

2.4.11 制备空白质粒（pIRES2-EGFP）作为阴性对照组

具体制备方法同上文所述，将空白质粒（pIRES2-EGFP）（图 6）与 Rfect 试剂构建复合物，转染方法参照 Rfect 质粒 DNA 转染试剂盒操作说明书进行。

3. 验证 NPY siRNA 和 NPY 过表达质粒对细胞 NPY 基因表达的影响

3.1 实验分组

Control 组：MC3T3-E1+PBS 组；scrambled siRNA 组：MC3T3-E1+scrambled siRNA-Rfect 组；NPY siRNA 组：MC3T3-E1+NPY siRNA-Rfect 组；NPY plasmid 组：MC3T3-E1+CMV-NPY-IRES2-EGFP-Rfect 组；empty vector 组：MC3T3-E1+pIRES2-EGFP-Rfect 组。

3.2 定量 PCR 法检测细胞第 4、7 天的 NPY 基因表达

方法同 2.4.2-2.4.5，检测细胞第 4 天及第 7 天的 NPY mRNA 表达。在此实验基础上，系统阐述了 NPY mRNA 在不同对照组的差异性表达的情况，实验结果及统计分析后发现 Control 组与 scrambled siRNA 组和 empty vector 组的 NPY mRNA 表达是基本一致的，所以我们后续实验选择了 Control 组作为对照来研究过表达及干扰 NPY 后的细胞蛋白表达及其生物学效应。

3.3 半定量蛋白检测（Western blot）法检测细胞第 4、7 天的 NPY 蛋白表达

3.3.1 细胞总蛋白的提取

（1）弃培养基，预冷的 PBS 洗涤 2 遍，将细胞用细胞刮板刮下，1000rpm×5min。

PBS 冲洗 3 遍后收集细胞沉淀。

(2) 轻轻吸去上清液, 加入 RIPA 细胞裂解液 150 μ l, 在裂解液用前加入 1mmol/L 苯甲基磺酰氟 (PMSF)。

(3) 4 $^{\circ}$ C, 15000rpm $\times$ 15min。留取离心后的上清到崭新的 EP 管中, -80 $^{\circ}$ C 存储。

3.3.2 BCA 法测定蛋白浓度

(1) 按说明将蛋白标准稀释至 0.5mg/ml, 按照 0, 1, 2, 4, 8, 12, 16, 20 μ l 加入 96 孔板中, PBS 补足至 20 μ l。

(2) 取 1 μ l 待测蛋白加入 96 孔板中, PBS 补足至 20 μ l, 每个样品做两个复孔。

(3) 按照 BCA 试剂盒中的 B 液与 A 液按照 1:50 的体积比混匀制备。每孔加入 BCA 工作液 0.2ml, 37 $^{\circ}$ C 培育 30min。

(4) 酶标仪测定 570nm 时的吸光度。

(5) 制作标准曲线并计算各组蛋白浓度。

3.3.3 SDS-PAGE 凝胶电泳

(1) 参照操作说明, 根据 NPY 蛋白的分子量, 配制相应浓度的分离胶。分离胶凝固后, 配制 10%的浓缩胶。

(2) 将凝胶置于电泳槽中, 加入 1 $\times$ 电泳液 1L, 轻拍直到玻璃板下的气体排出。

(3) 根据 NPY 蛋白浓度估算各标本的上样量, 振荡混匀后加入蛋白样本。

(4) 80V 电泳 $\times$ 30min 后, 将电压调至 120V, 电泳至溴酚蓝达玻璃板下缘结束。

3.3.4 转膜及显色

(1) 将 PVDF 膜裁剪合适后置于 4 $^{\circ}$ C 预冷的甲醇中浸泡。

(2) 按照黑色板-海绵垫-滤纸-凝胶-PVDF 膜-滤纸-海绵垫-白色板顺序在转膜夹中放置, 避免气泡发生。

(3) 将转膜夹置于转膜槽, 倒入转膜缓冲液, 槽外以冰水环绕降温。

(4) 接通电源, 以 0.2A 电流, 转膜 1h。

(5) 封闭: 关闭电源, 取出 PVDF 膜, 放入 5%BSA 中室温封闭 1h。

(6) 孵育一抗 (anti-NPY): 弃封闭液, 将 PVDF 膜置入稀释好的一抗 (NPY, 1:1000), 4 $^{\circ}$ C 孵育过夜。

(7) 洗涤: 0.05% TBST 洗膜, 5min $\times$ 3 次。

(8) 孵育二抗: 加入二抗 (horseradish peroxidase-linked guinea pig anti-rabbit IgG polyclonal) (1:2000), 室温下在摇床上震荡孵育 1h。

(9) 洗涤: TBST 洗膜, 5min×3 次。

(10) 显色: 将 ECL 的 A、B 液按 1:1 体积混匀。在蛋白面向上的 PVDF 膜上滴加发光液。

(11) 扫描成像: 将滴加过发光液的 PVDF 膜放入凝胶化学发光检测系统中, 进行曝光并保存图像。

(12) 图像分析: 使用 image J (Version 1.8.0; national institutes of Health) 软件, 检测不同图像条带的光密度值。

3.4 MC3T3-E1 成骨诱导及染色

取对数生长期细胞, 胰酶消化将细胞密度调整为 $1 \times 10^6/\text{ml}$, 置于 6 孔板内 (2ml/孔), 将板置于温箱中孵育。待细胞融合度达到 80 %~90 % 时, 加入成骨诱导培养基继续培养, 并按照 NPY siRNA、NPY plasmid 分组依次加入对应的干扰或过表达质粒, 设置 Control 组, 每 2 到 3d 换一次液。14d 后, 行茜素红、ALP、 β -catenin 免疫荧光染色。

3.4.1 茜素红染色

(1) 诱导 14d 后, 小心拿取培养板, 弃上清, PBS 清洗 2 次。

(2) 滴入 4% 多聚甲醛固定 10min, PBS 洗涤 2-3 次。

(3) 滴入配置好的茜素红染液, 在湿盒中放置, 避光孵育 15min, PBS 清洗 2 到 3 次。

(4) 显微镜下检测, 拍照。

3.4.2 ALP 染色

(1) 诱导 14d 后, 小心拿取培养板, 弃上清, PBS 洗涤 2 次。

(2) 加入 4% 多聚甲醛固定 10min, PBS 洗涤 2-3 次。

(3) 滴入配制好的 ALP 孵育液, 避光, 在湿盒中放置培育 15min, PBS 清洗 2 到 3 次。

(4) 显微镜下检测, 拍照。

3.4.3 β -catenin 免疫荧光染色

(1) 用 PBS 将铺满细胞的载玻片在培养板中洗涤, 3min/次×3 次。

(2) 用 4% 的多聚甲醛将爬片固定 15min, PBS 洗涤, 3min/次×3 次。

(3) 5% Triton X-100 室温通透 MC3T3-E1 细胞 20min。

(4) PBS 浸洗玻片, 3min/次×3 次。将 PBS 液吸干后滴入山羊血清, 室温封闭 30

min。

(5) 吸干封闭液，每张玻片滴加配好的一抗 β -catenin 并置于湿盒内，4℃孵育过夜。

(6) 加荧光二抗，PBST 浸洗爬片，3min/次×3 次。吸干爬片后加入稀释的荧光二抗，30℃培育 60min，PBST 洗涤，3min/次×3 次。

(7) 滴入 DAPI 浸染细胞核，避光孵育 5min，PBST 浸洗，5min/次×4 次。

(8) 将爬片用吸水纸吸干，用抗荧光淬灭剂的封片液封片，在荧光显微镜下收集图像。

4. 统计学分析

所有实验重复 3 次，数据结果以均数±标准差(means±SD)表示。使用 SPSS 18.0 软件中 One-way ANOVA 方法行各组间总体差异检验，Scheffe multiple comparisons test (图 1 和图 5) 或 Uncorrected Fisher's LSD (图 6) 方法进行各组间两两比较， $P<0.05$ 为差异有统计学意义。使用 GraphPad Prism v.6.0 软件行柱状图绘图。

实验结果

1. 成功筛选出干扰效率最佳的 NPY siRNA

为了检测 NPY siRNA 对 MC3T3-E1 细胞中 NPY 基因的影响，我们将 NPY siRNA 转染入细胞 48h 后，通过 RT-qPCR 方法检测 NPY 的 mRNA 表达，进而评估 NPY siRNA 干扰效果，筛选出干扰效率最高的 siRNA。应用单因素方差分析方法，整体有差异性。Scheffe multiple comparisons test 和 Uncorrected Fisher's LSD 方法进行各组间两两比较，siRNA1, siRNA2 与 siRNA3 组分别与对照组相比，具有统计学意义(** $P<0.01$ or *** $P<0.001$)。NPY siRNA3 组具有最高的干扰效率，达到了 75%。所以，我们选择 NPY siRNA3 完成后续生物学实验（图 1）。

2. 成功构建 NPY 过表达质粒

2.1 克隆详细资料

克隆流水号:	H13261	基因名称:	NPY	
GenBank ID:	NM_023456	基因大小:	294	
物种:	Mouse	上下游克隆酶切位点	NheI	BamHI
原核抗性:	Kan			
空载体信息:	H316 pIRES2-EGFP			
载体信息	pIRES2-EGFP (表达框 CMV-Npy-IRES2-EGFP)			
正向测序引物	CMV-F-1 GCACCAAAATCAACGGGAC			
反向测序引物	IRES-R CCTCACATTGCCAAAAGACG			
其它说明				

2.2 NPY 过表达质粒载体图谱（图 2）

2.3 NPY 基因合成序列编号（K99669-G0143325-1_N-5），测序比对情况（图 3）

2.4 空白质粒载体图谱（图 4）

3. 过表达及干扰 NPY 后检测细胞 NPY 基因表达的影响

为了更好了解构建的 NPY 过表达质粒及 NPY siRNA 对 MC3T3-E1 细胞 NPY mRNA 水平的影响，我们 RT-PCR 方法检测了转染后第 4、7 天 NPY mRNA 细胞表达情况。通过观察发现，NPY mRNA 可以在测定时间点恒定表达，并可被 NPY 过表达质粒及 NPY siRNA 明显促进或抑制，这为我们研究 NPY 基因的后续生物学效应提供了研究基础。

结果显示：NPY 过表达质粒组与空白质粒组对比，在第 4 天增加了 3.9 倍，第 7 天增加了 4.3 倍，差异有显著统计学意义 ($P<0.001$ ，图 5)。NPY siRNA 组与对

照组及 scrambled siRNA 组相比, NPY mRNA 表达在第 4 天下降了 75% ($P<0.05$), 第 7 天下降了 55% (图 5)。

从而从基因 mRNA 水平进一步验证了 NPY 过表达质粒及 NPY siRNA 可对 MC3T3-E1 细胞的 NPY 产生影响, 为我们后续研究 NPY 的生物学效应及机制打下了一定的基础。

4. 过表达及干扰 NPY 后检测细胞 NPY 蛋白表达的影响

为了更好了解构建的 NPY 过表达质粒及 NPY siRNA 对 MC3T3-E1 细胞 NPY 蛋白水平的影响, 我们检测了转染后第 4、7 天 MC3T3-E1 细胞 NPY 蛋白表达情况。通过观察发现, NPY 不光在 mRNA 水平恒定表达, 在蛋白水平也恒定表达。NPY 过表达质粒及 NPY siRNA 明显促进或抑制 MC3T3-E1 细胞 NPY 表达, 为我们研究 NPY 基因的后续生物学效应提供了一定基础。

结果显示: NPY 过表达组与对照组相比, 转染后第 4 天和第 7 天, NPY 蛋白分别增加了 1.2 倍和 1.5 倍($P<0.05$; 图 6 C 和 D)。NPY siRNA 组与对照组相比, 转染后第 4 天和第 7 天, NPY 蛋白分别降低了 45%和 35% ($P<0.05$; 图 6 C 和 D)。

总之, NPY 基因可被 MC3T3-E1 细胞不同时间点恒定表达, 并可被构建的 NPY 过表达质粒和 NPY siRNA 促进或抑制, 为后续研究 NPY 对 MC3T3-E1 细胞的生物学机制创造了必要条件。

5. 过表达及干扰 NPY 后检测细胞成骨分化的影响

5.1 茜素红 S (Alizarin Red S) 染色

研究表明, 茜素红 S 是蒽醌类衍生物, 可与钙形成橙红色螯合物, 可以反馈细胞成骨钙沉积的多少^[10]。为了了解过表达及干扰 NPY 对 MC3T3-E1 细胞钙质沉积的效应, 我们选用茜素红 S 染色的方法进行了观测。结果显示: NPY 过表达组 MC3T3-E1 细胞数目增多, 体积较对照组增大, 核染色较深, 胞内粒子增多, 可见较多的紫红色钙结节 (图 7.B)。NPY 干扰组 MC3T3-E1 细胞数目较少, 核染色较浅, 可见较少的紫红色钙结节 (图 7.C)。

5.2 碱性磷酸酶 (Alkaline phosphatase, ALP) 染色

哺乳动物机体内所蕴含的碱性磷酸酶 (ALP) 是由多种基因家族编码的含有金属锌的酶, 其含有的骨同工酶可能参与哺乳动物的骨钙化及骨沉积^[11]。通过检测 ALP 钙沉积和细胞数量可以用来定性或定量间充质干细胞的成骨分化^[12]。为了检测过表达及干扰 NPY 后 MC3T3-E1 细胞成骨分化的变化, 我们也采用 ALP 染

色的方法进行了观察。镜下观察发现, NPY 过表达组 MC3T3-E1 细胞数目增多, 体积较对照组增大, 核染色较深, 胞内黄色钙盐沉积较多, 可见较多蓝色颗粒或块状沉淀 (图 8.B)。NPY 干扰组 MC3T3-E1 细胞数目较少, 核染色较浅, 蓝色颗粒较少 (图 8.C)。

5.3 β -catenin 免疫荧光染色

在早期的骨折修复阶段, β -catenin 基因在骨髓间充质干细胞和前成骨细胞转化为成骨、软骨细胞中起非常重要的作用^[13, 14]。并且, β -catenin 蛋白被用来作为骨折修复、骨重建的重要标志物^[13]。为了观测过表达及干扰 NPY 后 MC3T3-E1 细胞成骨分化的变化, 我们选用了 β -catenin 蛋白进行了观察。镜下观察 NPY 过表达组 MC3T3-E1 细胞核数目增多 (图 9.D), MC3T3-E1 细胞质内绿色荧光较多 (图 9.E), 反应出 NPY 过表达组 β -catenin 蛋白高表达。镜下观察 NPY 干扰组 MC3T3-E1 细胞核数目较少 (图 9.G), MC3T3-E1 细胞质内绿色荧光较少 (图 9.H), 提示 NPY 干扰组 β -catenin 蛋白低表达。

综上所述, 过表达或干扰 NPY 能明显影响 MC3T3-E1 细胞成骨分化, 有助于我们进一步研究 MC3T3-E1 细胞成骨分化相关机制。

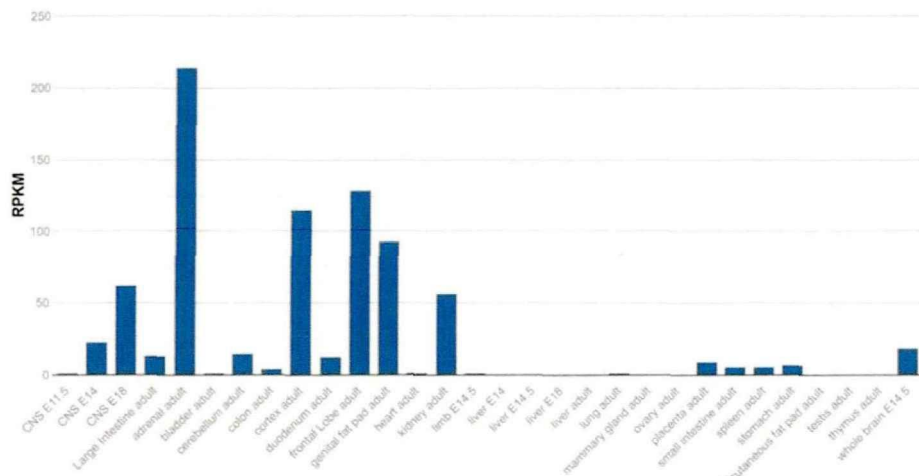
讨 论

近年来随着再生医学的发展和干细胞的研究进展,具备分化潜力的前成骨细胞移植对于修复骨缺损、促进骨折愈合具有非常重要的临床意义,逐渐成为实验研究的热点。

研究表明,MC3T3-E1 细胞具有定向分化为成骨细胞、骨细胞转化并钙化成骨的能力,对于研究细胞成骨机制具有重要的意义。早在 1983 年, H Sudo 博士^[15]就深入研究了前成骨细胞 MC3T3-E1,该细胞取材于新生 C57/BL/6 小鼠头骨,其高浓度碱性磷酸酶刺激下可发生融合,并定向分化为成骨细胞,最终矿化成骨组织。MC3T3-E1 细胞呈多层状生长,呈现成纤维细胞形态。在第 21 天,嗜碱性结节区发现成团细胞,呈现典型的成骨细胞形态。随着孵育时间的延长,结节的数量和大小显著增加,到 40~50 天时,肉眼较易识别。高度钙化的骨基质分布在发育良好的结节内。骨细胞嵌于骨基质内,成纤维细胞及成骨细胞在基质周边分布。微量元素无机盐物质最后结成羟基磷灰石晶体,沉积于骨表面。成骨细胞、幼年成骨细胞和富溶酶体细胞的细胞质膜对 ALP 和钙离子的细胞化学染色呈现强阳性反应。这些文献结果表明,MC3T3-E1 细胞具有定向分化并钙化成骨的能力。本部分实验中同样发现 MC3T3-E1 细胞呈纤维细胞样贴壁生长;ALP 染色见胞浆蓝色强阳性表达;茜素红 S 染色,可见橘红色钙结节沉积。染色结果提示 MC3T3-E1 细胞具有成骨细胞分化能力。

K. Tatemoto 教授于 1982 年率先从猪脑中提取了 NPY^[16]。NPY 由 36 个氨基酸构成,其分子结构与消化系统的 YY 肽(peptide YY, PYY)和胰多肽(pancreatic polypeptide, PP)为同一家族,均具有两个反向螺旋结构区(1 个多脯氨酸螺旋结构的 N 端和 1 个螺旋结构的 C 端),形成具有发卡样的三维立体结构^[17]。NPY 在心血管、神经、免疫和内分泌系统参与调节机体的多种生理和病理生理反应,其中包括神经元兴奋^[18]、抑郁^[19]、焦虑^[20]、神经传递^[21]、神经源性内分泌^[22, 23]、血管收缩^[24-26]、细胞生长^[27]或摄食和能量平衡^[3, 17, 28-31]。同时, NPY 在干细胞疾病治疗中的作用备受关注^[32, 33]。

NPY 广泛存在于人体各类组织中,人类个体 27 种不同组织样本 NPY 基因测序表达量如下图所示^[34-38]。下图反应的是人类个体 27 种不同组织样本 NPY 基因测序表达量, NPY 在肾上腺最高,大脑额叶和皮质层中次之。Eitner 等研究发现,正常大鼠和绵羊膝关节髌旁滑膜内可见大量含有感觉纤维和酪氨酸羟化酶阳性的



交感神经纤维。交感神经纤维中富含 NPY 和血管活性肠肽（vasoactive intestinal peptide，VIP），对膝关节成骨细胞的增生、分化等具有重要的促进作用，是维持骨量稳定的重要神经营养因子^[39]。研究表明，外源性 NPY 注射可促进造血干细胞动员，并显著改善卵巢切除骨质疏松模型小鼠骨量丢失，促进小鼠骨髓成骨细胞明显增加^[7, 40]。Ana F Nunes 等研究发现，在甲状腺素运载蛋白(transthyretin, TTR)敲除小鼠模型^[6]中，其外周成骨细胞、骨细胞和软骨细胞均可合成 NPY，随着 NPY 水平增高，小鼠骨密度及骨小梁体积明显增加。然而，在中枢系统下丘脑 NPY 敲除鼠模型^[41, 42]中，中枢性 NPY 表达降低时，小鼠外周骨量反而增加，提示小鼠骨量的调节存在外周相对独立的调节系统。

通过我们的研究表明，NPY 基因可促进 MC3T3-E1 细胞成骨分化，对促进骨折愈合中起非常重要的作用。由于哺乳动物鼠类基因与人类基因高度同源，研究 NPY 在小鼠 MC3T3-E1 细胞的生物学机制，对揭示 NPY 在人体内骨折成骨机制具有重要研究价值。此外，本部分研究中由于未定量检测 MC3T3-E1 经干扰或转染后的细胞增殖数据和成骨表现指标，因而对 NPY 的成骨能力的评估稍有不足，有待于今后进一步的研究。

结 论

1. 干扰 NPY 可抑制 MC3T3-E1 细胞成骨分化。
2. 过表达 NPY 可促进 MC3T3-E1 细胞成骨分化。

附 图

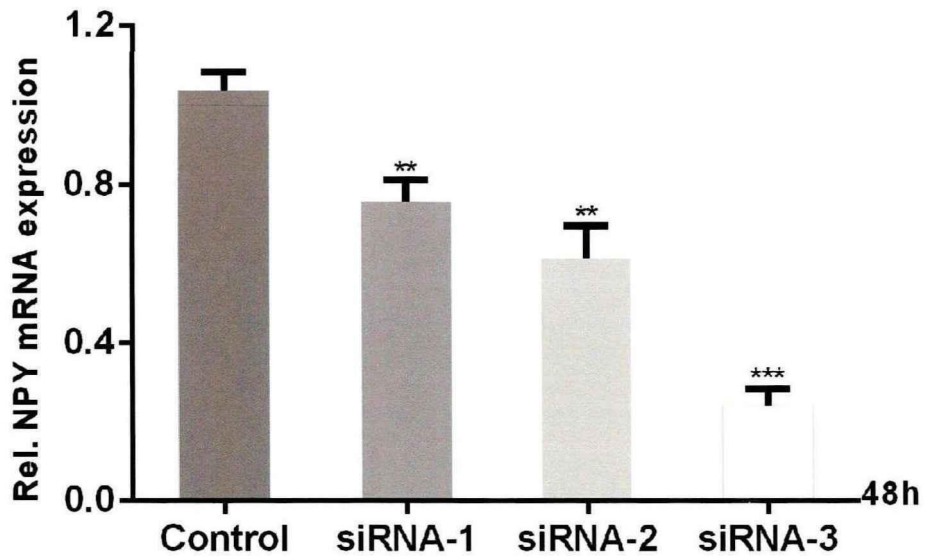


图 1 NPY siRNA 干扰效果

NPY siRNA 转染 MC3T3-E1 细胞 48 小时后,Q-PCR 反应检测目的基因 NPY 的表达，评价 NPY siRNA 对目的基因 NPY 的干扰效果。 NPY siRNA3 具有最高的干扰效率， >75%。

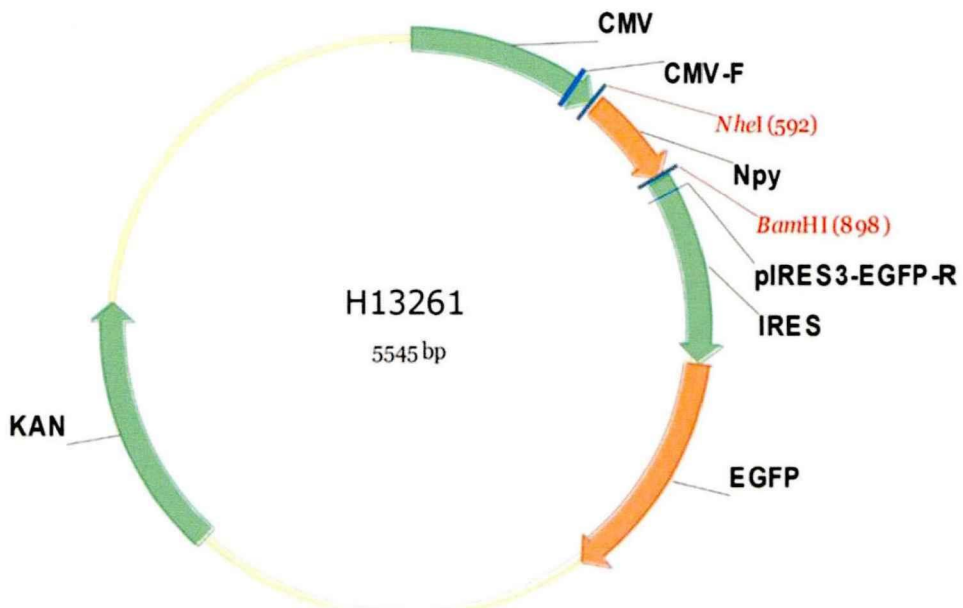


图 2 过表达质粒载体图谱（CMV-NPY-IRES2-EGFP）

	1	50
(1)	GATCCGCTAGCGCCACC	ATGCTAGGTAACAAGCGAATGGGGCTGTGTGGA
(1)	-----	ATGCTAGGTAACAAGCGAATGGGGCTGTGTGGA
	51	100
(51)	CTGACCCTCGCTCTATCTCTGCTCGTGTGTTGGGCATTCTGGCTGAGGG	
(34)	CTGACCCTCGCTCTATCTCTGCTCGTGTGTTGGGCATTCTGGCTGAGGG	
	101	150
(101)	GTACCCCTCCAAGCCGGACAATCCGGGCGAGGACGCGCCAGCAGAGGACA	
(84)	GTACCCCTCCAAGCCGGACAATCCGGGCGAGGACGCGCCAGCAGAGGACA	
	151	200
(151)	TGGCCAGATACTACTCCGCTCTGCGACACTACATCAATCTCATCACCAGA	
(134)	TGGCCAGATACTACTCCGCTCTGCGACACTACATCAATCTCATCACCAGA	
	201	250
(201)	CAGAGATATGGCAAGAGATCCAGCCCTGAGACACTGATTTTCAGACCTCTT	
(184)	CAGAGATATGGCAAGAGATCCAGCCCTGAGACACTGATTTTCAGACCTCTT	
	251	300
(251)	AATGAAGGAAAGCACAGAAAACGCCCCCAGAACAAGGCTTGAAGACCCCTT	
(234)	AATGAAGGAAAGCACAGAAAACGCCCCCAGAACAAGGCTTGAAGACCCCTT	
	301	350
(301)	CCATGTGGTGA	GGATCCGCCCTCTCCCTCCCCCCCCCTAACGTTACTG
(284)	CCATGTGGTGA	-----
	351	400
(351)	GCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCTATATGTTATTT	
(295)	-----	
		401
	SEQ (401)	TCCAC
	Npy (295)	-----

图 3 NPY 基因测序比对图谱

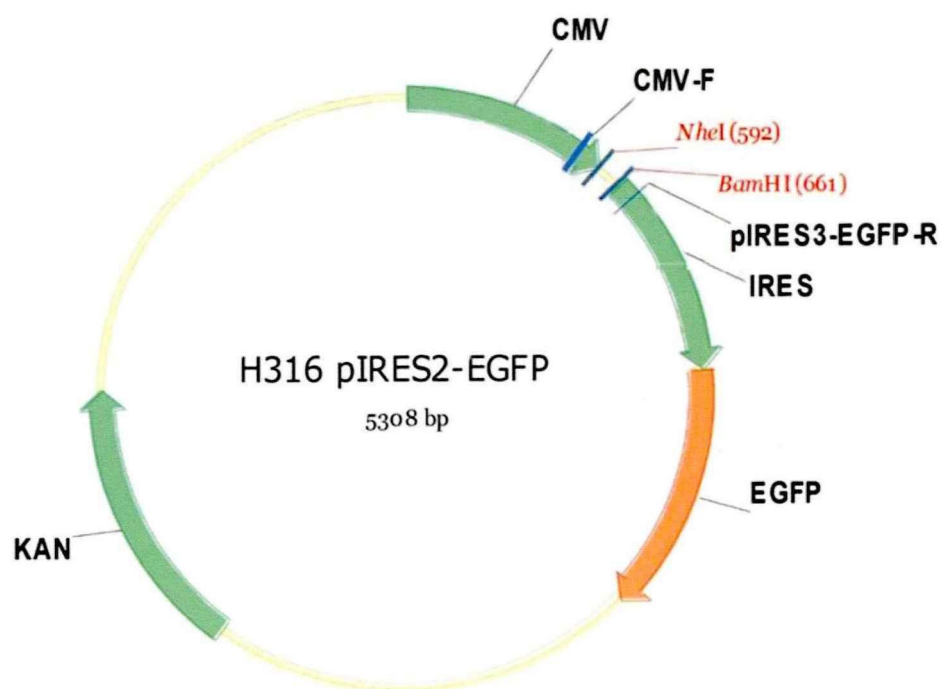


图 4 空白质粒载体图谱 (pIRES2-EGFP)

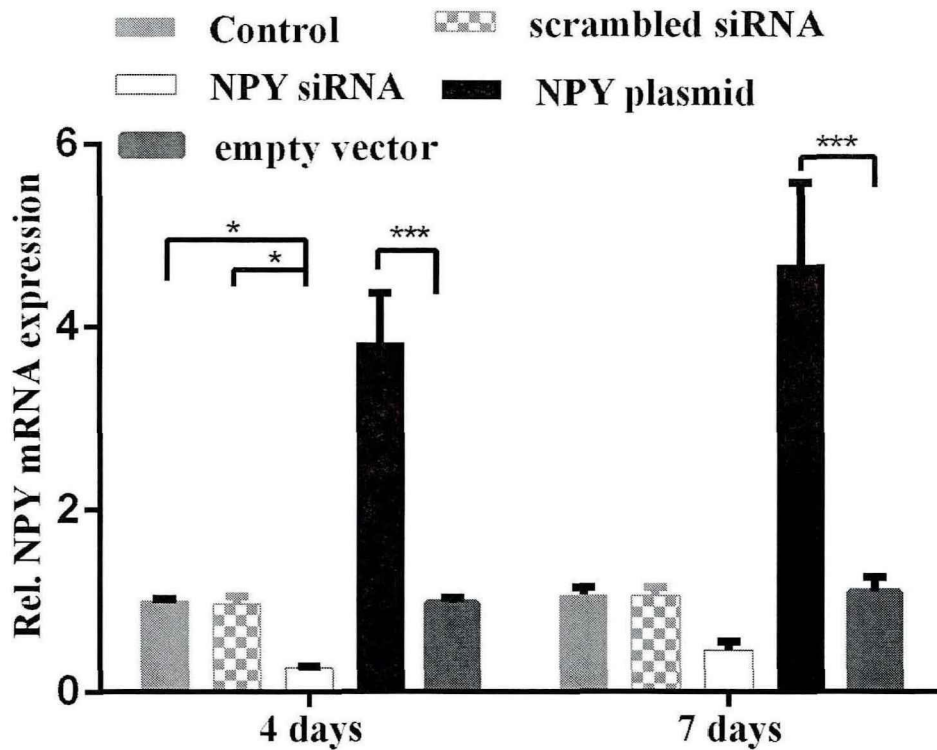


图 5 MC3T3-E1细胞成骨诱导第4、7天NPY基因表达情况

NPY 过表达质粒组与空白质粒组对比，在第 4 天增长了 3.9 倍，第 7 天增长了 4.3 倍，差异有显著统计学意义 ($P<0.001$)。NPY siRNA 组与对照组及 scrambled siRNA 组相比，NPY mRNA 表达在第 4 天下降了 75% ($P<0.05$)，第 7 天下降了 55%。(* $P<0.05$ ，*** $P<0.001$)。

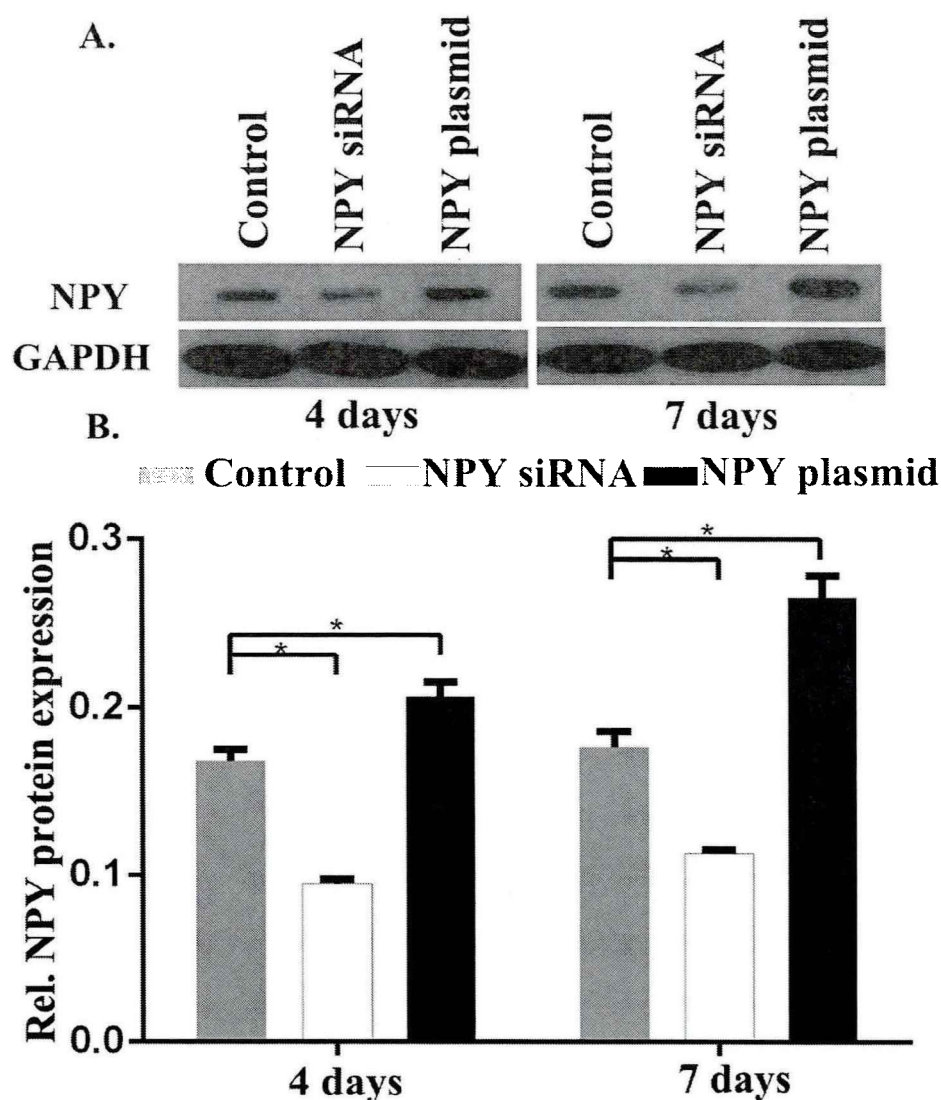


图 6 MC3T3-E1 细胞成骨诱导第 4、7 天 NPY 蛋白表达情况

NPY 过表达组与对照组相比，转染后第 4 天和第 7 天，NPY 蛋白分别增加了 1.2 倍和 1.5 倍($P < 0.05$; 图 C 和 D)。NPY siRNA 组与对照组相比，转染后第 4 天和第 7 天，NPY 蛋白分别降低了 45% 和 35% ($P < 0.05$)。(* $P < 0.05$, *** $P < 0.001$)。

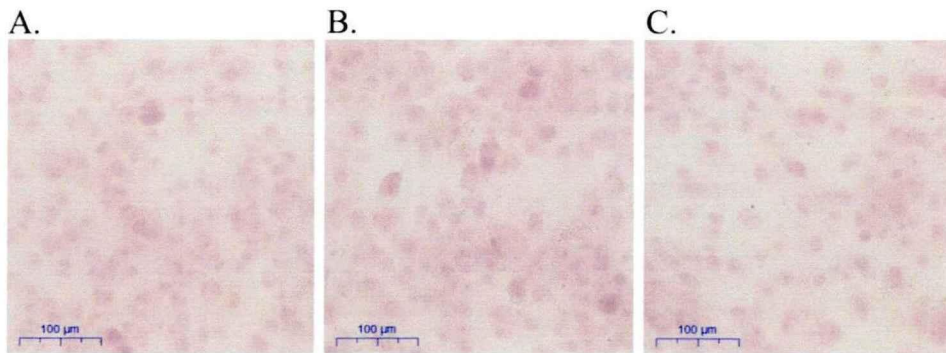


图 7 MC3T3-E1 细胞成骨诱导 14 天不同干预组茜素红染色 (标尺 100μm)

A.Control 组; B.NPY 过表达组; C.NPY siRNA 组。MC3T3-E1 细胞成骨诱导培养 14 天后, 镜下观察过表达组细胞数目增多, 体积较对照组增大, 核染色较深, 胞内粒子增多, 可见较多较大紫红色钙结节。镜下观察干扰组细胞数目较少, 核染色较浅, 紫红色钙结节较少。

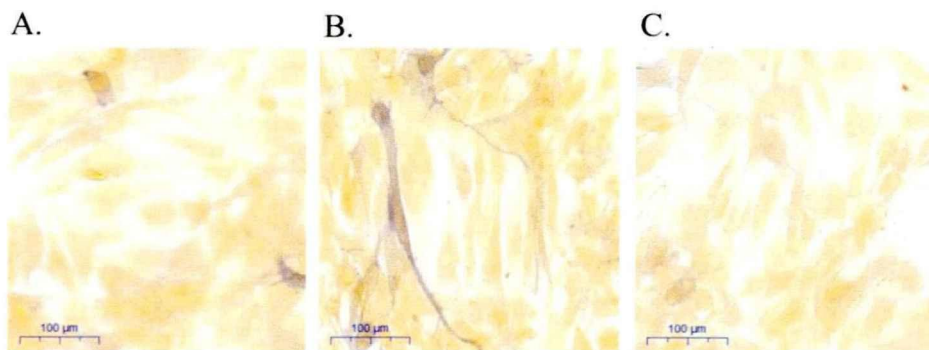


图 8 MC3T3-E1 细胞成骨诱导 14 天 ALP 染色 (标尺 100μm)

A.Control 组; B.NPY 过表达组; C.NPY siRNA 组。镜下观察过表达组细胞数目增多, 体积较对照组增大, 核染色较深, 胞内黄色钙盐沉积较多, 可见较多蓝色颗粒或块状沉淀。镜下观察干扰组较对照组细胞数目较少, 核染色较浅, 蓝色颗粒较少。

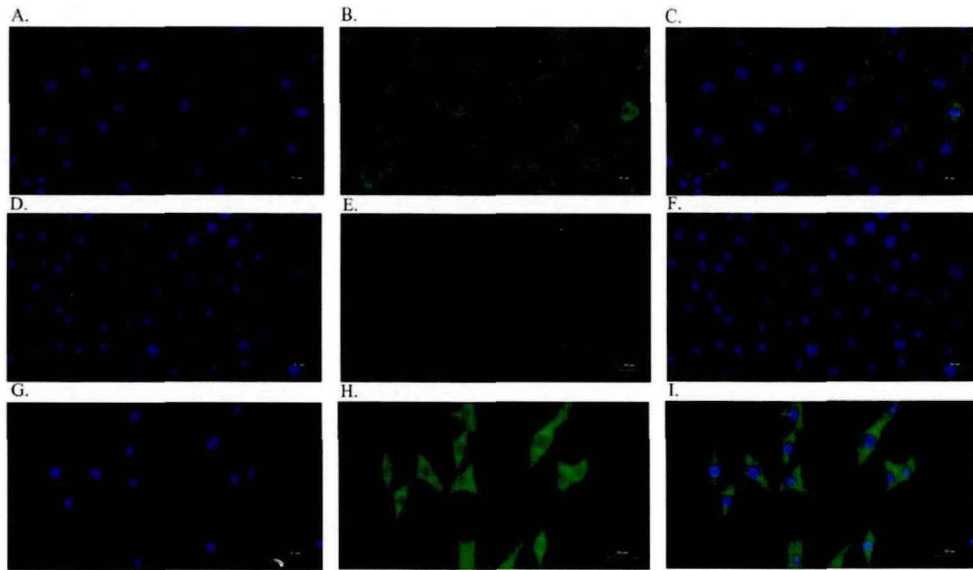


图 9 MC3T3-E1 细胞成骨诱导 14 天 β -catenin 染色 (标尺 40 μ m)

A. Control Dapi; B. Control β -catenin; C. Control Merge; D. NPY 过表达 Dapi; E. NPY 过表达 β -catenin; F. NPY 过表达 Merge; G. NPY siRNA Dapi; H. NPY siRNA β -catenin; I. NPY siRNA Merge。镜下观察 NPY 过表达组细胞核数目增多 (图 9. D), 细胞质内绿色荧光较多 (图 9.E), 反应出 NPY 过表达组 β -catenin 蛋白高表达。镜下观察 NPY siRNA 组较对照组细胞核数目较少 (图 9.G), 细胞质内绿色荧光较少 (图 9.H), 提示 NPY siRNA 组 β -catenin 蛋白低表达。

附表

表1 逆转录定量PCR的引物序列

Name	Sequence
NPY	5'-CTCGTGTGTTTGGGCATTC-3'
NM_023456	3'-TAGTGTCGCAGAGCGGAGTA-5'
siRNA1	5'-CCAAACAAUAGACAUGCUUdTdT-3'
NM_000909.6	3'-AAGCAUGUCUAUUGUUUGGdTdT-5'
siRNA2	5'-GCCC UUUGUGAUCUAUCAAdTdT-3'
NM_000909.6	3'-UUGAUAGAUCACAAAGGGCdTdT-5'
siRNA3	5'-CCAAGCGAAUCAACAUCAUdTdT-3'
NM_000909.6	3'-AUGAUGUUGAUUCGCUUGGdTdT-5'
siRNA 对照反义链	5'-UUCGUCUGUACUCCACAUAATTdTdT-3'
(Scrambled siRNA)	3'-GAUGUCUUCUACAGUCCGATTdTdT-5'
GAPDH	5'-TCCACCACCCTGTTGCTGTA-3'
NM_002046.3	3'-ACCACAGTCCATGCCATCAC-5'

参考文献

- [1] F. Yang, J. Wang, J. Hou, H. Guo, C. Liu, Bone regeneration using cell-mediated responsive degradable PEG-based scaffolds incorporating with rhBMP-2, *Biomaterials*, 34 (2013) 1514-1528.
- [2] A. Ho-Shui-Ling, J. Bolander, L.E. Rustom, A.W. Johnson, F.P. Luyten, C. Picart, Bone regeneration strategies: Engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives, *Biomaterials*, 180 (2018) 143-162.
- [3] S. Grässel, D. Muschter, Do Neuroendocrine Peptides and Their Receptors Qualify as Novel Therapeutic Targets in Osteoarthritis?, *Int J Mol Sci*, 19 (2018).
- [4] A. Zengin, L. Zhang, H. Herzog, P.A. Baldock, A. Sainsbury, Neuropeptide Y and sex hormone interactions in humoral and neuronal regulation of bone and fat, *Trends in endocrinology and metabolism: TEM*, 21 (2010) 411-418.
- [5] L. Teixeira, D.M. Sousa, A.F. Nunes, M.M. Sousa, H. Herzog, M. Lamghari, NPY revealed as a critical modulator of osteoblast function in vitro: new insights into the role of Y1 and Y2 receptors, *J Cell Biochem*, 107 (2009) 908-916.
- [6] A.F. Nunes, M.A. Liz, F. Franquinho, L. Teixeira, V. Sousa, C. Chenu, M. Lamghari, M.M. Sousa, Neuropeptide Y expression and function during osteoblast differentiation--insights from transthyretin knockout mice, *FEBS J*, 277 (2010) 263-275.
- [7] M.H. Park, N. Kim, H.K. Jin, J.S. Bae, Neuropeptide Y-based recombinant peptides ameliorate bone loss in mice by regulating hematopoietic stem/progenitor cell mobilization, *BMB reports*, 50 (2017) 138-143.
- [8] X. Yang, Y. Yang, B.F. Sun, Y.S. Chen, J.W. Xu, W.Y. Lai, A. Li, X. Wang, D.P. Bhattarai, Wang, Y.P. Sun, Y.G. Yang, 5-methylcytosine promotes mRNA export - NSUN2 as the methyltransferase and ALYREF as an m(5)C reader, *Cell Res*, 27 (2017) 606-625.
- [9] J. Liu, H. Fang, Z. Chi, Z. Wu, D. Wei, D. Mo, K. Niu, A.S. Balajee, T.K. Hei, L. Nie, Y. Zhao, XPD localizes in mitochondria and protects the mitochondrial genome from oxidative DNA damage, *Nucleic Acids Research*, 43 (2015) 5476-5488.
- [10] A. Bensimon-Brito, J. Carreira, G. Dionísio, A. Huysseune, M.L. Cancela, P.E. Witten, Revisiting in vivo staining with alizarin red S--a valuable approach to analyse zebrafish skeletal mineralization during development and regeneration, *BMC developmental biology*, 16 (2016) 2.

- [11] U. Sharma, D. Pal, R. Prasad, Alkaline phosphatase: an overview, *Indian journal of clinical biochemistry* : IJCB, 29 (2014) 269-278.
- [12] E. Birmingham, G.L. Niebur, P.E. McHugh, L.M. McNamara, Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche, *European cells & materials*, 23 (2012) 13-27.
- [13] Y. Chen, H.C. Whetstone, A.C. Lin, P. Nadesan, Q. Wei, R. Poon, B.A. Alman, Beta-catenin signaling plays a disparate role in different phases of fracture repair: implications for therapy to improve bone healing, *PLoS medicine*, 4 (2007) e249.
- [14] Y.H. Hsu, D.P. Kiel, Clinical review: Genome-wide association studies of skeletal phenotypes: what we have learned and where we are headed, *The Journal of clinical endocrinology and metabolism*, 97 (2012) E1958-1977.
- [15] H. Sudo, H.A. Kodama, Y. Amagai, S. Yamamoto, S. Kasai, In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria, *J Cell Biol*, 96 (1983) 191-198.
- [16] K. Tatemoto, Neuropeptide Y: complete amino acid sequence of the brain peptide, *Proc Natl Acad Sci U S A*, 79 (1982) 5485-5489.
- [17] C. Wahlestedt, D.J. Reis, Neuropeptide Y-related peptides and their receptors--are the receptors potential therapeutic drug targets?, *Annual review of pharmacology and toxicology*, 33 (1993) 309-352.
- [18] A. Vezzani, G. Sperk, W.F. Colmers, Neuropeptide Y: emerging evidence for a functional role in seizure modulation, *Trends in neurosciences*, 22 (1999) 25-30.
- [19] J.P. Redrobe, Y. Dumont, R. Quirion, Neuropeptide Y (NPY) and depression: from animal studies to the human condition, *Life Sci*, 71 (2002) 2921-2937.
- [20] A. Kask, J. Harro, S. von Hörsten, J.P. Redrobe, Y. Dumont, R. Quirion, The neurocircuitry and receptor subtypes mediating anxiolytic-like effects of neuropeptide Y, *Neuroscience and biobehavioral reviews*, 26 (2002) 259-283.
- [21] C.D. Stretton, P.J. Barnes, Modulation of cholinergic neurotransmission in guinea-pig trachea by neuropeptide Y, *Br J Pharmacol*, 93 (1988) 672-678.
- [22] E. Solcia, L. Usellini, R. Buffa, G. Rindi, L. Villani, C. Zampatti, E. Silini, Endocrine cells producing regulatory peptides, *Experientia*, 43 (1987) 839-850.
- [23] M.J. Morris, J.M. Pavia, Stimulation of neuropeptide Y overflow in the rat paraventricular nucleus by corticotropin-releasing factor, *J Neurochem*, 71 (1998) 1519-1524.

- [24] C. Wahlestedt, L. Edvinsson, E. Ekblad, R. Håkanson, Neuropeptide Y potentiates noradrenaline-evoked vasoconstriction: mode of action, *The Journal of pharmacology and experimental therapeutics*, 234 (1985) 735-741.
- [25] J. Pernow, J. Schwieler, T. Kahan, P. Hjendahl, J. Oberle, B.G. Wallin, J.M. Lundberg, Influence of sympathetic discharge pattern on norepinephrine and neuropeptide Y release, *The American journal of physiology*, 257 (1989) H866-872.
- [26] P. Walker, E. Grouzmann, M. Burnier, B. Waeber, The role of neuropeptide Y in cardiovascular regulation, *Trends in pharmacological sciences*, 12 (1991) 111-115.
- [27] C. Pellieux, T. Sauthier, A. Domenighetti, D.J. Marsh, R.D. Palmiter, H.R. Brunner, T. Pedrazzini, Neuropeptide Y (NPY) potentiates phenylephrine-induced mitogen-activated protein kinase activation in primary cardiomyocytes via NPY Y5 receptors, *Proc Natl Acad Sci U S A*, 97 (2000) 1595-1600.
- [28] S.W. Lee, B.G. Stanley, NMDA receptors mediate feeding elicited by neuropeptide Y in the lateral and perifornical hypothalamus, *Brain Res*, 1063 (2005) 1-8.
- [29] W. Xie, F. Li, Y. Han, Z. Li, J. Xiao, Neuropeptides are associated with pain threshold and bone microstructure in ovariectomized rats, *Neuropeptides*, 81 (2020) 101995.
- [30] F. Coluzzi, M.S. Scerpa, M. Centanni, The Effect of Opiates on Bone Formation and Bone Healing, *Curr Osteoporos Rep*, 18 (2020) 325-335.
- [31] N.C. Laddha, M. Dwivedi, M.S. Mansuri, M. Singh, H.H. Patel, N. Agarwal, A.M. Shah, R. Begum, Association of neuropeptide Y (NPY), interleukin-1B (IL1B) genetic variants and correlation of IL1B transcript levels with vitiligo susceptibility, *PLoS One*, 9 (2014) e107020.
- [32] M.H. Park, I.K. Jung, W.K. Min, J.H. Choi, J.S. Bae, Neuropeptide Y improves cisplatin-induced bone marrow dysfunction without blocking chemotherapeutic efficacy in a cancer mouse model, *BMB reports*, 50 (2017) 417-422.
- [33] S. Fujiwara, M. Hoshizaki, Y. Ichida, D. Lex, E. Kuroda, K.J. Ishii, S. Magi, M. Okada, H. Takao, M. Gandou, H. Imai, R. Hara, H. Herzog, A. Yoshimura, H. Okamura, J.M. Penninger, A.S. Slutsky, S. Uhlig, K. Kuba, Y. Imai, Pulmonary phagocyte-derived NPY controls the pathology of severe influenza virus infection, *Nat Microbiol*, 4 (2019) 258-268.

- [34] M. Alshalalfa, P.L. Nguyen, H. Beltran, W.S. Chen, E. Davicioni, S.G. Zhao, T.R. Rebbeck, E.M. Schaeffer, T.L. Lotan, F.Y. Feng, B.A. Mahal, Transcriptomic and Clinical Characterization of Neuropeptide Y Expression in Localized and Metastatic Prostate Cancer: Identification of Novel Prostate Cancer Subtype with Clinical Implications, *European urology oncology*, 2 (2019) 405-412.
- [35] R. Aller, J.J. López-Gomez, O. Izaola, D. de Luis, Role of neuropeptide Y gene variant (rs161477) in liver histology in obese patients with non-alcoholic fatty liver disease, *Endocrinologia, diabetes y nutricion*, 66 (2019) 217-222.
- [36] A.L. Appelt, R.F. Andersen, A. Jakobsen, Prognostic Value of Serum NPY Hypermethylation in Neoadjuvant Chemoradiotherapy for Rectal Cancer: Secondary Analysis of a Randomized Trial, *American journal of clinical oncology*, 43 (2020) 9-13.
- [37] W. Dai, Y. Liu, Y. Zhang, Y. Sun, C. Sun, Y. Zhang, X. Lv, Expression of neuropeptide Y is increased in an activated human HSC cell line, *Scientific reports*, 9 (2019) 9500.
- [38] N. Wang, D. Wang, H. Zhou, C. Xu, X. Hu, Z. Qian, X. Xu, Serum Neuropeptide Y Level is Associated with Post-Ischemic Stroke Epilepsy, *Journal of stroke and cerebrovascular diseases : the official journal of National Stroke Association*, 30 (2021) 105475.
- [39] A. Eitner, J. Pester, S. Nietzsche, G.O. Hofmann, H.G. Schaible, The innervation of synovium of human osteoarthritic joints in comparison with normal rat and sheep synovium, *Osteoarthritis and cartilage*, 21 (2013) 1383-1391.
- [40] M.H. Park, J.K. Lee, N. Kim, W.K. Min, J.E. Lee, K.T. Kim, H. Akiyama, H. Herzog, E.H. Schuchman, H.K. Jin, J.S. Bae, Neuropeptide Y Induces Hematopoietic Stem/Progenitor Cell Mobilization by Regulating Matrix Metalloproteinase-9 Activity Through Y1 Receptor in Osteoblasts, *Stem Cells*, 34 (2016) 2145-2156.
- [41] S. Lin, D. Boey, H. Herzog, NPY and Y receptors: lessons from transgenic and knockout models, *Neuropeptides*, 38 (2004) 189-200.
- [42] P.A. Baldock, N.J. Lee, F. Driessler, S. Lin, S. Allison, B. Stehrer, E.J. Lin, L. Zhang, R.F. Enriquez, I.P. Wong, M.M. McDonald, M. During, D.D. Pierroz, K. Slack, Y.C. Shi, E. Yulyaningsih, A. Aljanova, D.G. Little, S.L. Ferrari, A. Sainsbury, J.A. Eisman, H. Herzog, Neuropeptide Y knockout mice reveal a central role of NPY in the coordination of bone mass to body weight, *PLoS One*, 4 (2009) e8415.

第三部分 神经肽Y上调Runx2、Osterix表达促进小鼠前成骨细胞MC3T3-E1成骨的机制研究

前言

MC3T3-E1 细胞是一种前成骨细胞, 拥有增殖快、易培养、成骨分化时间短等优点, 在骨组织工程支架中是理想植入的种子细胞。它可在成骨诱导下定向分化为成骨细胞, 而成骨细胞在中轴及外周骨组织中有维持骨骼构造, 调节骨沉积, 参与骨折后骨合成及骨重塑等重要功能。

近来, 体内、外细胞学研究表明, 神经肽 Y (NPY) 在调控骨再生、骨形成、骨重塑机制中非常关键。但是, NPY 对 MC3T3-E1 细胞系的作用机制及生物学效应仍不明了。因此, 探讨 NPY 参与并促进细胞骨合成的机制, 对于改善严重创伤性骨折的预后具有重要的临床意义。

NPY 基因是神经超级通路中 341 个基因之一, 但它在神经超级通路调控骨代谢及骨平衡中的作用却不是很清楚。最近, NPY 及其 NPY1R 受体在机体外周组织(成骨细胞、骨细胞)表面表达^[1]。研究表明, 外源性阻断细胞 NPY1R 受体信号通路会上调细胞内源性成骨转录因子(Runx2 and Osterix)的表达^[2], 进而成骨能力增强, 骨量增多^[3]。王钊等于 2011 年研究表明, 骨髓间充质干细胞(bone marrow-derived stromal cells, BMSCs)表达 NPY 及其 NPY Y1 受体, 并且 NPY Y1 mRNA 随成骨诱导时间增高而增高^[4]。在这个过程中, 揭示了 NPY 及其 NPY Y1 受体可能参与诱导 BMSCs 向成骨细胞分化。这与 Teixeira 等^[5]和 Hosaka 等^[6]的研究结果一致。然而, 直到最近, 人们对 MC3T3-E1 细胞系核内 NPY 基因调控的成骨机制知之甚少。

为了研究这一机制, 我们将 NPY 过表达质粒和 NPYsiRNA 转染到 MC3T3-E1 细胞中, 观察其对细胞成骨的生物学效应。在第二部分中我们已经成功制备了 NPY 过表达质粒及 NPY siRNA, 本部分将选取小鼠前成骨 MC3T3-E1 细胞并成骨诱导培养, 观察过表达或干扰 NPY 后对 MC3T3-E1 成骨细胞成骨相关基因 Runx2、Osterix、ALP、OCN 表达的影响, 为 NPY 在骨折愈合及修复的临床应用中提供实验依据。

材料与方法

1. 材料

1.1 细胞株

MC3T3-E1 细胞购于协和医学院细胞库。

1.2 主要药品和试剂

DMEM 试剂	美国 Gibco 公司
RFect 小核酸转染试剂盒	常州百代生物科技有限公司
细胞培养板	广州洁特生物过滤股份有限公司
RFect 质粒 DNA 转染试剂盒	常州百代生物科技有限公司
10% Fetal Bovine Serum (FBS)	美国 Gibco 公司
minimum essential medium (MEM)	美国 Gibco 公司
异丙醇	Sinopharm chemical Co.Ltd.
三氯甲烷	Sinopharm chemical Co.Ltd.
RNA 提取液	天根生化科技北京有限公司
FastStart Universal SYBR Green Master (Rox)	瑞士 Roche 公司
RevertAid First Strand cDNA Synthesis Kit	Thermo Fisher
引物(NPY, Runx2, OCN, ALP, Osterix 和 GADPH)	武汉擎科创新生物科技有限公司
胰蛋白酶	中国碧云天生物公司
HyPure TMMolecular Biology Grade Water	美国 HyClone 公司
Osterix 抗体 (cat. no. bs-1110R)	Bioss Bio.
ALP 抗体(cat. no. 11187-1-AP)	ProteinTech Group
OCN 抗体 (cat. no. Paa471Mu01)	Cloud-Clone Corp.
Runx2 抗体(cat. no. PAB011Mu01)	Cloud-Clone Corp.
NPY 抗体(cat. no. DF6431)	Affinity Biosciences

二 抗 (horseradish peroxidase-linked guinea pig anti-rabbit IgG polyclonal)(cat. no. SAA544Rb59)	Cloud-Clone Corp.
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1.3 主要仪器设备

台式高速冷冻微量离心机	上海 DragonLab 有限公司
荧光定量 PCR 仪	瑞士 Roche 公司
超微量分光光度计	Thermo Fisher
标准试剂型纯水仪	青岛富勒姆科技有限公司
离心管	美国 Axygen Biosciences 公司
TIP 头	美国 Axygen Biosciences 公司
超声波清洗器 CX-250	北京医疗设备二厂
电磁搅拌仪 ETS-D4	德国 IKA 公司
微量注射泵 KDS100	美国 KDS 公司
精密电子天平 AL104-1C	瑞士梅特勒公司
移液器	德国 Eppendorf 公司
旋涡混合器 VORTEX-5	江苏海门其林贝尔仪器公司
超净工作台	苏州安泰空气技术有限公司
电恒温水浴箱	上海精宏实验设备有限公司
荧光显微镜	日本 Olympus 公司
灌胶架、玻璃板、样品梳	美国 Bio-Rad 公司
培养板	美国 Corning 公司
电恒温水浴箱	上海精宏实验设备有限公司
紫外分光光度计	日本日立公司
超低温冰箱	日本 SANYO 公司
荧光分光光度计 Hitachi850	日本 Hitachi 公司
普通冰箱	青岛 Haier 公司
二氧化碳培养箱	德国 Heraeus 公司
凝胶化学发光检测系统	Thermo Fisher
全自动高压灭菌器	德国 Heraeus 公司

多样品研磨珠均质仪	Omni International
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1.4 主要试剂的配制

- (1) 1×转膜缓冲液：同第一部分 1.4。
- (2) 1×TBST 溶液：同第一部分 1.4。
- (3) 5%脱脂奶粉：同第一部分 1.4。

2. 方法

2.1 细胞转染及细胞培养

制备 NPY siRNA 及 NPY 过表达质粒及相应转染方法及细胞培养同第二部分，分别于转染后第 4 天和第 7 天取样，做进一步检测。

2.2 荧光定量 PCR 法检测过表达或干扰 NPY 后对细胞 Runx2, Osterix, OCN, ALP 的表达影响

2.2.1 总 RNA 抽提

操作步骤同第一部分 2.5.1。

2.2.2 反转录

操作步骤同第一部分 2.5.2。

2.2.3 定量 PCR 检测目的基因（Runx2, Osterix, OCN, ALP）表达情况

操作步骤同第一部分 2.5.3。

2.2.4 结果处理

数据处理步骤同第一部分 2.5.4。

2.3 Western blot 检测过表达或干扰 NPY 后对 MC3T3-E1 细胞 Runx2, Osterix, OCN, ALP 的相关蛋白表达影响

2.3.1 细胞总蛋白的提取

操作步骤同第二部分 3.3.1。

2.3.2 BCA 法测定蛋白浓度

操作步骤同第二部分 3.3.2。

2.3.3 SDS-PAGE 凝胶电泳

根据待测目的蛋白（Runx2, Osterix, OCN, ALP）的分子量，配制相应浓度的分离胶及浓缩胶，其余步骤参照第二部分 3.3.3 部分操作进行。。

2.3.4 转膜及显色

操作步骤同第二部分 3.3.4。

3. 统计学分析

所有实验重复 3 次，数据以均数 $\pm$ 标准差(means $\pm$ SD)表示。使用 SPSS 18.0 软件中 One-way ANOVA 方法进行各组总体差异检验， 各组间两两比较采用 Uncorrected Fisher's LSD 方法， $P < 0.05$ 为差异有统计学意义。使用 GraphPad Prism v.6.0 软件行柱状图绘图。

实验结果

1. NPY 上调细胞成骨特异性转录因子 Runx2、Osterix 基因的表达

1.1 过表达或干扰 NPY 后检测细胞 Runx2 和 Osterix 基因表达的影响

研究表明, Runx2 是一个不可替代的成骨早期转录因子, 参与调节间充质干细胞向成骨细胞的分化及成骨细胞特异性基因的表达^[7]。此外, Runx2 在成骨细胞的终末分化中也起主要作用^[8]。为了进一步从体外研究揭示 NPY 对 MC3T3-E1 细胞成骨的影响, 本部分将 NPY 过表达质粒和 NPY siRNA 转染 MC3T3-E1 细胞, 检测细胞中 Osterix 和 Runx2 表达的变化, 揭示 NPY 的相应生物学机制。

结果显示: NPY 过表达质粒可明显上调 Runx2 和 Osterix mRNA 的表达。Runx2 mRNA 与对照组相比, 第 4 天升高了 5.4 倍, 第 7 天升高了 6 倍 (图 1)。Osterix mRNA 与对照组相比, 第 4 天升高了 2.7 倍, 第 7 天升高了 3 倍。有趣的是 Runx2 mRNA 表达是 Osterix mRNA 的 2 倍 ($P<0.001$; 图 1 C and D)。

为充分了解 NPY 对 Runx2 和 Osterix mRNA 表达的影响, 我们将设计好的 NPY siRNA 转染入 MC3T3-E1 细胞系中, 检测了不同时间点 Runx2 和 Osterix mRNA 的表达。Runx2 mRNA 第 4 天较对照组下降了 50%, 第 7 天下降了 35%。Osterix mRNA 第 4 天较对照组下降了 80% ($P<0.001$), 第 7 天下降了 55% (图 1 C and D)。

1.2 过表达或干扰 NPY 后检测细胞 Runx2 和 Osterix 蛋白表达的影响

为更好了解 NPY 对 Runx2 和 Osterix 表达的影响, 我们从蛋白水平做了进一步的研究。结果表明: NPY 过表达质粒可促进 Runx2 和 Osterix 蛋白表达, 与对照组相比, Runx2 蛋白第 4 天增长了 1.6 倍, 第 7 天增长了 1.2 倍; Osterix 蛋白表达第 4 天增长了 1.6 倍, 第 7 天增长了 2.1 倍 ($P<0.05$; 图 2 A-C)

为充分了解 NPY 对 Runx2 和 Osterix 蛋白表达的影响, 我们将设计好的 NPY siRNA 转染入 MC3T3-E1 细胞系中, 与对照组相比, Runx2 蛋白表达第 4 天下降了接近 40% ($P<0.05$), 第 7 天下降了 55% ($P<0.05$) (图 2 A-C)。Osterix 蛋白表达第 4 天下降了接近 60% ($P<0.05$), 第 7 天下降了 55% ($P<0.05$) (图 2 A-C)。

综上所述, NPY 在基因和蛋白水平均可上调 MC3T3-E1 细胞 Runx2 和 Osterix 的表达, 从而促进 MC3T3-E1 细胞成骨分化。

2. NPY 促进 MC3T3-E1 细胞成骨分化

2.1 过表达或干扰 NPY 后检测细胞 ALP 和 OCN 基因表达的影响

NPY 对成骨细胞分化至关重要。骨形成因子如 ALP、OCN 的表达水平取决于

成骨分化的不同阶段^[9]。为了进一步从体外研究揭示 NPY 对细胞成骨的影响, 本部分将 NPY 过表达质粒和 NPY siRNA 转染小鼠前成骨 MC3T3-E1 细胞后, 检测细胞中 ALP 和 OCN 表达的变化, 揭示 NPY 的相应生物学机制。

结果显示: NPY 过表达质粒可明显促进 MC3T3-E1 细胞成骨。与对照组相比, ALP mRNA 在第 4 天和第 7 天分别增加了 3.7 倍和 4.9 倍, OCN mRNA 在第 4 天和第 7 天分别增加了 4.0 倍和 4.3 倍 ($P<0.001$; 图 1 A and B)。

为了进一步确定 NPY 对 MC3T3-E1 细胞中 ALP 和 OCN mRNA 的影响, 我们将 NPY siRNA 转染入 MC3T3-E1 细胞系中, 检测了不同时间点基因 ALP 和 OCN mRNA 的表达。ALP mRNA 第 4 天和第 7 天较对照组分别降低了 56% ($P<0.05$) 和 26%, OCN mRNA 第 4 天和第 7 天较对照组分别降低了 60% ($P<0.05$) 和 35%。

2.2 过表达或干扰 NPY 后检测细胞 ALP 和 OCN 蛋白表达的影响

为更好了解 NPY 对 ALP 和 OCN 表达的影响, 我们从蛋白水平做了进一步的研究。结果显示: NPY 过表达质粒可促进 ALP 和 OCN 蛋白表达, 与对照组相比, ALP 蛋白第 4 天和第 7 天均增加了 1.6 倍; OCN 蛋白第 4 天增加了 1.4 倍, 第 7 天增加了 1.6 倍, 均有统计学意义 ($P<0.05$; 图 2 A-C)。

为了进一步确定 NPY 对 MC3T3-E1 细胞中 ALP 和 OCN 蛋白的影响, 我们将设计好的 NPY siRNA 转染入 MC3T3-E1 细胞系中, 检测了不同时间点基因 ALP 和 OCN 蛋白的表达, 与对照组相比, ALP 蛋白第 4 天和第 7 天均减少了 60%; OCN 蛋白第 4 天减少了 64%, 第 7 天减少了 78%, 均有统计学意义 ($P<0.05$; 图 2 A-C)。

综上所述, NPY 在基因和蛋白水平均可促进 ALP 和 OCN 的表达, 从而促进 MC3T3-E1 细胞成骨分化。

讨 论

NPY 是由 36 个氨基酸构成的多肽, 属于一个神经肽的庞大家族。该家族还包括 YY 肽和胰腺多肽^[10, 11]。NPY 首先在机体中枢神经系统被检测到, 尤其在下丘脑弓状核高表达^[10]。Baldock 等^[12]对敲除 NPY1R 或 NPY2R 受体的小鼠检测后发现小鼠皮质骨和骨小梁的骨密度增高。但是, 在骨微环境中, NPY 在基因水平上的调控机制尚不清楚。因此, 本部分的研究目的是在 MC3T3-E1 细胞系中对 NPY 的相关成骨机制进行了较为深入的探讨。

NPY 是一种具有调节能量消耗和骨量平衡双重效能的神经递质^[13]。目前已知关于 NPY 的神经通路有以下几种: 1.抗精神病药途径 (Antipsychotics Pathway), 相关度分值 0.893; 2.交感神经通路(Sympathetic Nerve Pathway), 相关度分值 0.765; 3.内皮素通路, (Endothelin Pathways), 相关度分值 0.638; 4.脂肪细胞脂降解调节通路 (Regulation of lipolysis in adipocytes), 相关度分值 0.510; 5.脂肪细胞因子信号通路 (Adipocytokine signaling pathway), 相关度分值 0.446; 6.环磷酸腺苷信号通路 (cAMP signaling pathway), 相关度分值 0.255; 7.神经生物学信号通路 (Neuroscience signaling pathway), 相关度分值 0.191; 8.活化的蛋白激酶 N1(PKN1)刺激 AR (雄激素受体) 调节激肽释放酶 2 和激肽释放酶 3 (KLK2 和 KLK3) 基因转录信号通路[Activated PKN1 stimulates transcription of AR (androgen receptor) regulated genes KLK2 and KLK3], 相关度分值 0.159; 9.肽配体结合受体信号通路 (Peptide ligand-binding receptors), 相关度分值 0.128; 10. G 蛋白偶联受体信号通路 (Signaling by GPCR), 相关度分值 0.080。最新研究表明, NPY 是成骨相应的神经通路的关键因子, 并成为研究热点^[14-18], 有必要研究 NPY 与成骨相关因子作用机制, 并对不同成骨相关因子的作用强度做进一步对照分析, 有助于对 NPY 的促成骨机制做更深入了解。

Igwe 等^[19]发现, NPY 诱导的小鼠颅骨成骨细胞分化相关标志物 OCN、牙本质基质蛋白 1 (dentin matrix protein 1, DMP1)和骨涎蛋白(bone sialoprotein, BSP)的表达降低, 相应矿化程度也降低。其研究结果表明, 外源性 NPY 处理小鼠颅骨成骨细胞后, 细胞内环磷酸腺苷 (cyclic 3',5'-adenosinemonophosphate, cAMP) 和分化标志物(OCN、BSP 和 DMP1)水平降低^[19]。骨形态发生蛋白 (bone morphogenetic protein, BMP) 信号通路是关键的成骨相关通路, BMP 可上调两种关键的成骨转录因子 Osterix 和 Runx2 来刺激成骨细胞分化和维持成骨细胞活性^[8, 20]。本研究中证

实了 NPY 通过 Osterix 和 Runx2 基因参与成骨, 揭示了 NPY 参与 BMP 信号通路调节骨代谢。Runx2 基因中具有两种亚型, 分别为骨相关 Runx2-II 和非骨相关 Runx2-I, 他们仅在各自的 N 端不同, 由不同的启动子启动转录程序^[21]。为了阐明 Runx2 骨相关亚型的功能, Xiao, Z. S. 等通过靶向敲除远端启动子和外显子 1, 成功建立了敲除 runx2-II 基因(Runx2-II(-/-))的小鼠模型^[21]。Runx2-II(-/-)小鼠意外形成了由骨胶原间充质细胞膜内骨化衍生出来的中轴骨、阑尾和颅面骨^[21]。虽然其软骨细胞成熟表现正常, 但肥大软骨细胞带不能骨化成干骺骨, 导致生长板增宽^[21]。Runx2-II(-/-)小鼠中 Runx2-I 表达的代偿性增加, 但不足以促进成骨细胞成熟或使其转录活性正常^[21]。Xiao, Z. S. 等研究表明, Runx2-I 是早期骨生成和膜内骨化所必需的, 而 Runx2-II 则是成骨细胞完全成熟和软骨成骨所必须的^[21]。Runx2 作为成骨的关键因子, 缺失 Runx2 基因可能会导致人类和哺乳动物的颅骨、锁骨发育缺陷^[22]。Runx2 和 Osterix 被认为是成骨细胞成熟乃至钙化成骨的重要调节因子^[23]。本部分研究同样验证了 Runx2 基因在调节 MC3T3-E1 细胞骨发生中的重要生物学机能, 并受 NPY 的表达的影响。

最新研究表明, Osterix 不仅在骨折后膜内成骨起作用, 也在软骨终末分化中起重要作用^[24]。Osterix 在成骨细胞、多种癌组织及软骨细胞中都有表达^[25]。Osterix 蛋白高度保守, 其氨基酸序列在人类和小鼠之间的同源性达 95%。Osterix 诱导多种成熟成骨细胞基因的表达, 如胶原 I 型 $\alpha 1$ (collagen type I $\alpha 1$, Col1a1)、骨连蛋白 (Osteonectin)、骨桥蛋白 (Osteopontin, OPN)、骨钙素 (Osteocalcin, OCN) 和骨形态发生蛋白 (Bone sialoprotein, Bsp), 这些都是成骨细胞成骨化骨过程中产生所必需的^[26]。Osterix 由骨形态发生蛋白 2 (bone morphogenetic protein2, BMP2) 所诱导, 是成骨细胞转录诱导、成骨分化和钙盐沉积的关键因子^[27]。Osterix 基因是成骨细胞成骨所需的 Runx2 重要下游效应因子。Nakashima 等发现 Osterix 基因在 Runx2-II(-/-)的小鼠中显著降低, 同时, 其转录的 ALP、OCN、OPN 和细胞外基质磷酸化糖蛋白 (matrix extracellular phosphoglycoprotein, MEPE) 表达降低^[27]。但是, 这些成骨标记物的水平在杂合突变小鼠中是正常或轻微下降^[22]。Xiao, Z. S. 等的研究也证实 Runx2 是 Osterix 基因的上游调控基因, Runx2-I 还参与 Runx2-II 沉默小鼠 1/2 的 Osterix 基因表达; 换言之, 1/2 的 Osterix 表达可能受到上游 Runx2 基因的调控^[21]。本研究(图 2C 和 2D)同样提示, Osterix 基因与 Runx2 基因表达一致, Osterix mRNA 几乎是 Runx2 mRNA 表达水平的一半, 与上述学者的研究基本一致。

据文献报导, ALP 是成骨细胞分化早期的重要表型标志物^[21, 22], 其内含子 1 的核心位点受 Runx2 调控, 所以 ALP 的表达及其活性直接受到 Runx2 的影响^[23]。固然, 我们较为系统的研究了 NPY 对 Runx2、Osterix、ALP 和 OCN mRNA 和蛋白的影响, 部分阐明了 NPY 基因可上调 Runx2 和 Osterix 表达促进成骨细胞分化的潜在生物学机制。但是我们也认识到细胞成骨信号通路的复杂性, 受多种成骨相关基因的影响, 今后可进一步研究 NPY 对其他成骨相关因子如 OPN、cAMP、MEPE、BSP、 β -catenin 的生物学作用, 以进一步明确 NPY 与成骨之间的相互关系, 为将来 NPY 投入临床应用创造条件。

随着交通事故造成的骨创伤的日益增多, 骨折合并巨大骨缺损给临床工作带来了许多挑战。生物活性分子与骨人工支架相结合, 将有助于解决这一临床难题, 具有重要的研究价值。

结 论

1. NPY 通过上调 Runx2、Osterix 基因的表达促进 MC3T3-E1 细胞成骨。
2. NPY 有望成为创伤后促进骨折愈合的靶点。

附图

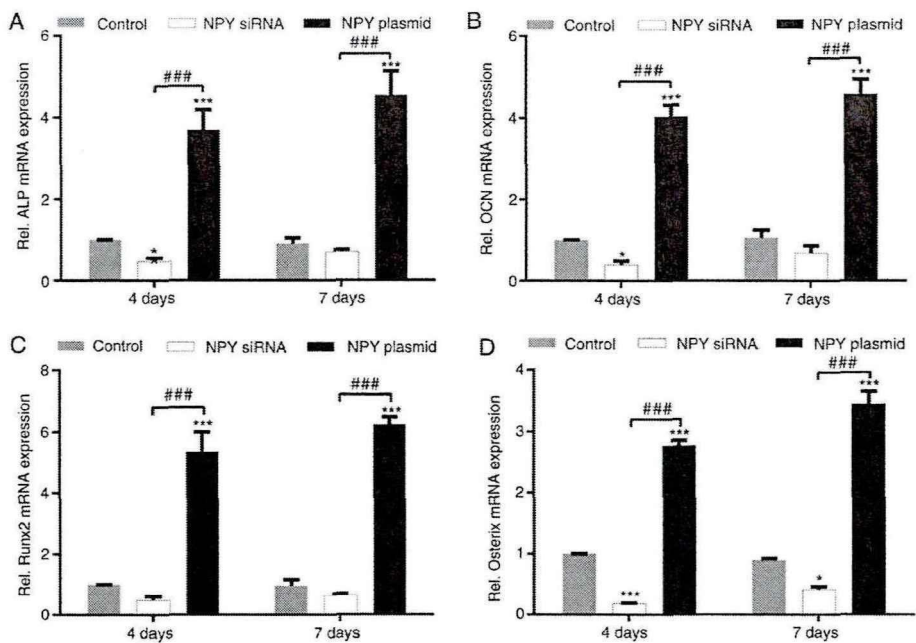


图 1 MC3T3-E1 细胞 ALP、OCN、Runx2 和 Osterix mRNA 的表达

过表达 NPY 后, ALP mRNA 与对照组相比在第 4 天和第 7 天分别增加 3.7 倍和 4.9 倍 ($P<0.001$, 图 A); OCN mRNA 与对照组相比在第 4 天和第 7 天分别增加 4.0 倍和 4.3 倍 ($P<0.001$, 图 B); Runx2 mRNA 与对照组相比, 第 4 天升高 5.4 倍, 第 7 天升高 6 倍 ($P<0.001$, 图 C); Osterix mRNA 与对照组相比, 第 4 天升高 2.7 倍, 第 7 天升高 3 倍 ($P<0.001$, 图 D)。干扰 NPY 后, ALP mRNA 第 4 天和第 7 天较对照组分别降低 56% ($P<0.05$)和 26% (图 A); OCN mRNA 第 4 天和第 7 天较对照组分别降低 60% ($P<0.05$)和 35% (图 B); Runx2 mRNA 第 4 天较对照组下降 50%, 第 7 天下降 35% (图 C); Osterix mRNA 第 4 天较对照组下降 80% ($P<0.001$), 第 7 天下降 55% (图 D)。(* $P<0.05$, *** $P<0.001$ vs. 对照组; ### $P<0.001$ vs. siRNA 组)

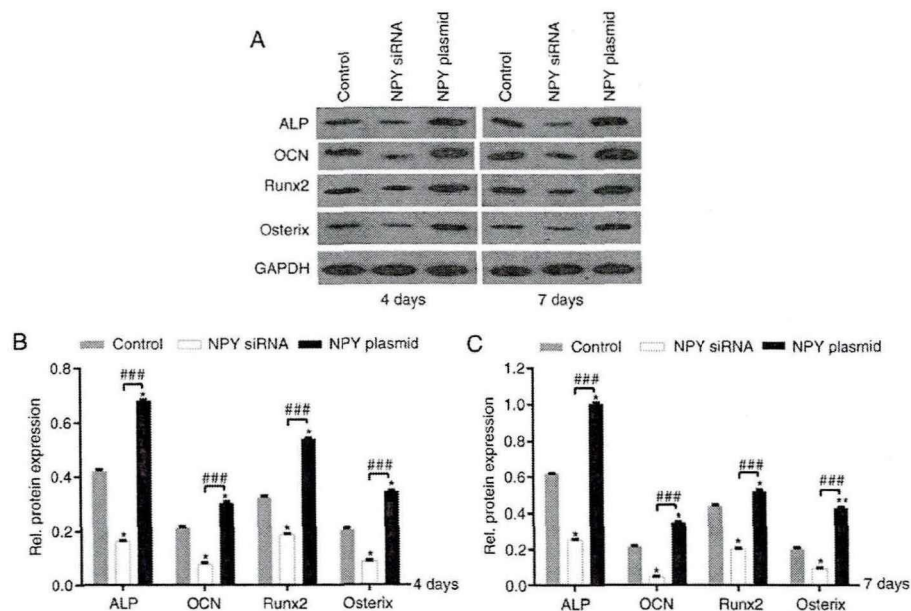


图 2 MC3T3-E1 细胞 ALP、OCN、Runx2 和 Osterix 蛋白的表达

过表达 NPY 后, ALP 蛋白的表达与对照组相比第 4 天和第 7 天均增加 1.6 倍 ($P<0.05$, 图 A-C); OCN 蛋白与对照组相比第 4 天增加 1.4 倍, 第 7 天增加 1.6 倍 ($P<0.05$, 图 A-C); Runx2 蛋白与对照组相比第 4 天增长 1.6 倍, 第 7 天增长 1.2 倍 ($P<0.05$, 图 A-C); Osterix 蛋白表达与对照组相比第 4 天增长 1.6 倍, 第 7 天增长 2.1 倍 ($P<0.05$, 图 A-C)。干扰 NPY 后, ALP 蛋白表达较对照组第 4 天和第 7 天均减少 60% ($P<0.05$, 图 A-C); OCN 蛋白表达较对照组第 4 天减少 64%, 第 7 天减少 78% ($P<0.05$, 图 A-C); Runx2 蛋白表达较对照组第 4 天下降接近 40% ($P<0.05$, 图 A-C), 第 7 天下降 55% ($P<0.05$, 图 2 A-C); Osterix 蛋白表达第 4 天下降接近 60% ($P<0.05$, 图 A-C), 第 7 天下降 55% ($P<0.05$, 图 2 A-C)。(* $P<0.05$, *** $P<0.001$ vs. 对照组; ### $P<0.001$ vs. siRNA 组)

附表

表 1 逆转录定量 PCR 的引物序列

Name	Sequence
NPY	5'-CTCGTGTGTTTGGGCATTC-3'
NM_023456	3'-TAGTGTCGCAGAGCGGAGTA-5'
siRNA	5'-CCAAGCGAAUCAACAUCAUdTdT-3'
NM_000909.6	3'-AUGAUGUUGAUUCGCUUGGdTdT-5'
Runx2	5'-GACGAGGCAAGAGTTTCACC-3'
NM_009820	3'-GGACCGTCCACTGTCACTTT-5'
Osterix	5'-GATGGCGTCCTCTCTGCTTG-3'
NM_130458.3	3'-TCTTTGTGCCCTCCTTCCCC-5'
OCN	5'-CAAGCAGGGAGGCAATAAGG-3'
NM_007541	3'-CGTCACAAGCAGGGTTAAGC-5'
ALP	5'-TGGTCACAGCAGTTGGTAGC-3'
NM_001287172.1	3'-CTGAGATTCGTCCCTCGCTG-5'
GAPDH	5'-TCCACCACCCTGTTGCTGTA-3'
NM_002046.3	3'-ACCACAGTCCATGCCATCAC-5'

参考文献

- [1] H. Horsnell, P.A. Baldock, Osteoblastic Actions of the Neuropeptide Y System to Regulate Bone and Energy Homeostasis, *Curr Osteoporos Rep*, 14 (2016) 26-31.
- [2] N.J. Lee, K.L. Doyle, A. Sainsbury, R.F. Enriquez, Y.J. Hort, S.J. Riepler, P.A. Baldock, H. Herzog, Critical role for Y1 receptors in mesenchymal progenitor cell differentiation and osteoblast activity, *J Bone Miner Res*, 25 (2010) 1736-1747.
- [3] D.M. Sousa, P.A. Baldock, R.F. Enriquez, L. Zhang, A. Sainsbury, M. Lamghari, H. Herzog, Neuropeptide Y Y1 receptor antagonism increases bone mass in mice, *Bone*, 51 (2012) 8-16.
- [4] Z. Wang, D. Jin, Y. Tuo, X. Guo, J. Wen, J. Zhou, L. Xia, Y. Zhang, [Calcitonin gene-related peptide promoting migration of rat bone marrow mesenchymal stem cells and stimulating expression of vascular endothelial growth factor], *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi*, 25 (2011) 1371-1376.
- [5] L. Teixeira, D.M. Sousa, A.F. Nunes, M.M. Sousa, H. Herzog, M. Lamghari, NPY revealed as a critical modulator of osteoblast function in vitro: new insights into the role of Y1 and Y2 receptors, *J Cell Biochem*, 107 (2009) 908-916.
- [6] H. Hosaka, A. Nagata, T. Yoshida, T. Shibata, T. Nagao, T. Tanaka, Y. Saito, I. Tatsuno, Pancreatic polypeptide is secreted from and controls differentiation through its specific receptors in osteoblastic MC3T3-E1 cells, *Peptides*, 29 (2008) 1390-1395.
- [7] J. Han, J. Zuo, D. Zhu, C. Gao, The correlation between SNPs within the gene of adrenergic receptor and neuropeptide Y and risk of cervical vertigo, *J Clin Lab Anal*, 32 (2018) e22366.
- [8] L. Li, M. Sapkota, M. Gao, H. Choi, Y. Soh, Macrolactin F inhibits RANKL-mediated osteoclastogenesis by suppressing Akt, MAPK and NFATc1 pathways and promotes osteoblastogenesis through a BMP-2/smad/Akt/Runx2 signaling pathway, *Eur J Pharmacol*, 815 (2017) 202-209.
- [9] J. Chen, G. He, Y. Wang, D. Cai, MicroRNA-223 promotes osteoblast differentiation of MC3T3-E1 cells by targeting histone deacetylase 2, *International Journal of Molecular Medicine*, (2018).

- [10] J.M. Allen, T.E. Adrian, K. Tatemoto, J.M. Polak, J. Hughes, S.R. Bloom, Two novel related peptides, neuropeptide Y (NPY) and peptide YY (PYY) inhibit the contraction of the electrically stimulated mouse vas deferens, *Neuropeptides*, 3 (1982) 71-77.
- [11] K. Tatemoto, M. Carlquist, V. Mutt, Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide, *Nature*, 296 (1982) 659-660.
- [12] P.A. Baldock, A. Sainsbury, S. Allison, E.J. Lin, M. Couzens, D. Boey, R. Enriquez, M. During, H. Herzog, E.M. Gardiner, Hypothalamic control of bone formation: distinct actions of leptin and y_2 receptor pathways, *J Bone Miner Res*, 20 (2005) 1851-1857.
- [13] S. Lin, D. Boey, H. Herzog, NPY and Y receptors: lessons from transgenic and knockout models, *Neuropeptides*, 38 (2004) 189-200.
- [14] K. Wolf, E. Höcherl, T. Farkas, C. Pfister, [Expression and dynamics of peptidergic nerve fibers in granulation tissue after distance osteosynthesis], *Der Unfallchirurg*, 104 (2001) 948-954.
- [15] P. Dong, X. Gu, G. Zhu, M. Li, B. Ma, Y. Zi, Melatonin Induces Osteoblastic Differentiation of Mesenchymal Stem Cells and Promotes Fracture Healing in a Rat Model of Femoral Fracture via Neuropeptide Y/Neuropeptide Y Receptor Y1 Signaling, *Pharmacology*, 102 (2018) 272-280.
- [16] S. Liu, J.Q. Wu, J.J. Hu, L. Wang, Z. Wang, H. Meng, L.J. Zhuo, J.X. Zheng, [Neuropeptide Y Y1 receptor antagonist PD160170 promotes osteogenic differentiation of rat bone marrow mesenchymal stem cells in vitro and femoral defect repair in rats], *Nan Fang Yi Ke Da Xue Xue Bao*, 38 (2018) 669-676.
- [17] F. Coluzzi, M.S. Scerpa, M. Centanni, The Effect of Opiates on Bone Formation and Bone Healing, *Curr Osteoporos Rep*, 18 (2020) 325-335.
- [18] W. Xie, F. Li, Y. Han, Y. Qin, Y. Wang, X. Chi, J. Xiao, Z. Li, Neuropeptide Y1 receptor antagonist promotes osteoporosis and microdamage repair and enhances

osteogenic differentiation of bone marrow stem cells via cAMP/PKA/CREB pathway, *Aging* (Albany NY), 12 (2020) 8120-8136.

[19] J.C. Igwe, X. Jiang, F. Paic, L. Ma, D.J. Adams, P.A. Baldock, C.C. Pilbeam, I. Kalajzic, Neuropeptide Y is expressed by osteocytes and can inhibit osteoblastic activity, *J Cell Biochem*, 108 (2009) 621-630.

[20] J.S. Moon, S.H. Kim, S.H. Oh, Y.W. Jeong, J.H. Kang, J.C. Park, H.J. Son, S. Bae, B.I. Park, M.S. Kim, J.T. Koh, H.M. Ko, Relaxin augments BMP-2-induced osteoblast differentiation and bone formation, *J Bone Miner Res*, 29 (2014) 1586-1596.

[21] Z.S. Xiao, A.B. Hjelmeland, L.D. Quarles, Selective deficiency of the "bone-related" Runx2-II unexpectedly preserves osteoblast-mediated skeletogenesis, *J Biol Chem*, 279 (2004) 20307-20313.

[22] J. Hecht, V. Seitz, M. Urban, F. Wagner, P.N. Robinson, A. Stiege, C. Dieterich, U. Kornak, U. Wilkening, N. Brieske, C. Zwingman, A. Kidess, S. Stricker, S. Mundlos, Detection of novel skeletogenesis target genes by comprehensive analysis of a Runx2(-/-) mouse model, *Gene Expr Patterns*, 7 (2007) 102-112.

[23] P. Ducy, R. Zhang, V. Geoffroy, A.L. Ridall, G. Karsenty, Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation, *Cell*, 89 (1997) 747-754.

[24] Q. Liu, M. Li, S. Wang, Z. Xiao, Y. Xiong, G. Wang, Recent Advances of Osterix Transcription Factor in Osteoblast Differentiation and Bone Formation, *Front Cell Dev Biol*, 8 (2020) 601224.

[25] S. Qu, J. Wu, Q. Bao, B. Yao, R. Duan, X. Chen, L. Li, H. Yuan, Y. Jin, C. Ma, Osterix promotes the migration and angiogenesis of breast cancer by upregulation of S100A4 expression, *J Cell Mol Med*, 23 (2019) 1116-1127.

[26] J. Renn, C. Winkler, Osterix-mCherry transgenic medaka for in vivo imaging of bone formation, *Dev Dyn*, 238 (2009) 241-248.

[27] K. Nakashima, X. Zhou, G. Kunkel, Z. Zhang, J.M. Deng, R.R. Behringer, B. de Crombrughe, The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation, *Cell*, 108 (2002) 17-29.

综 述

神经肽 Y 在骨与能量合成中的生物学功能及研究进展

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摘 要

神经肽 Y (neuropeptide Y, NPY)，一种含有 36 个氨基酸的肽，在人类及哺乳动物中枢及外周神经系统普遍表达，在维持骨稳态、能量合成、摄食反射等多种生理进程中发挥了重要的作用^[1,2]。NPY 具有促进骨折愈合、治疗膝关节骨性关节炎、改善骨质疏松症的预后等广泛的临床应用前景。本文对 NPY 在骨与能量合成中生物学功能进行了综述，为未来的相关临床应用研究提供新的思路。

关键词：神经肽 Y，骨折，骨性关节炎、骨质疏松症

前 言

K. Tatemoto 教授于 1982 年率先从猪脑中提取出 NPY^[3]。NPY 广泛存在于人体各类组织中，NPY 在人体肾上腺最高，大脑额叶和皮质层次之^[4-8]。NPY 结构与消化系统的 YY 肽 (peptide YY, PYY) 和胰多肽 (pancreatic polypeptide, PP) 为同一家族，均具有 1 个多脯氨酸螺旋结构的 N 端和 1 个螺旋结构的 C 端的反向平行的螺旋结构区，形成具有发卡样的三维立体结构^[9]。NPY 与 PYY 及 PP 有很多类似的主要生理作用，如刺激摄食反射、升高血压、能量稳态、骨稳态等^[9-14]。近年来，NPY 在干细胞疾病治疗中的作用也备受关注^[15, 16]。NPY 参与调节破骨细胞介导的骨吸收、成骨细胞介导的骨合成及骨重塑，是骨折愈合的关键因子^[3]。

本文综述了 NPY 在改善骨性关节炎、促进脑损伤后骨折愈合、治疗骨质疏松症、调节摄食反射中的作用及研究进展，为未来进一步探索 NPY 的分子生物学机制提供参考。

1. NPY 改善骨性关节炎的预后

骨性关节炎 (osteoarthritis, OA) 目前是世界第 11 位致残疾病。世界的 10%

人口,超过 65 岁的患者几乎都会出现髌、膝关节疼痛等骨性关节炎的临床症状^[17,18]。OA 不光对患者的生活水平造成很大的影响,同时还造成严重的社会经济负担^[18-20]。OA 会造成患者行为能力下降,心脑血管疾患,进而影响患者寿命及生存质量^[18]。OA 患者较正常人群具有更高的死亡率。因此,通过积极的一级和二级预防来降低 OA 患者的发病率刻不容缓^[20]。

NPY 对关节软骨修复、骨代谢平衡具有双重作用^[11-13]。研究表明,关节的滑膜、软骨、半月板和软骨下骨等髌、膝等关节的组织都含有丰富的神经肽^[14]。这些组织的细胞分布了多种神经内分泌衍生肽的受体,包括前阿片黑皮素(proopiomelanocortin, POMC)、 α -促黑色素细胞激素(α -melanocyte-stimulating hormone, α -MSH)、 β -内啡肽(β -endorphin, β -ED)、促肾上腺皮质激素(adreno-cortico-tropic-hormone, ACTH)、血管活性肠肽(vasoactive intestinal peptide, VIP)和 NPY^[14]。NPY 和 VIP 是重要的交感神经肽,其对髌、膝等关节软骨的神经滋养作用正受到越来越多的关注。NPY 和 VIP 被认为是改善 OA 的潜在治疗靶点^[14]。

Eitner 等研究发现,正常大鼠和绵羊膝关节髌旁区滑膜可见大量含有感觉纤维和酪氨酸羟化酶阳性的交感神经(NPY 和 VIP)纤维,其对大鼠和绵羊膝关节成骨细胞的增殖、分化等具有重要的推动作用^[21]。而在失交感神经支配的 OA 模型中,其滑膜组织不能够对损伤产生炎症反应,不能修复 OA 软骨缺损^[21],从而更容易发生膝关节骨性关节炎。在患有大鼠和绵羊 OA 动物模型中,应用 α -MSH、ACTH 和 NPY,能明显缓解 OA 的进展^[14]。NPY 和 VIP 在骨细胞、成骨细胞、破骨细胞中可通过直接和间接激活多个通路来促进骨代谢。NPY 可抑制破骨细胞增生并增强成骨细胞的活性,从而起到维持骨稳态的作用。

综上所述,神经肽 Y 能明显促进关节软骨修复,减轻关节局部炎症反应,从而改善患者预后。

2. NPY促进脑损伤患者骨折愈合

Garland, D. E 教授早在 1980 年临床研究发现,骨折合并脑损伤病人愈合显著加快^[22]。随后的研究也进一步证实了上述观点^[23,24]。P V Giannoudis 教授于 2006 年系统研究了 67 例长骨骨折病人,其中合并脑损伤 17 人,无脑损伤病人 50 人。研究发现脑损伤病人骨折愈合时间平均 10.5 周,而无脑损伤病愈合时间 20.5-26.9 周。

脑损伤病人的骨折愈合时间明显减少, 几乎是无脑损伤病人时间的1/2[25]。临床中我们同样发现类似的现象, 这与文献报道相一致[26, 27]。

McIntosh, T. K.教授在1992年采用大鼠液体撞击(fluid percussion, FP)脑损伤模型证实了在颅脑损伤15min后NPY-免疫反应纤维即可出现在受损的皮质和邻近的海马区, 创伤后脑脊液中也会出现NPY浓度增高[28]。创伤性脑损伤后脑脊液及外周骨组织特别是骨膜中NPY明显增高, Wistar大鼠骨折模型中骨愈合速度明显加快[14, 29, 30]。由此可见, NPY可通过脑脊液途径对骨代谢产生影响, 显著提高骨折愈合速度, 具有重要的临床应用前景。

褪黑素(Melatonin, MLT)是由大脑间脑顶部松果体分泌的激素之一。褪黑素在脑损伤蛛网膜下腔出血患者中大量聚集, 可清除氧自由基, 起到脑损伤保护作用[31, 32]。MLT可促进NPY、NPY1R、锌转运体1(zinc transporter, Zip1)表达[32]。并且, NPY/NPY1R信号通路介导褪黑素诱导间充质干细胞成骨分化, 促进大鼠股骨骨折愈合[31]。

3. NPY是治疗骨质疏松症的潜在靶点

骨质疏松症已成为一个全球性的健康问题, 影响广大的老年群体[33-36]。骨质疏松导致的脆性骨折包括髋关节、脊柱和手腕等部位的骨折, 以髋部骨折的预后最差[33]。早在1990年, 全世界髋骨骨折的直接和间接年费用估计为348亿美元, 预计在今后50年中将大幅增加[33]。研究表明, NPY参与调控成骨细胞、骨细胞、破骨细胞等多种细胞代谢[2, 37-39]。NPY因具有改善成骨细胞活力的特点, 故被认为是治疗骨质疏松症导致脆性骨折的潜在靶点。

长期口服非甾体抗炎药物、阿片类药物、质子泵抑制剂、降糖药物、抗抑郁药、抗精神病药、抗惊厥药等会导致病人脆性骨折, 这些药物是人体骨折的高危因素[40]。Vestergaard, P 教授2011年研究了10年服用非甾体抗炎药的病人与未服用非甾体抗炎药病人的随访调查, 服用NSAID药患者骨折风险显著增加(HR = 1.44, 95% CI 1.07-1.93); 阿片类药物会明显抑制体内细胞的骨代谢, 增加脆性骨折风险[41, 42]。同时, 阿片类药物通过阻断交感神经肽(VIP和NPY), 能明显抑制骨合成代谢[12, 43]。此外, 阿片类药物抑制成骨细胞骨合成、促进破骨细胞骨吸收、影响骨细胞骨代谢, 发挥骨抑制作用[44, 45], 增加骨质疏松脆性骨折的风险。

动物骨质疏松模型可通过多种模式来构建。结扎大鼠胆管可造成动物缓慢肝硬化,进而出现肝源性骨质疏松^[46];切除大鼠卵巢(ovariectomized, OVX)造成动物雌激素缺乏,进而出现激素性骨质疏松^[11, 47]。Mona Moradi 等教授于2019年研究中做了进一步的验证,即在结扎大鼠胆管导致的肝硬化动物模型中,骨量显著降低,骨质疏松明显^[46]。最新研究表明, NPY可对不同源性的骨质疏松症具有治疗作用^[12]。Weixin Xie等进行的OVX动物模型的研究表明,胫骨表面的相应基因—降钙素基因相关肽(calcitonin gene-related peptide, CGRP)、P物质(substance P, SP)、VIP、NPY表达上调,而NPY1R的表达下调^[11]。提示SP、CGRP、VIP和NPY参与了OVX大鼠骨质疏松骨重建过程。

总之, NPY可作为治疗绝经后骨质疏松(Postmenopausal osteoporosis, PMO)、药物性骨质疏松、肝源性骨质疏松、激素性骨质疏松等的潜在靶点^[11],深入研究 NPY具有重要的临床意义。

4. NPY在调节动物摄食反射和能量合成方面的作用

NPY可通过阿片信号通路或NPY及其受体通路影响动物摄食反射。NPY诱导摄食的阿片受体涉及几乎所有主要的阿片受体,其方式与观察到的缺糖或缺脂摄食反应相似^[48]。NPY可通过中枢mu和kappa阿片受体来诱导动物摄食行为^[48]。与β-内啡肽(beta-endorphin, β-ED)、阿片肽(opioid peptides)和强啡肽A(dynorphin A)可通过μ和kappa阿片受体来诱导动物摄食行为类似^[48]。应用kappa阿片受体拮抗剂或kappa反义探针的预处理, NPY诱导的摄食反射呈剂量依赖性减少^[48]。此外, NPY还可在中枢神经系统中通过多种NPY受体调节动物摄食反射。NPY Y1受体(NPY receptor Y1, NPY1R)和NPY Y5受体(NPY receptor Y5, NPY5R)会促进动物进食行为^[49-52],而NPY Y2受体(NPY receptor Y2, NPY2R)和NPY Y4受体(NPY receptor Y4, NPY4R)会抑制进食行为^[53, 54]。

NPY在维持体内能量平衡方面也发挥了非常重要的作用^[55]。NPY及NPY1R受体广泛位于肠粘膜下和肠肌丛神经节细胞内^[56],并被检测到NPY参与调控胃肠粘膜肠内神经元。1 μmol/L浓度的NPY即能显著促进胃肠粘膜肠内神经元增殖,减少细胞凋亡^[57]。在NPY-siRNA、NPY-Y1受体拮抗剂和NPY(-/-)小鼠体内培养的肠神经细胞中,胶质细胞源性神经营养因子(GDNF)介导的神经细胞增殖和活性明显降低^[57]。与野生型小鼠相比, NPY(-/-)小鼠胃排空加快,肠

道转运延迟。同时, NPY及其NPY1R和NPY2R受体也影响脂肪细胞代谢, NPY在外周交感神经元/肾上腺兴奋期间可直接作用于其的NPY1R和NPY2R受体, 刺激体内脂肪累积合成^[58]。

展 望

NPY 具有多种重要的生物学功能, 是重要的骨骼神经营养因子。在治疗关节骨性关节炎、促进骨折愈合、能量合成、摄食反射等多种生理进程中发挥独特作用。因此, NPY 具有巨大的临床应用前景和重要的研究价值。然而, 目前 NPY 发挥作用的生物学功能及相应机制仍不明了, 尚需进一步全面而深入的研究。

参考文献

- [1] P.A. Baldock, N.J. Lee, F. Driessler, S. Lin, S. Allison, B. Stehrer, E.J. Lin, L. Zhang, R.F. Enriquez, I.P. Wong, M.M. McDonald, M. During, D.D. Pierroz, K. Slack, Y.C. Shi, E. Yulyaningsih, A. Aljanova, D.G. Little, S.L. Ferrari, A. Sainsbury, J.A. Eisman, H. Herzog, Neuropeptide Y knockout mice reveal a central role of NPY in the coordination of bone mass to body weight, *PLoS One*, 4 (2009) e8415.
- [2] I. Matic, B.G. Matthews, T. Kizivat, J.C. Igwe, I. Marijanovic, S.T. Ruohonen, E. Savontaus, D.J. Adams, I. Kalajzic, Bone-specific overexpression of NPY modulates osteogenesis, *J Musculoskelet Neuronal Interact*, 12 (2012) 209-218.
- [3] K. Tatemoto, Neuropeptide Y: complete amino acid sequence of the brain peptide, *Proc Natl Acad Sci U S A*, 79 (1982) 5485-5489.
- [4] M. Alshalalfa, P.L. Nguyen, H. Beltran, W.S. Chen, E. Davicioni, S.G. Zhao, T.R. Rebbeck, E.M. Schaeffer, T.L. Lotan, F.Y. Feng, B.A. Mahal, Transcriptomic and Clinical Characterization of Neuropeptide Y Expression in Localized and Metastatic Prostate Cancer: Identification of Novel Prostate Cancer Subtype with Clinical Implications, *European urology oncology*, 2 (2019) 405-412.
- [5] R. Aller, J.J. López-Gomez, O. Izaola, D. Primo, D. de Luis, Role of neuropeptide Y gene variant (rs161477) in liver histology in obese patients with non-alcoholic fatty liver disease, *Endocrinologia, diabetes y nutricion*, 66 (2019) 217-222.
- [6] A.L. Appelt, R.F. Andersen, J. Lindebjerg, A. Jakobsen, Prognostic Value of Serum NPY Hypermethylation in Neoadjuvant Chemoradiotherapy for Rectal Cancer: Secondary Analysis of a Randomized Trial, *American journal of clinical oncology*, 43 (2020) 9-13.
- [7] W. Dai, Y. Liu, Y. Zhang, Y. Sun, C. Sun, Y. Zhang, X. Lv, Expression of neuropeptide Y is increased in an activated human HSC cell line, *Scientific reports*, 9 (2019) 9500.
- [8] N. Wang, D. Wang, H. Zhou, C. Xu, X. Hu, Z. Qian, X. Xu, Serum Neuropeptide Y Level is Associated with Post-Ischemic Stroke Epilepsy, *Journal of stroke and cerebrovascular diseases : the official journal of National Stroke Association*, 30

(2021) 105475.

- [9] C. Wahlestedt, D.J. Reis, Neuropeptide Y-related peptides and their receptors--are the receptors potential therapeutic drug targets?, *Annual review of pharmacology and toxicology*, 33 (1993) 309-352.
- [10] S.W. Lee, B.G. Stanley, NMDA receptors mediate feeding elicited by neuropeptide Y in the lateral and perifornical hypothalamus, *Brain Res*, 1063 (2005) 1-8.
- [11] W. Xie, F. Li, Y. Han, Z. Li, J. Xiao, Neuropeptides are associated with pain threshold and bone microstructure in ovariectomized rats, *Neuropeptides*, 81 (2020) 101995.
- [12] F. Coluzzi, M.S. Scerpa, M. Centanni, The Effect of Opiates on Bone Formation and Bone Healing, *Current osteoporosis reports*, 18 (2020) 325-335.
- [13] N.C. Laddha, M. Dwivedi, M.S. Mansuri, M. Singh, H.H. Patel, N. Agarwal, A.M. Shah, R. Begum, Association of neuropeptide Y (NPY), interleukin-1B (IL1B) genetic variants and correlation of IL1B transcript levels with vitiligo susceptibility, *PLoS One*, 9 (2014) e107020.
- [14] S. Grässel, D. Muschter, Do Neuroendocrine Peptides and Their Receptors Qualify as Novel Therapeutic Targets in Osteoarthritis?, *International journal of molecular sciences*, 19 (2018).
- [15] M.H. Park, I.K. Jung, W.K. Min, J.H. Choi, G.M. Kim, H.K. Jin, J.S. Bae, Neuropeptide Y improves cisplatin-induced bone marrow dysfunction without blocking chemotherapeutic efficacy in a cancer mouse model, *BMB reports*, 50 (2017) 417-422.
- [16] S. Fujiwara, M. Hoshizaki, Y. Ichida, D. Lex, E. Kuroda, K.J. Ishii, S. Magi, M. Okada, H. Takao, M. Gandou, H. Imai, R. Hara, H. Herzog, A. Yoshimura, H. Okamura, J.M. Penninger, A.S. Slutsky, S. Uhlig, K. Kuba, Y. Imai, Pulmonary phagocyte-derived NPY controls the pathology of severe influenza virus infection, *Nat Microbiol*, 4 (2019) 258-268.
- [17] A. Litwic, M.H. Edwards, E.M. Dennison, C. Cooper, Epidemiology and burden

of osteoarthritis, *British medical bulletin*, 105 (2013) 185-199.

[18] T.W. O'Neill, P.S. McCabe, J. McBeth, Update on the epidemiology, risk factors and disease outcomes of osteoarthritis, *Best practice & research. Clinical rheumatology*, 32 (2018) 312-326.

[19] M. Cross, E. Smith, D. Hoy, S. Nolte, I. Ackerman, M. Fransen, L. Bridgett, S. Williams, F. Guillemin, C.L. Hill, L.L. Laslett, G. Jones, F. Cicuttini, R. Osborne, T. Vos, R. Buchbinder, A. Woolf, L. March, The global burden of hip and knee osteoarthritis: estimates from the global burden of disease 2010 study, *Ann Rheum Dis*, 73 (2014) 1323-1330.

[20] C. Palazzo, C. Nguyen, M.M. Lefevre-Colau, F. Rannou, S. Poiraudau, Risk factors and burden of osteoarthritis, *Annals of physical and rehabilitation medicine*, 59 (2016) 134-138.

[21] A. Eitner, J. Pester, S. Nietzsche, G.O. Hofmann, H.G. Schaible, The innervation of synovium of human osteoarthritic joints in comparison with normal rat and sheep synovium, *Osteoarthritis and cartilage*, 21 (2013) 1383-1391.

[22] D.E. Garland, L. Toder, Fractures of the tibial diaphysis in adults with head injuries, *Clinical orthopaedics and related research*, (1980) 198-202.

[23] R. Perkins, A.P. Skirving, Callus formation and the rate of healing of femoral fractures in patients with head injuries, *The Journal of bone and joint surgery. British volume*, 69 (1987) 521-524.

[24] R.J. Newman, M.H. Stone, S.K. Mukherjee, Accelerated fracture union in association with severe head injury, *Injury*, 18 (1987) 241-246.

[25] P.V. Giannoudis, S. Mushtaq, P. Harwood, S. Kambhampati, M. Dimoutsos, Z. Stavrou, H.C. Pape, Accelerated bone healing and excessive callus formation in patients with femoral fracture and head injury, *Injury*, 37 Suppl 3 (2006) S18-24.

[26] W. Huang, Z. Li, Z. Li, R. Yang, Does traumatic brain injury result in accelerated mandibular fracture healing?, *J Oral Maxillofac Surg*, 70 (2012) 2135-2142.

[27] J. Morley, S. Marsh, E. Drakoulakis, H.C. Pape, P.V. Giannoudis, Does traumatic brain injury result in accelerated fracture healing?, *Injury*, 36 (2005) 363-368.

- [28] T.K. McIntosh, D. Ferriero, Changes in neuropeptide Y after experimental traumatic brain injury in the rat, *J Cereb Blood Flow Metab*, 12 (1992) 697-702.
- [29] K. Morioka, Y. Marmor, J.A. Sacramento, A. Lin, T. Shao, K.R. Mclau, D.R. Clark, M.S. Beattie, R.S. Marcucio, T. Mclau, 3rd, A.R. Ferguson, J.C. Bresnahan, C.S. Bahney, Differential fracture response to traumatic brain injury suggests dominance of neuroinflammatory response in polytrauma, *Sci Rep*, 9 (2019) 12199.
- [30] M. Arık, Y. Ekinici, K. Gürbüz, S. Batın, The effects of focal brain damage on fracture healing: An experimental rat study, *Eklemler hastalıkları ve cerrahisi = Joint diseases & related surgery*, 30 (2019) 267-274.
- [31] C. Rodriguez, J.C. Mayo, R.M. Sainz, I. Antolín, F. Herrera, V. Martín, R.J. Reiter, Regulation of antioxidant enzymes: a significant role for melatonin, *Journal of pineal research*, 36 (2004) 1-9.
- [32] D. Bonnefont-Rousselot, F. Collin, D. Jore, M. Gardès-Albert, Reaction mechanism of melatonin oxidation by reactive oxygen species in vitro, *Journal of pineal research*, 50 (2011) 328-335.
- [33] N. Harvey, E. Dennison, C. Cooper, Osteoporosis: impact on health and economics, *Nature reviews. Rheumatology*, 6 (2010) 99-105.
- [34] J.M. Lane, L. Russell, S.N. Khan, Osteoporosis, *Clinical orthopaedics and related research*, (2000) 139-150.
- [35] P.N. Sambrook, Osteoporosis, *The Medical journal of Australia*, 165 (1996) 332-336.
- [36] M. Srivastava, C. Deal, Osteoporosis in elderly: prevention and treatment, *Clinics in geriatric medicine*, 18 (2002) 529-555.
- [37] J. Kaludjerovic, W.E. Ward, Bone-specific gene expression patterns and whole bone tissue of female mice are programmed by early life exposure to soy isoflavones and folic acid, *The Journal of nutritional biochemistry*, 26 (2015) 1068-1076.
- [38] S.J. Allison, P.A. Baldock, H. Herzog, The control of bone remodeling by neuropeptide Y receptors, *Peptides*, 28 (2007) 320-325.
- [39] N.J. Lee, K.L. Doyle, A. Sainsbury, R.F. Enriquez, Y.J. Hort, S.J. Riepler, P.A.

- Baldock, H. Herzog, Critical role for Y1 receptors in mesenchymal progenitor cell differentiation and osteoblast activity, *J Bone Miner Res*, 25 (2010) 1736-1747.
- [40] P. Vestergaard, P. Hermann, J.E. Jensen, P. Eiken, L. Mosekilde, Effects of paracetamol, non-steroidal anti-inflammatory drugs, acetylsalicylic acid, and opioids on bone mineral density and risk of fracture: results of the Danish Osteoporosis Prevention Study (DOPS), *Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA*, 23 (2012) 1255-1265.
- [41] R.B. Waade, E. Molden, M.I. Martinsen, M. Hermann, A.H. Ranhoff, Psychotropics and weak opioid analgesics in plasma samples of older hip fracture patients - detection frequencies and consistency with drug records, *British journal of clinical pharmacology*, 83 (2017) 1397-1404.
- [42] T. Buchheit, R. Zura, Z. Wang, S. Mehta, G.J. Della Rocca, R.G. Steen, Opioid exposure is associated with nonunion risk in a traumatically injured population: An inception cohort study, *Injury*, 49 (2018) 1266-1271.
- [43] C. Mattia, E. Di Bussolo, F. Coluzzi, Non-analgesic effects of opioids: the interaction of opioids with bone and joints, *Current pharmaceutical design*, 18 (2012) 6005-6009.
- [44] P.A. Baldock, F. Driessler, S. Lin, I.P. Wong, Y. Shi, E. Yulyaningsih, L. Castillo, S. Janmaat, R.F. Enriquez, A. Zengin, B.L. Kieffer, C. Schwarzer, J.A. Eisman, A. Sainsbury, H. Herzog, The endogenous opioid dynorphin is required for normal bone homeostasis in mice, *Neuropeptides*, 46 (2012) 383-394.
- [45] F. Aerts-Kaya, B. Ullum, A. Mammadova, S. Köse, G. Aydin, P. Korkusuz, D. Uçkan-Çetinkaya, Neurological Regulation of the Bone Marrow Niche, *Advances in experimental medicine and biology*, 1212 (2020) 127-153.
- [46] M. Moradi, A.H. Doustimotlagh, A.R. Dehpour, N. Rahimi, A. Golestani, The influence of TRAIL, adiponectin and sclerostin alterations on bone loss in BDL-induced cirrhotic rats and the effect of opioid system blockade, *Life Sci*, 233 (2019) 116706.

- [47] C.M. Francucci, E. Ghigo, M. Boscaro, Endocrinopathy-induced osteoporosis. Impact on health and economics of osteoporosis, *J Endocrinol Invest*, 33 (2010) 2-3.
- [48] Y. Israel, Y. Kandov, E. Khaimova, A. Kest, S.R. Lewis, G.W. Pasternak, Y.X. Pan, G.C. Rossi, R.J. Bodnar, NPY-induced feeding: pharmacological characterization using selective opioid antagonists and antisense probes in rats, *Peptides*, 26 (2005) 1167-1175.
- [49] H. Higuchi, [Neuropeptide Y (NPY)], *Nihon Yakurigaku Zasshi*, 132 (2008) 310-312.
- [50] H. Higuchi, T. Niki, T. Shiiya, Feeding behavior and gene expression of appetite-related neuropeptides in mice lacking for neuropeptide Y Y5 receptor subclass, *World journal of gastroenterology*, 14 (2008) 6312-6317.
- [51] J.C. Erickson, K.E. Clegg, R.D. Palmiter, Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y, *Nature*, 381 (1996) 415-421.
- [52] A. Kanatani, S. Mashiko, N. Murai, N. Sugimoto, J. Ito, T. Fukuroda, T. Fukami, N. Morin, D.J. MacNeil, L.H. Van der Ploeg, Y. Saga, S. Nishimura, M. Ihara, Role of the Y1 receptor in the regulation of neuropeptide Y-mediated feeding: comparison of wild-type, Y1 receptor-deficient, and Y5 receptor-deficient mice, *Endocrinology*, 141 (2000) 1011-1016.
- [53] S. Lin, Y.C. Shi, E. Yulyaningsih, A. Aljanova, L. Zhang, L. Macia, A.D. Nguyen, E.J. Lin, M.J. During, H. Herzog, A. Sainsbury, Critical role of arcuate Y4 receptors and the melanocortin system in pancreatic polypeptide-induced reduction in food intake in mice, *PLoS One*, 4 (2009) e8488.
- [54] H. Higuchi, Molecular analysis of central feeding regulation by neuropeptide Y (NPY) neurons with NPY receptor small interfering RNAs (siRNAs), *Neurochem Int*, 61 (2012) 936-941.
- [55] P.A. Baldock, S.J. Allison, P. Lundberg, N.J. Lee, K. Slack, E.J. Lin, R.F. Enriquez, M.M. McDonald, L. Zhang, M.J. During, D.G. Little, J.A. Eisman, E.M. Gardiner, E. Yulyaningsih, S. Lin, A. Sainsbury, H. Herzog, Novel role of Y1 receptors in the coordinated regulation of bone and energy homeostasis, *J Biol Chem*,

282 (2007) 19092-19102.

[56] A.E. Peaire, A. Krantis, W.A. Staines, Distribution of the NPY receptor subtype Y1 within human colon: evidence for NPY targeting a subpopulation of nitrergic neurons, *J Auton Nerv Syst*, 67 (1997) 168-175.

[57] M. Anitha, B. Chandrasekharan, J.R. Salgado, E. Grouzmann, S. Mwangi, S.V. Sitaraman, S. Srinivasan, Glial-derived neurotrophic factor modulates enteric neuronal survival and proliferation through neuropeptide Y, *Gastroenterology*, 131 (2006) 1164-1178.

[58] Y.C. Shi, P.A. Baldock, Central and peripheral mechanisms of the NPY system in the regulation of bone and adipose tissue, *Bone*, 50 (2012) 430-436.

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1. Neuropeptide Y upregulates Runx2 and osterix and enhances osteogenesis in mouse MC3T3-E1 cells via an autocrine mechanism. Bo Zhang, Xiaolei Zhang, Juan Xiao, Xuguang Zhou, Yuan Chen and Chunzheng Gao. Molecular Medicine Reports. 2020; 22: 4376-4382. (SCI, IF:2.154, 第一作者)
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学位论文评阅及答辩情况表

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英文文章一

Neuropeptide Y upregulates Runx2 and osterix and enhances osteogenesis in mouse MC3T3-E1 cells via an autocrine mechanism

Abstract

The neuropeptide Y (NPY) system is considered one of the primary neural signaling pathways. NPY, produced by osteoblasts and other peripheral tissues, is known to inhibit biological functions of osteoblasts. However, until recently, little was known of the autocrine mechanism by which NPY is regulated. To investigate this mechanism, overexpression plasmids and small interfering RNA (siRNA) targeting NPY were transfected into the MC3T3-E1 cell line to observe its effects on osteogenesis. NPY overexpression was found to markedly enhance the osteogenic ability of MC3T3-E1 cells by an autocrine mechanism, coincident with the upregulation of osterix and runt-related transcription factor 2 (Runx2). Furthermore, NPY increased the activities of alkaline phosphatase (ALP) and osteocalcin (OCN) by upregulating their osteoblastic expression *in vitro* (as well as that of osterix and Runx2). Following transfection with NPY-siRNA, the osteoblastic ability of MC3T3-E1 cells was markedly decreased, and NPY deficiency inhibited the protein expression of osterix, Runx2, OCN and ALP in primary osteoblasts. Collectively, these results indicated that NPY played an important role in osteoblast differentiation by regulating the osterix and Runx2 pathways.

Key words

neuropeptide Y, MC3T3-E1 cell line, runt-related transcription factor 2, osterix, small interfering RNA

Introduction

Severe trauma, tumor resection, cancer or congenital diseases can result in large segmental bone defects [1]. Synthetic bone graft substitutes combined with bioactive

molecules, such as growth factors, peptides and small molecules, can improve the delivery of bone precursor cells, as well as subsequent bone formation and metabolism [2]. Bioactive molecules are required in patients with larger bone defects (>2 cm) [2]. For this purpose, growth factors, peptides and small molecules are currently being evaluated at the pre-clinical and clinical levels [2]. Based on previous findings, combining Neuropeptide Y (NPY) with the appropriate corresponding bone scaffold may bring about effective clinical translations and novel insights into the future of bone transplantation.

NPY, a 36-amino acid peptide, is a neurotransmitter located in the brain and autonomic nervous system [3]. As a typical regulator of appetite and energy balance, NPY serves an important role in the central nervous system [4], as well as a protective role in chronic bone loss-induced stress [5]. NPY, which is stored and released together with noradrenaline in the process of nerve stimulation, is part of the sympathetic arm of the peripheral nervous system [4]. NPY has been identified in several cell types, including osteoblasts and adipocytes [6-8], and has previously been demonstrated to regulate the balance of bone mass in both the central and peripheral regions [9, 10].

NPY receptors are members of the G protein-coupled receptor superfamily, and five subtypes (Y1, 2, 4, 5 and 6) are currently acknowledged in humans [11]. However, in rodents, bone mass is regulated by the Y1 and Y2 receptors only [12]. In previous studies, inhibiting the Y1 receptor increased the mRNA expression levels of osterix and runt-related transcription factor 2 (Runx2), two transcription factors essential for osteoblastic differentiation and bone formation [13]. Y1 inhibition can also promote the activity of Runx2 and osterix, and thus may contribute to osteoblastic differentiation and the expression of osteocalcin (OCN), alkaline phosphatase (ALP), bone sialoprotein (BSP) and collagen 1 α [14]. However, whether NPY itself is able to modulate osteoblasts via Runx2, osterix and bone morphogenetic protein (BMP) signaling remains to be elucidated.

Igwe et al [15] revealed that NPY is expressed in osteoblasts and serves an

important role in osteogenic differentiation. They also identified higher NPY mRNA expression in bone fractions highly abundant in mature osteoblasts and osteocytes. In a previous study, treatment of osteoblasts with NPY decreased the expression of markers of osteoblast differentiation [9]. Collectively, the aforementioned studies focused on cellular proliferation and osteogenesis as a result of the exogenous administration of NPY. However, little is known of the effects of NPY alterations at the genetic level. In the present study, the aim of the study was to investigate the effects of regulating NPY by an autocrine mechanism (coincident with the regulation of Runx2 and osterix) using the MC3T3-E1 cell line.

In a previous report, non-tumorous MC3T3-E1 cells were extracted from the skull and underwent a typical osteoblastic differentiation process *in vitro* [16]. In the present study, experiments were conducted by transfecting MC3T3-E1 cells with NPY small interfering RNA (siRNA) or overexpression plasmids to alter its expression level; this method demonstrated the autocrine, rather than the exocrine mechanism by which NPY regulates osteoblastic function. The osteoblastic ability of MC3T3-E1 cells was significantly enhanced following NPY overexpression, resulting in an increase in the expression of ALP, OCN, Runx2 and osterix mRNA levels at different time points, compared with those in untreated cells. In conclusion, this study demonstrated that NPY signaling serves a positive role in osteogenic differentiation of MC3T3-E1 cells by upregulating Runx2 and osterix *in vitro*. This will help to solve the clinical problem of large bone defects caused by severe trauma, tumor resection, cancer or congenital diseases in the future.

Materials and methods:

Cell isolation, culture and treatment. The MC3T3-E1 cell line was purchased from the Cell Bank of Union Medical University (<http://sbm.pumc.edu.cn/>). MC3T3-E1 cells were cultured in α -minimum essential medium (MEM with 10% Fetal Bovine Serum, both Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10 mmol β -glycerophosphate, 50 μ g/ml ascorbic acid and 10 mmol dexamethasone, and maintained at 37°C in 5% CO₂. The culture medium was renewed every 2-3 days.

Expression plasmids and transfection. MC3T3-E1 cells (2.0×10^6 cells/well) were seeded into 6-well plates (2 ml/well) and cultured at 37°C (5% CO₂) until 70-80% confluence. In order to study the subsequent biological effects of NPY on MC3T3-E1 cells, three different groups of siRNA (final concentration: 10 nmol/l) and an NPY overexpression plasmid (final concentration: 25 nmol/l) were designed by Wuhan Qingke Innovation Biotech Co., Ltd, along with a scrambled siRNA group (Wuhan Qingke Innovation Biotech Co., Ltd) and an empty vector group (Wuhan Qingke Innovation Biotech Co., Ltd), which acted as the negative controls. The three NPY siRNA target sequences (siRNA1, siRNA2 and siRNA3) are displayed in Table I. Then, untransfected cells were used as the control group for subsequent experiments. The cells were transfected with NPY siRNA and overexpression plasmids; NPY siRNA transfection was conducted for 48h using RFect siRNA transfection reagent (Changzhou Baidai Biotechnology Co., Ltd.), and the NPY overexpression plasmid was transfected using RFect DNA transfection reagent (Changzhou Baidai Biotechnology Co., Ltd), also for 48 h. Material synthesis and reagent preparation used in RFect siRNA and plasmid DNA transfection were performed according to the instructions of the transfection kits. Statistical analysis results showed that NPY siRNA3 had the highest interference efficiency, and as a result, siRNA3 was used for subsequent experimentation. The cells were then cultured for 7 days in the same conditions as aforementioned.

Reverse transcription-quantitative (RT-q)PCR analysis. At 4 and 7 days post-transfection, MC3T3-E1 cells were collected and gene expression was detected by RT-qPCR. Total RNA was isolated using TRIzol[®] reagent (Tiangen Biotech Co., Ltd.) and quantified using the NanoDrop™ 2000 (Thermo Fisher Scientific, Inc.). The RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol; the primer sequences for NPY, Runx2, OCN, ALP, osterix and GADPH (Wuhan Qingke Innovation Biotechnology Co., Ltd.) are displayed in Table I. The FastStart Universal SYBR-Green Master (Rox) was then used to perform qPCR with

the LightCycler® 480 Instrument II system (both Roche Diagnostics), under the following thermocycling conditions: 95°C for 5 min, 44 cycles at 95°C for 15 sec, and then 72°C for 20 sec. Melting curve analysis was used to confirm the specificity of transcription amplification. Fold changes in target gene expression were quantified using the $2^{-\Delta\Delta C_q}$ method [17] after normalization to the internal reference gene (GAPDH).

Western blot analysis. The cells were harvested and lysed with RIPA lysis buffer containing phenylmethane sulfonyl fluoride and protease inhibitors (both Sigma-Aldrich; Merck KGaA). The concentration of each sample was determined by a bicinchoninic acid protein assay. Proteins from each group (30 µg/lane) were resolved via SDS-PAGE on a 10% gel, and subsequently transferred to PVDF membranes. After blocking with 5% BSA (Sigma-Aldrich; Merck KGaA) at room temperature for 1 h in a phosphate buffered solution (PBS with 0.1% Tween-20; pH 7.5) and washing in TBS with 0.05% Tween-20 (TBST), the membranes were incubated with anti-NPY (1:1,000; cat. no. DF6431; Affinity Biosciences), anti-OCN (1:500; cat. no. PAA471Mu01; Cloud-Clone Corp.), anti-ALP (1:1,500; cat. no. 11187-1-AP; ProteinTech Group, Inc.), anti-osterix (1:1,000; cat. no. bs-1110R; BIOSS) and anti-Runx2 (1:500; cat. no. PAB011Mu01; Cloud-Clone Corp.) antibodies overnight at 4°C. After rinsing with TBST, the membranes were all incubated with a secondary antibody (horseradish peroxidase-linked guinea pig anti-rabbit IgG polyclonal; 1:2,000; cat. no. SAA544Rb59; Cloud-Clone Corp.) at 4°C for 1 h, and then developed using a chemiluminescence detection system (Pierce; Thermo Fisher Scientific, Inc.). The data were analyzed using ImageJ software (Version 1.8.0; National Institutes of Health); the relative protein expression level was calculated as the grey value ratio of the target protein to GAPDH.

Statistical analysis. The experimental data are presented as the mean±SD of three independently performed experiments. One-way ANOVA was used to analyze the overall differences between multiple groups. The Scheffe (Fig. 1A and B) or least significant difference post hoc tests were used to evaluate the differences between

groups using SPSS 18.0 software (SPSS, Inc.); representative bar graphs were created in GraphPad Prism v.6.0 (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Screening of NPY-specific siRNA. Comparison of the control, siRNA1, siRNA2 and siRNA3 groups was conducted by one-way ANOVA followed by Scheffe post hoc test, and the differences among the groups were statistically significant ($P < 0.01$ or $P < 0.001$). NPY siRNA3 had the highest interference efficiency of $> 75\%$, and as a result, siRNA3 was used for subsequent experimentation (Fig. 1A).

Secretion and expression of NPY in MC3T3-E1 cells. First, NPY secretion from MC3T3-E1 cells was verified at 4 and 7 days; NPY was consistently expressed at all time points, which provided the basis for the following experiments (Fig. 1B). In addition, NPY mRNA was sustainably expressed in both the experimental and control groups (Fig. 1B). Furthermore, the overexpression plasmids significantly promoted mRNA expression in MC3T3-E1 cells, which was increased by ~ 3.9 -fold at day 4 and 4.3 -fold at day 7, in comparison with the empty vector group ($P < 0.001$; Fig. 1B).

In order to more fully understand the role of NPY, MC3T3-E1 cells were transfected with NPY siRNA and cultured for 48 h, and changes in NPY mRNA expression were observed over time. NPY mRNA expression was decreased by ~ 0.75 -fold at day 4 and ~ 0.55 -fold at day 7, compared with the control and scrambled siRNA groups; there was statistical significance at day 4 ($P < 0.05$; Fig. 1B) and no statistical significance at day 7. Conversely, semi-quantitative analysis revealed that NPY overexpression increased NPY protein expression by 1.2 -fold at day 4 and 1.5 -fold at day 7, compared with the control group ($P < 0.05$; Fig. 1C and D). NPY siRNA was then used to further elucidate the role of NPY in MC3T3-E1 cells. NPY protein expression was decreased by ~ 0.45 -fold and 0.35 -fold at days 4 and 7 post-transfection, respectively ($P < 0.05$; Fig. 1C and D).

NPY is critical for osteoblastic differentiation. The expression levels of bone-formation factors, such as ALP and OCN, are determined by the different stages

of bone differentiation [18]. In the present study, ALP and OCN mRNA was consistently expressed in the three siRNA groups at days 4 and 7 post-transfection (Fig. 2A and 2B). The effects of NPY overexpression were then investigated using the MC3T3-E1 cell line, and NPY overexpression was found to significantly increase ALP mRNA expression by ~3.7 and 4.9-fold at days 4 and 7, and OCN mRNA expression by ~4.0 and 4.3-fold at days 4 and 7, respectively, compared with the control group ($P<0.001$; Fig. 2A and B).

To further determine the effects of NPY on ALP and OCN mRNA, MC3T3-E1 cells were transfected with NPY siRNA for 48 h, and the effects were observed over a subsequent time period. At days 4 and 7, ALP mRNA was decreased by ~0.56-fold and 0.26-fold, and OCN mRNA was decreased by ~0.60-fold and 0.35-fold, respectively, compared with the control group. The differences were statistically significant at day 4 ($P<0.05$; Fig. 2A and B). The ALP mRNA was also compared between the siRNA and overexpression groups, and overexpression was found to increase these levels ~7.9-fold (compared with siRNA inhibition) at day 4 and ~6.5-fold at day 7, and OCN mRNA levels ~10.1-fold (compared with siRNA inhibition) at day 4 and ~6.7-fold at day 7, respectively ($P<0.001$; Fig. 2A and B).

Semi-quantitative analysis of protein expression revealed that OCN protein expression was increased by 1.4-fold at day 4, and 1.6-fold at day 7 following NPY overexpression, compared with the control group; ALP protein expression was increased by 1.6-fold at days 4 and 7 ($P<0.05$; Fig. 3A-C).

Following NPY siRNA interference, ALP and OCN protein expression was also assessed; the level of ALP at days 4 and 7 was decreased by ~0.60-fold, and the level of OCN protein expression was decreased by ~0.64-fold at day 4 and ~0.78-fold at day 7, compared with the control group ($P<0.05$; Fig. 3A-C).

NPY regulates Runx2 and osterix expression in MC3T3-E1 cells. To the best of our knowledge, Runx2 is an important early transcription factor involved in the expression of osteoblast-specific genes, and the differentiation of mesenchymal stem cells into osteoblasts [19]. In addition, Runx2 is hypothesized to serve a primary role

in the terminal differentiation of osteoblasts [20].

Therefore in the present study, the effects of NPY on Runx2 and osterix expression were determined using MC3T3-E1 cells. NPY overexpression was found to significantly increase Runx2 and osterix mRNA expression. Compared with the control group, Runx2 mRNA expression increased by ~5.4-fold at day 4, and ~6-fold at day 7. Additionally, osterix mRNA was increased by ~2.7-fold at day 4 and ~3-fold at day 7, compared with the control group. Of note, the level of Runx2 mRNA expression was twice that of osterix ($P < 0.001$; Fig. 2C and D).

To further understand the effects of NPY on Runx2 and osterix mRNA expression, MC3T3-E1 cells were transfected with NPY siRNA and the expression levels of Runx2 and osterix mRNA were determined at subsequent time points. At day 4, Runx2 mRNA expression was decreased by ~0.50-fold compared with the control group, and ~0.35-fold at day 7. Osterix mRNA expression decreased by ~0.80-fold at day 4 and 0.55-fold at day 7, compared with the control group. The differences in osterix mRNA expression were statistically significant at day 4 ($P < 0.001$; Fig. 2C and D).

Next, the levels of Runx2 and osterix mRNA expression were compared between the siRNA and overexpression groups. In the overexpression group, Runx2 mRNA expression was 10-fold higher than that of the siRNA group at days 4 and 7, and osterix mRNA expression was ~15-fold greater than that observed in the siRNA group at day 4, and ~8-fold higher at day 7 ($P < 0.001$; Fig. 2C and D).

Semi-quantitative analysis of Runx2 and osterix expression revealed that compared with the control group, NPY overexpression increased Runx2 protein expression by 1.6-fold at day 4, and 1.2-fold at day 7. Similarly, osterix protein expression was increased by 1.6-fold at day 4, and 2.1-fold at day 7, compared with the control group ($P < 0.05$; Fig. 3A-C).

In the siRNA group, Runx2 protein expression was decreased by ~0.40-fold and 0.55-fold at days 4 and 7, respectively, compared with the control group. There was no statistical difference in Runx2 mRNA expression in the siRNA group in Fig. 2A,

whereas the difference in Runx2 protein expression was statistically significant in Fig. 3A-C ($P<0.05$). Likewise, osterix protein expression was decreased by ~ 0.60 -fold and 0.55 -fold at days 4 and 7, respectively, compared with the control group ($P<0.05$; Fig. 3A-C).

In conclusion, these findings indicated that NPY may be a positive regulator of osteogenic differentiation in mouse MC3T3-E1 cells.

Discussion:

NPY is a polypeptide of 36 amino acids, belonging to a larger family of neuropeptides that also includes the YY peptide and the pancreatic polypeptide [21, 22]. Baldock et al [23] found that Y1 or Y2 receptor knockout in mice resulted in increased trabecular and cortical bone mass. However, within the bone microenvironment, the regulatory mechanisms of NPY at the genetic level remain unclear. As a result, the aim of the present study was to conduct an in-depth assessment of these hypotheses in the MC3T3-E1 cell line.

NPY is a neurotransmitter that has dual functions in the regulation of energy consumption and bone mass [11]. A previous study determined that SNPs within NPY and neuraminidase (NA) were closely associated with the susceptibility and prognosis of patients with cervical vertigo [19]. Therefore, NPY and NA could be used as diagnostic markers and therapeutic targets for cervical vertigo [19]. Igwe et al [15] revealed that mouse calvarial osteoblasts fostered in the presence of NPY decreased the expression of differentiation-related markers, including OCN, BSP and dentin matrix acidic phosphoprotein 1 (DMP1), and also reduced the extent of mineralization. Their findings indicated that the levels of intracellular cAMP and differentiation markers (OCN, BSP and DMP1) in mouse calvarial osteoblasts were reduced by exogenous NPY treatment [15]. In the present study, NPY was overexpressed in MC3T3-E1 cells to observe its effects on osteogenic ability. Overexpressing NPY was found to markedly enhance the osteogenic ability of mouse MC3T3-E1 cells via an autocrine mechanism, coincident with the upregulation of Runx2 and osterix. In addition, the mRNA expression levels of ALP, OCN, osterix and Runx2 were

increased at different time points. In contrast to Igwe et al [15], the present study reported that endogenous NPY enhanced the expression of MC3T3-E1 cell differentiation markers, including ALP, OCN, Runx2 and osterix.

BMP signaling stimulates osteoblast differentiation and osteogenic activity by upregulating osterix and Runx2, which are essential osteogenic transcription factors [20, 24]. Runx2 is an important factor for bone growth, and the heterozygous loss of Runx2 results in clavicular dysplasia in humans, and the corresponding phenotype in mice [25]. It has also been confirmed that homozygous Runx2-II^{-/-} mice possess lower levels of bone mass and hypertrophic cartilage [25]. Runx2-I is necessary for early bone growth and intramembranous ossification, whereas Runx2-II is needed for the complete maturation of osteoblasts and osteogenesis in cartilage [26]. Osterix, a downstream effector of Runx2 required for osteoblast differentiation, is significantly reduced in selective Runx2 II^{-/-} mice [27]. An associated decrease in the expression of bone ALP, OCN, osteopontin and matrix extracellular phosphoglycoprotein was also observed. However, the levels of these markers were normal or slightly decreased in heterozygous mutant mice [25]. Despite this, the relationship between Runx2 and osterix in osteogenic differentiation (in the MC3T3-E1 cell line specifically) remains to be elucidated. Nakashima et al [27] found that osterix was not expressed in Runx2-II^{-/-} mice. In the present study, it was hypothesized that NPY may regulate osteoblast differentiation via the upregulation of Runx2 and osterix, and the role of NPY in promoting the cell differentiation and maturation of MC3T3-E1 cells via the Runx2 and osterix pathways was further validated. In a previous study based on the findings of Xiao et al [26], Runx2 was revealed to be the upstream regulatory gene of osterix. Runx2-I is also involved in maintaining 50% of osterix gene expression in Runx2-II-deficient (Runx2-II^{-/-}) mice; thus, 50% of osterix mRNA may be regulated by upstream Runx2 mRNA [26]. In the present study (Fig. 2C and 2D), osterix gene expression was found to be consistent with that of Runx2, and the level of osterix gene expression was almost half that of Runx2. It was therefore hypothesized that osterix is a target gene of Runx2, by which it can be positively regulated.

The RFect siRNA and plasmid DNA transfection reagents were specifically designed by Changzhou Baidai Biotechnology Co., Ltd (Patent application number: 20100022618) for transfecting siRNA and DNA into eukaryotic cells; this is a novel method derived from animal-origin free lipid transfection reagents, and the technique can be used on a wide range of adherent cell lines. It has numerous useful features; for example, the efficiency of transfection is >90%, and the effect of gene knockdown is markedly apparent [28]. Furthermore, minimal cytotoxicity reduces non-specific effects and cellular stress [28, 29]. Low concentrations of siRNA and DNA can be used to obtain high levels of knockdown and overexpression, respectively [28, 29]. In the present study, the interference efficiency of NPY mRNA at day 4 reached ~75% and 80%, respectively. In addition, the overexpression of NPY mRNA was ~4- and 6-fold at days 4 and 7, respectively. However, it was also found that Runx2 gene and protein expression were not synchronized at the same time point. Following NPY knockdown, the expression of Runx2 mRNA only showed a slight decrease, whereas the alteration in Runx2 protein expression was statistically significant. One reason could be that the expression of Runx2 protein determined the behavior of the mouse MC3T3-E1 cells, whereas Runx2 mRNA detection had temporal and spatial specificity, which may mean that the expression of Runx2 mRNA and protein are not in sync at the same time point. This mechanism was also supported by previous articles [24, 25]. Nevertheless, there were numerous limitations of the present study. One limitation is that the osteogenic differentiation was performed with the use of a single cell line for only 7 days, which also increases the uncertainty underlying the function of NPY in Runx2. Adding extra cell lines and prolonging the experiment time would increase the accuracy and validity of the experiment. Furthermore, the mechanism of action of NPY on Runx2 is not clear, and further research is required in the future. Additional cell lines and more time points for osteogenic differentiation are needed in further experiments. However, overall, the RFect plasmid DNA and siRNA transfection technique, which was adopted in the present study, was found to be reliable for use with the MC3T3-E1 cell line.

Based on the results of previous studies [2, 30-32], combining bioactive molecules, such as growth factors, peptides and small molecules with corresponding bone scaffold may lead to effective clinical transformation and provide new insights into the future of bone transplantation. Notably, this study demonstrated that NPY signaling directly promotes osteogenic differentiation of MC3T3-E1 cells by upregulating Runx2 and osterix in vitro. Additionally, NPY serves a positive role in bone formation, suggesting that NPY may be a therapeutic target for large bone defects caused by severe trauma, tumor resection, cancer or congenital diseases in the future.

Figures

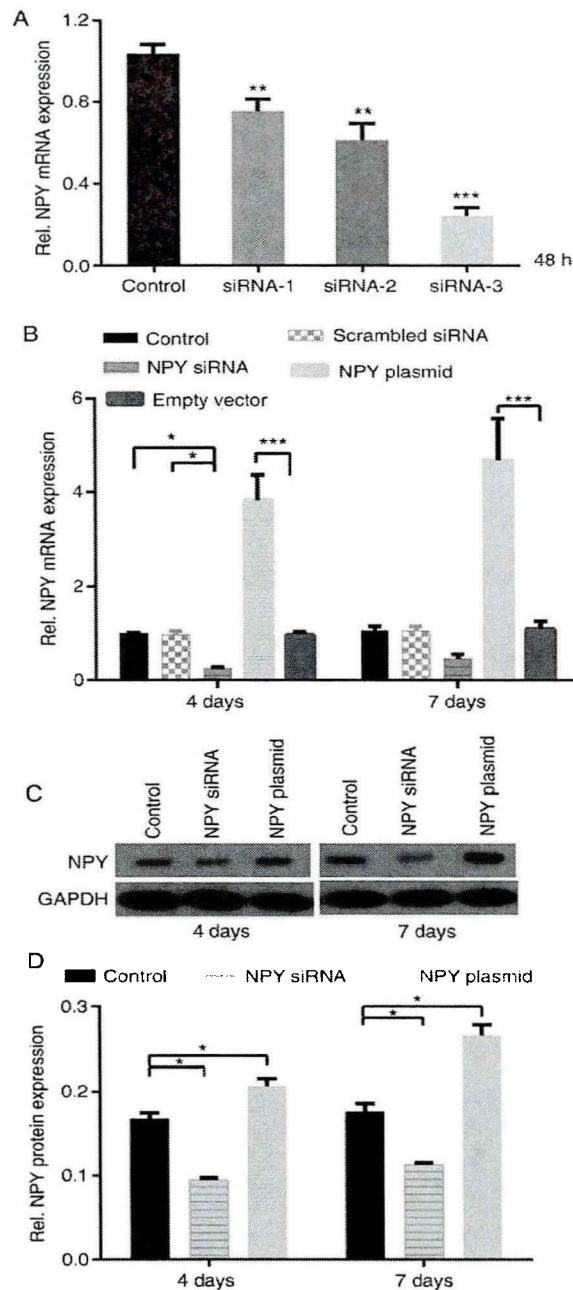


Figure 1. Expression of NPY mRNA and protein in MC3T3-E1 cells.

(A) Effects of siRNA inhibition on the expression of NPY mRNA at 48 h. ** $P < 0.01$, *** $P < 0.001$ vs. Control. NPY (B) mRNA expression at days 4 and 7. NPY protein expression at days 4 and 7 (C) evaluated by western blotting and (D) semi-quantified. * $P < 0.05$, *** $P < 0.001$.

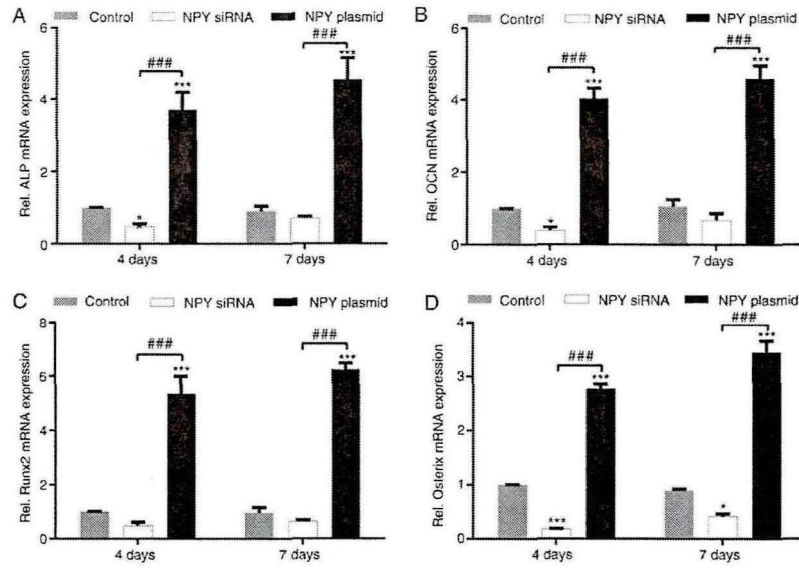


Figure 2. Expression of ALP, OCN, Runx2 and osterix mRNA in MC3T3-E1 cells.

Expression of (A) ALP, (B) OCN, (C) Runx2 and (D) osterix mRNA at days 4 and 7. * $P < 0.05$, *** $P < 0.001$ vs. control group; ### $P < 0.001$ vs. siRNA group.

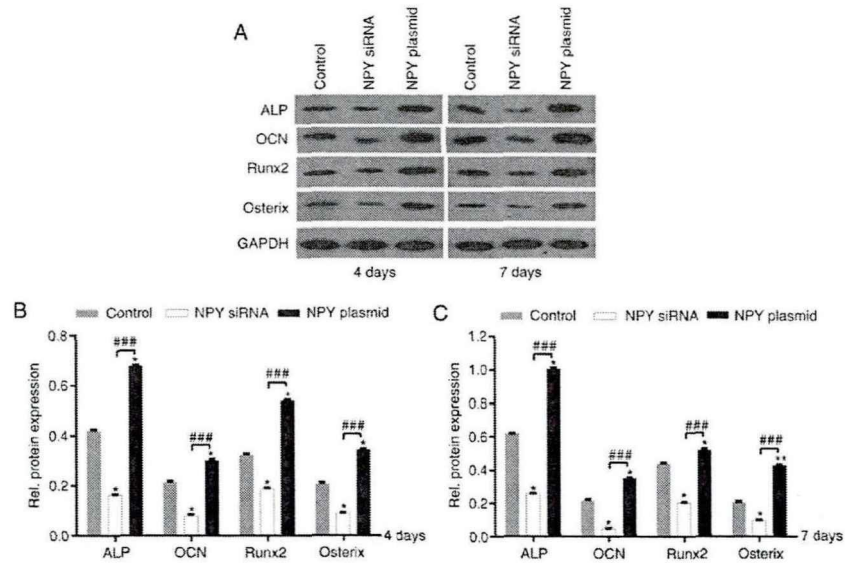


Figure 3. Expression of ALP, OCN, Runx2 and osterix protein in MC3T3-E1 cells.

(A) Western blotting analysis of ALP, OCN, Runx2 and osterix protein expression at days 4 and 7. Semi-quantification of protein expression at (B) day 4 and (C) day 7.

* $P < 0.05$, ** $P < 0.01$ vs. control group; ### $P < 0.001$ vs. siRNA group.

Tables

Table I. Primers used for reverse transcription-quantitative PCR.

Gene name	Primer sequences (5'→3')	GenBank Number
NPY	Sense: CTCGTGTGTTTGGGCATTC	NM_023456
	Antisense: TAGTGTCGCAGAGCGGAGTA	
siRNA1	Sense: CCAAACAAUAGACAUGCUUdTdT	NM_000909.6
	Antisense: AAGCAUGUCUAUUGUUUGGdTdT	
siRNA2	Sense: GCCCUUUGUGAUCUAUCAAdTdT	NM_000909.6
	Antisense: UUGAUAGAUCACAAAGGGCdTdT	
siRNA3	Sense: CCAAGCGAAUCAACAUCAUdTdT	NM_000909.6
	Antisense: AUGAUGUUGAUUCGCUUGGdTdT	
Runx2	Sense: GACGAGGCAAGAGTTTCACC	NM_009820
	Antisense: GGACCGTCCACTGTCACTTT	
OCN	Sense: CAAGCAGGGAGGCAATAAGG	NM_007541
	Antisense: CGTCACAAGCAGGGTTAAGC	
GAPDH	Sense: TCCACCACCCTGTTGCTGTA	NM_002046.3
	Antisense: ACCACAGTCCATGCCATCAC	
ALP	Sense: TGGTCACAGCAGTTGGTAGC	NM_001287172.1
	Antisense: CTGAGATTCGTCCCTCGCTG	
Osterix	Sense: GATGGCGTCCTCTCTGCTTG	NM_130458.3
	Antisense: TCTTTGTGCCTCCTTTCCCC	

References

- [1] F. Yang, J. Wang, J. Hou, H. Guo, C. Liu, Bone regeneration using cell-mediated responsive degradable PEG-based scaffolds incorporating with rhBMP-2, *Biomaterials*, 34 (2013) 1514-1528.
- [2] A. Ho-Shui-Ling, J. Bolander, L.E. Rustom, A.W. Johnson, F.P. Luyten, C. Picart, Bone regeneration strategies: Engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives, *Biomaterials*, 180 (2018) 143-162.
- [3] S.C. Baraban, Neuropeptide Y and limbic seizures, *Rev Neurosci*, 9 (1998) 117-128.
- [4] A.P. Silva, C. Cavadas, E. Grouzmann, Neuropeptide Y and its receptors as potential therapeutic drug targets, *Clin Chim Acta*, 326 (2002) 3-25.
- [5] P.A. Baldock, S. Lin, L. Zhang, T. Karl, Y. Shi, F. Driessler, A. Zengin, B. Horner, N.J. Lee, I.P. Wong, E.J. Lin, R.F. Enriquez, B. Stehrer, M.J. During, E. Yulyaningsih, S. Zolotukhin, S.T. Ruohonen, E. Savontaus, A. Sainsbury, H. Herzog, Neuropeptide y attenuates stress-induced bone loss through suppression of noradrenaline circuits, *J Bone Miner Res*, 29 (2014) 2238-2249.
- [6] L.E. Kuo, J.B. Kitlinska, J.U. Tilan, L. Li, S.B. Baker, M.D. Johnson, E.W. Lee, M.S. Burnett, S.T. Fricke, R. Kvetnansky, H. Herzog, Z. Zukowska, Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome, *Nat Med*, 13 (2007) 803-811.
- [7] P.A. Baldock, S.J. Allison, P. Lundberg, N.J. Lee, K. Slack, E.J. Lin, R.F. Enriquez, M.M. McDonald, L. Zhang, M.J. During, D.G. Little, J.A. Eisman, E.M. Gardiner, E. Yulyaningsih, S. Lin, A. Sainsbury, H. Herzog, Novel role of Y1 receptors in the coordinated regulation of bone and energy homeostasis, *J Biol Chem*, 282 (2007) 19092-19102.
- [8] K. Yang, H. Guan, E. Arany, D.J. Hill, X. Cao, Neuropeptide Y is produced in visceral adipose tissue and promotes proliferation of adipocyte precursor cells via the Y1 receptor, *FASEB J*, 22 (2008) 2452-2464.
- [9] E.C. Khor, P. Baldock, The NPY system and its neural and neuroendocrine

regulation of bone, *Curr Osteoporos Rep*, 10 (2012) 160-168.

[10] H. Horsnell, P.A. Baldock, Osteoblastic Actions of the Neuropeptide Y System to Regulate Bone and Energy Homeostasis, *Curr Osteoporos Rep*, 14 (2016) 26-31.

[11] S. Lin, D. Boey, H. Herzog, NPY and Y receptors: lessons from transgenic and knockout models, *Neuropeptides*, 38 (2004) 189-200.

[12] S.J. Allison, P.A. Baldock, H. Herzog, The control of bone remodeling by neuropeptide Y receptors, *Peptides*, 28 (2007) 320-325.

[13] M. Yahara, K. Tei, M. Tamura, Inhibition of neuropeptide Y Y1 receptor induces osteoblast differentiation in MC3T3E1 cells, *Mol Med Rep*, 16 (2017) 2779-2784.

[14] G. Karsenty, Transcriptional control of skeletogenesis, *Annu Rev Genomics Hum Genet*, 9 (2008) 183-196.

[15] J.C. Igwe, X. Jiang, F. Paic, L. Ma, D.J. Adams, P.A. Baldock, C.C. Pilbeam, I. Kalajzic, Neuropeptide Y is expressed by osteocytes and can inhibit osteoblastic activity, *J Cell Biochem*, 108 (2009) 621-630.

[16] H. Sudo, H.A. Kodama, Y. Amagai, S. Yamamoto, S. Kasai, In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria, *J Cell Biol*, 96 (1983) 191-198.

[17] K.J. Livak, T.D. Schmittgen, Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method, *Methods*, 25 (2001) 402-408.

[18] J. Chen, G. He, Y. Wang, D. Cai, MicroRNA-223 promotes osteoblast differentiation of MC3T3-E1 cells by targeting histone deacetylase 2, *International Journal of Molecular Medicine*, (2018).

[19] J. Han, J. Zuo, D. Zhu, C. Gao, The correlation between SNPs within the gene of adrenergic receptor and neuropeptide Y and risk of cervical vertigo, *J Clin Lab Anal*, 32 (2018) e22366.

[20] L. Li, M. Sapkota, M. Gao, H. Choi, Y. Soh, Macrolactin F inhibits RANKL-mediated osteoclastogenesis by suppressing Akt, MAPK and NFATc1 pathways and promotes osteoblastogenesis through a BMP-2/smad/Akt/Runx2 signaling pathway, *Eur J Pharmacol*, 815 (2017) 202-209.

- [21] K. Tatemoto, M. Carlquist, V. Mutt, Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide, *Nature*, 296 (1982) 659-660.
- [22] J.M. Allen, T.E. Adrian, K. Tatemoto, J.M. Polak, J. Hughes, S.R. Bloom, Two novel related peptides, neuropeptide Y (NPY) and peptide YY (PYY) inhibit the contraction of the electrically stimulated mouse vas deferens, *Neuropeptides*, 3 (1982) 71-77.
- [23] P.A. Baldock, A. Sainsbury, S. Allison, E.J. Lin, M. Couzens, D. Boey, R. Enriquez, M. During, H. Herzog, E.M. Gardiner, Hypothalamic control of bone formation: distinct actions of leptin and y2 receptor pathways, *J Bone Miner Res*, 20 (2005) 1851-1857.
- [24] J.S. Moon, S.H. Kim, S.H. Oh, Y.W. Jeong, J.H. Kang, J.C. Park, H.J. Son, S. Bae, B.I. Park, M.S. Kim, J.T. Koh, H.M. Ko, Relaxin augments BMP-2-induced osteoblast differentiation and bone formation, *J Bone Miner Res*, 29 (2014) 1586-1596.
- [25] J. Hecht, V. Seitz, M. Urban, F. Wagner, P.N. Robinson, A. Stiege, C. Dieterich, U. Kornak, U. Wilkening, N. Brieske, C. Zwingman, A. Kidess, S. Stricker, S. Mundlos, Detection of novel skeletogenesis target genes by comprehensive analysis of a Runx2(-/-) mouse model, *Gene Expr Patterns*, 7 (2007) 102-112.
- [26] Z.S. Xiao, A.B. Hjelmeland, L.D. Quarles, Selective deficiency of the "bone-related" Runx2-II unexpectedly preserves osteoblast-mediated skeletogenesis, *J Biol Chem*, 279 (2004) 20307-20313.
- [27] K. Nakashima, X. Zhou, G. Kunkel, Z. Zhang, J.M. Deng, R.R. Behringer, B. de Crombrughe, The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation, *Cell*, 108 (2002) 17-29.
- [28] X. Yang, Y. Yang, B.F. Sun, Y.S. Chen, J.W. Xu, W.Y. Lai, A. Li, X. Wang, D.P. Bhattarai, W. Xiao, H.Y. Sun, Q. Zhu, H.L. Ma, S. Adhikari, M. Sun, Y.J. Hao, B. Zhang, C.M. Huang, N. Huang, G.B. Jiang, Y.L. Zhao, H.L. Wang, Y.P. Sun, Y.G. Yang, 5-methylcytosine promotes mRNA export - NSUN2 as the methyltransferase

and ALYREF as an m(5)C reader, *Cell Res*, 27 (2017) 606-625.

[29] J. Liu, H. Fang, Z. Chi, Z. Wu, D. Wei, D. Mo, K. Niu, A.S. Balajee, T.K. Hei, L. Nie, Y. Zhao, XPD localizes in mitochondria and protects the mitochondrial genome from oxidative DNA damage, *Nucleic Acids Research*, 43 (2015) 5476-5488.

[30] C. Gao, S. Peng, P. Feng, C. Shuai, Bone biomaterials and interactions with stem cells, *Bone Res*, 5 (2017) 17059.

[31] C. Shuai, Y. Xu, P. Feng, G. Wang, S. Xiong, S. Peng, Antibacterial polymer scaffold based on mesoporous bioactive glass loaded with in situ grown silver, *Chemical Engineering Journal*, 374 (2019) 304-315.

[32] Y. Yang, J. Zan, W. Yang, F. Qi, C. He, S. Huang, S. Peng, C. Shuai, Metal organic frameworks as a compatible reinforcement in a biopolymer bone scaffold, *Materials Chemistry Frontiers*, 4 (2020) 973-984.

英文文章二

Effect and mechanism of neuropeptide Y on fracture healing of mice

Abstract

Neuropeptide Y (NPY) , a 36-amino acid peptide, has been identified in several cell types, including osteoblasts and adipocytes. NPY has previously been demonstrated to regulate the balance of bone mass in both the central and peripheral regions. In rodents, it has been shown that Y1 and Y2 receptors are involved in regulating the change of bone mass in mice only. However, it is yet to be fully elucidated how NPY and its Y1 receptor are involved in the regulation of bone fracture in mice, and whether they are regulated together with other genes, such as runt-related transcription factor 2 and osterix.

The present study aimed to investigate the effect and mechanism of NPY and neuropeptide Y1 receptor (NPY1R) genes on fracture healing of mice. The current findings will help to increase the understanding of the changes of NPY and NPY1R during the bone formation process in mice, and help to determine whether there was a synergistic effect with related osteogenic genes.

Key words

fracture, bone formation, neuropeptide Y, runt-related transcription factor 2, osterix

Introduction

Neuropeptide Y (NPY) is a neurotransmitter mainly distributed in the brain and gastrointestinal nervous system [1, 2]. NPY has been studied in osteoblasts, adipocytes and, preosteoblasts, amongst other cell types [3-6], and it has been shown to regulate bone balance in the central and peripheral regions [7, 8].

As early as 1992, McIntosh and Ferriero [9] used a model of fluid percussion brain injury in rats, and confirmed that NPY immunoreactive fibers could appear in the damaged cortex and adjacent hippocampus 15min after brain injury, and that the

concentration of NPY in cerebrospinal fluid was increased after trauma. In clinical practice, it is common that the fracture healing speed is significantly faster compared with that of simple fractures, and the related literature shows done extensive research on this area [10, 11]. It has been suggested that NPY may affect osteogenesis via the cerebrospinal fluid. Melatonin (MLT) is one of the hormones secreted by the pineal gland. The NPY/neuropeptide Y1 receptor (NPY1R) signaling pathway can be induced by a variety of factors, such as MLT, to induce the osteogenic differentiation of mesenchymal stem cells and promote the healing of femoral fracture in rats [12]. It has been reported that MLT promotes femoral fracture healing in rats, accompanied by increased expression of NPY and NPY1R, as well as enhanced expression of zinc uptake transporter zinc transporter 1 in nerve cells [13]. Therefore, it was suggested that NPY may not exert effects on fracture healing via the blood pathway, but via neural pathways, through the cerebrospinal fluid.

In a previous report, NPY signaling served a positive role in the osteogenic differentiation of MC3T3-E1 cells by upregulating runt-related transcription factor 2 (Runx2) and osterix expression in vitro [6]. The current report will further evaluate the correlation between NPY and its receptor and fracture in mice, as well as examine the biological effects of NPY and its receptor.

The aims of the present study were as follows: i) to investigate the serum expression level of NPY gene in a C57BL/6 mouse model of tibial closed fracture; ii) to detect the gene and protein expression levels of the osteogenic-related factors NPY, NPY1R, Runx2 and osterix at different time points of fracture healing in mice; and iii) to analyze the biological effect of NPY on fracture healing in mice. Moreover, this study aimed to determine the correlation between the osteogenesis mechanism and NPY and NPY1R genes in the tibial fractures of C57BL/6 mice. The finding could help to further understand the changes of NPY and NPY1R with bone fracture osteogenesis in mice and to determine whether there is a synergistic effect with related osteogenic genes. Thus, helping to solve the clinical problem of large bone defects caused by severe trauma, cancer, tumor resection or congenital diseases in the

future.

Materials and methods

Animals. C57BL/6 mice (age, 14 weeks; weight, 18-26g; average weight, 22.1 ± 3.8 g) were purchased from the Animal Experimental Center of Shandong University. All animal related experimental procedures were performed in accordance with an approved protocol of the Institutional Animal Care and Use Committee of the Second Hospital, Cheeloo College of Medicine, Shandong University [approval no. KYLL-2021(LW)019].

In total, 18 C57BL/6 male mice were selected as experimental subjects. Each mouse was placed into a container preloaded with 3% isoflurane and 60 ml/min oxygen for 5-10 min. After loss of postural reflexes, the mouse was removed from the anesthesia induction container and anesthesia was maintained via an inhalation mask providing 3% isoflurane and 60 ml/min oxygen. The breathing pattern and hind paw reflexes of mice were monitored during anesthesia, to ensure that the breathing rate was ~ 100 cycles/min and that the hind paw reflex was absent [14]. The unilateral tibial fracture was artificially broken without fixation. All animals were kept in a 12 h light/dark cycle in temperature ($22 \pm 2^\circ\text{C}$) and humidity ($55 \pm 5\%$) with standard rodent chow and water ad libitum, and these were used to establish a mouse model of a closed tibial fracture.

Serum analyses. The same dose of isoflurane for induction and anesthesia maintenance was applied as in the previous step. Blood samples were randomly collected from the left or right inner canthus of three mice, 0.1 ml each time at days 1, 7, and 14. Subsequently, the concentration of NPY in serum was detected via ELISA. The blood was used to clot at room temperature and serum were collected after centrifugation of blood samples at 5,000 rpm for 10 min in order to recover the serum. Serum aliquots were frozen before ELISA analysis for the NPY levels.

Reverse transcription-quantitative (RT-q)PCR analysis. The mice were euthanized under isoflurane anesthesia via cervical dislocation at predetermined time intervals (1, 7 and 14 days after fracture). On the 1st day the blood scab was extracted

from the fracture site of three mice. On the 7th day, the fibroblasts and chondrocytes were extracted from the callus from the fracture site of three mice. On the 14th day, the chondrocytes, osteoblasts, osteoclasts and the surrounding fibrous connective tissue cells were extracted from the callus from the fracture site of three mice. After these samples were collected, gene expression was detected via RT-qPCR. Total RNA was isolated using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified using the NanoDrop[™] 2000 system (Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA by the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The primer sequences for NPY, NPY1R, Runx2, osterix and GAPDH (Wuhan Qingke Innovation Biotechnology Co., Ltd.) are presented in Table I. FastStart Universal SYBR-Green Master (Rox) was then used to perform qPCR with the LightCycler[®] 480 Instrument II system (both Roche Diagnostics), under the following thermocycling conditions: Initial denaturation at 95°C for 5 min, followed by 44 cycles at 95°C for 15 sec, and 72°C for 20 sec. Melting curve analysis was allowed to confirm the specificity of transcription amplification. Fold changes in target gene expression were quantified using the $2^{-\Delta\Delta C_q}$ method [15] after normalization to the internal reference gene, GAPDH.

Immunohistochemistry analysis. The mice were killed as in the previous step at predetermined time intervals (1, 7 and 14 days after fracture). Fracture specimens of the tibia were stained and fixed with 70% ethanol. Next, NPY, NPY1R, Runx2 and osterix immunohistochemical staining was performed. The specific steps of the experiment were conducted as previously described [16].

The gray value of positive staining was measured automatically via the image analysis of the tissue measurement area using an AI image analysis system (Wuhan Servicebio Technology Co., Ltd.).

Statistical analysis. The experimental data are presented as the mean $\pm$ SD of three independently performed experiments. One-way ANOVA was used to analyze the overall differences between multiple groups, followed by least significant difference post hoc tests, which were used to evaluate the differences between groups. SPSS

18.0 software (SPSS, Inc.) was used for data analysis and representative bar graphs were created in GraphPad Prism v.6.0 (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Successful establishment of the closed tibial fracture model. Closed tibial fracture models were successfully constructed in 17/18 mouse models. All of the 17 mice could eat and drink water independently after the operation. The living state of the mice was observed, and it was found that after the operation the mice were safe and had no influences were observed in motor functions, sensory functions and social behaviors. On the 1st day after the operation, the mice did not move a great amount and may have shown a little pain, but it did not affect their eating and water intake. On the 2nd day after operation, the mice could move gradually. After 2 weeks, gradual weight bearing could be observed on the affected limbs, and sometimes the mice stood with both limbs at the same time.

Changing trend of serum NPY concentration in C57BL/6 mice after operation. Through the analysis of NPY concentration at different time points (1, 7 and 14 days after operation), it was found that the serum concentration of NPY was higher in the early stage of fracture. As the fracture healing time was increased, the concentration of NPY decreased temporarily at day 7, but then gradually increased at day 14 (Fig. 1). Moreover, there was no significant difference found in the ELISA results.

Expression levels of NPY genes and proteins during fracture healing in mice. The mRNA expression level of NPY was decreased by ~0.27-fold at day 7 and 0.09-fold at day 14, in comparison with day 1 ($P < 0.001$; Fig. 2A). Furthermore, the protein expression level of NPY was decreased by ~0.37-fold at day 7 and 0.55-fold at day 14, in comparison with day 1 ($P < 0.05$; Fig. 2B).

It has been suggested that the NPY gene may be highly expressed in inflammatory cells, pre-osteoblasts, vascular endothelial cells and adipocytes at the early stage of fracture [17, 18]. Immunohistochemistry showed positive staining for NPY on these cells at day 1 (Fig.). It was identified that, along with the proliferation of fibroblasts

and chondrocytes on the 7th day, the expression level of the NPY gene was decreased. Moreover, NPY gene expression in osteoblasts and osteoclasts on the surface of the new bone was gradually decreased 14 days after operation. These findings demonstrated the osteogenic mechanism after fracture and the mRNA expression level of NPY in different cell types.

Expression of NPY1R genes and proteins during fracture healing in mice. The mRNA expression level of NPY1R was decreased by ~ 0.36 -fold at day 7 and 0.55 -fold at day 14, in comparison with the day 1 ($P=0.059$; Fig. 2C). In addition, the protein expression level of NPY1R was decreased by ~ 0.08 -fold at day 7 and 0.21 -fold at day 14, in comparison with the day 1 ($P<0.001$; Fig. 2D).

It has been reported that the NPY1R gene is accumulated in the cytoplasm of mesenchymal stem cells and pre-osteoblasts at the early stage of inflammation [19]. The results demonstrated that, together the osteogenesis of cells, NPY1R expression was gradually increased on the surface of osteoblasts. The results revealed a trend involving an initial marked decrease, followed by a gradually increase, which was synchronized with the protein expression level of NPY. Thus, it was suggested that NPY may serve a role via cytoplasmic NPY1R.

Expression of the osteogenic specific transcription factor Runx2 gene and protein during fracture healing in mice. Runx2 is an important transcriptional regulator, which promotes mesenchymal cells transformation in pre-osteoblasts [20, 21]. The mRNA expression level of Runx2 was increased by ~ 7.88 -fold at day 7 and ~ 65.62 -fold at day 14, in comparison with day 1 ($P>0.05$; Fig. 3A). Moreover, the protein expression level of Runx2 was decreased by ~ 0.15 -fold at day 7 and 0.19 -fold at day 14, in comparison with day 1 ($P<0.001$; Fig. 3B).

It was found that, with the increase of Runx2 mRNA synthesis, the osteogenic ability of osteoblasts was gradually enhanced. Thus, the asynchronous expression level of Runx2 mRNA and protein maybe associated with regulatory mechanisms. It was suggested that the Runx2 and NPY genes may have a certain relationship in the regulation of osteogenesis, but this requires further investigation in follow-up studies.

Expression of the osteogenic specific transcription factor Osterix gene and protein during fracture healing in mice. The osterix gene is an osteogenic specific transcription factor [22-25]. The results demonstrated that the mRNA expression level of osterix was increased by ~123.98-fold at day 7 and ~775.06-fold at day 14 compared with day 1 ($P>0.05$; Fig. 3C). Furthermore, osterix protein expression was decreased by ~0.12-fold at day 7 and 0.10-fold at day 14, in comparison with day 1 ($P<0.001$; Fig. 3D).

It was identified that the expression trends of osterix and Runx2 at mRNA and protein levels were similar. This was a surprising finding, which indicated that osterix and Runx2 may be regulated by a common osteogenic transcription pathway or that there may be a potential relationship between them.

Discussion

NPY is a 36-amino acid peptide that belongs to a larger family of neuropeptides, including pancreatic peptides and YY peptides [26, 27]. NPY is a neurotransmitter with double functions in regulating bone mass and energy consumption [28]. Moreover, NPY is an important skeletal neurotrophic factor and serves a crucial role in promoting fracture healing, improving knee osteoarthritis, and treating osteoporotic brittle fractures [29, 30]. It has also been shown that NPY can promote bone metabolism via the direct and indirect activation of multiple pathways in osteoblasts, osteoblasts and osteoclasts [31]. Additionally, NPY enhances osteoblast mediated extracellular osteogenesis and inhibits osteoclast-mediated bone resorption and remodeling, which are key factors of cartilage repairing [32]. In the present study, NPY and NPY1R were highly expressed in inflammatory cells, bone marrow mesenchymal stem cells, preosteoblasts, vascular endothelial cells and adipocytes at the early stage of fracture in mice, suggesting that early NPY and NPY1R may have the ability to promote osteogenesis.

High expression levels of osterix and Runx2 in the bone morphogenetic protein signaling pathway can stimulate osteoblast differentiation and enhance osteogenic ability [33, 34]. The Runx2 gene is an important early osteogenic transcription factor,

which is involved in the differentiation of mesenchymal stem cells into osteoblasts and the expression levels of osteogenic specific genes [35-37]. In addition, the Runx2 gene is considered to serve a major role in the terminal differentiation of osteoblasts [38-41]. Nakashima et al [24] first discovered the osterix gene in 2002. Osterix, also known as Sp7, is a zinc finger transcription factor that is usually expressed in osteoblasts, and it regulates the differentiation and maturation of osteoblasts. Furthermore, the subcellular structure of osterix is mainly located in the nucleus [24]. However, recent study has shown that osterix gene was not only expressed in osteoblasts and chondrocytes, but was also upregulated in metastatic breast cancer cells and a variety of other cancer cells [42]. In the present study, Runx2 and osterix genes were highly expressed in various cell types, such as inflammatory cells, preosteoblasts, vascular endothelial cells and adipocytes during fracture healing in mice. However, it was also found that Runx2 gene and protein expression was not synchronized at the same time point. A possible reason could be that Runx2 mRNA expression detection sampling itself also has spatiotemporal specificity, and this mechanism also supported by previous articles [34, 43]. Whether the protein and mRNA expression levels of Runx2 are associated with additional regulatory mechanisms remains to be determined.

Under the initiative of animal protection organizations and animal welfare, the 4Rs (reduction, refinement, replacement and responsibility) advocated for animal experiments aim to reduce the number of experimental animals. Thus, only 18 mice were selected for these experiments, and, inevitably, there were individual differences in mice inevitably. Increasing the number of experiments may increase the accuracy and validity of the data.

The combination of bone scaffold with bioactive molecules, such as growth factors, small molecules and polypeptides, can provide novel ideas for the development of bone transplantation methods [44-47]. In previous studies, upregulation of Runx2 and osterix by NPY directly promoted the osteogenic differentiation of MC3T3-E1 cells in vitro [6]. Moreover, NPY in the periosteum and callus of fracture healing site was

significantly increased and could promote fracture healing [32]. In the current experiments, NPY and NPY1R genes were increased to promote fracture healing in C57BL/6 mice with early tibial fracture. It was identified that NPY could maintain bone homeostasis in vivo and promote fracture healing in mice. Furthermore, the mechanism of osteogenesis in mice was associated with osteogenic genes such as osterix and Runx2.

Figures

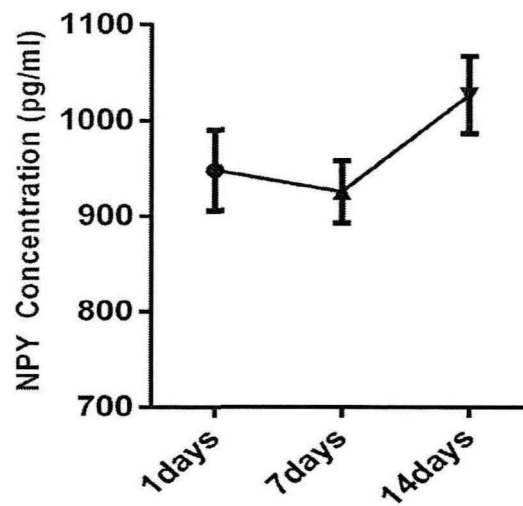


Figure 1. Serum NPY concentration in mouse fracture model.

There was no significant difference in serum NPY concentration in the mouse fracture model ($P>0.05$).

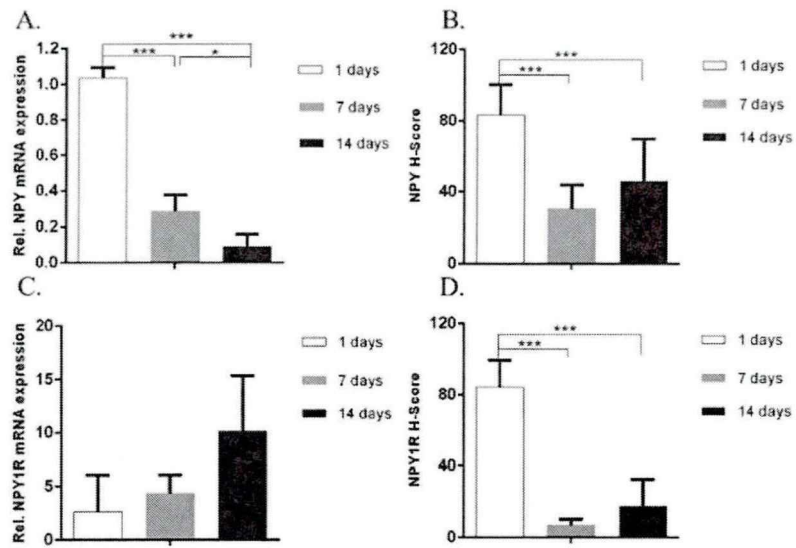


Figure 2. Expression of NPY and NPY1R mRNA and protein in the mouse fracture model

A. NPY mRNA expression at days 1, 7 and 14. B. NPY protein expression at days 1, 7 and 14. C. NPY1R mRNA expression at days 1, 7 and 14. D. NPY1R protein expression at days 1, 7 and 14. * $P<0.05$, *** $P<0.001$.

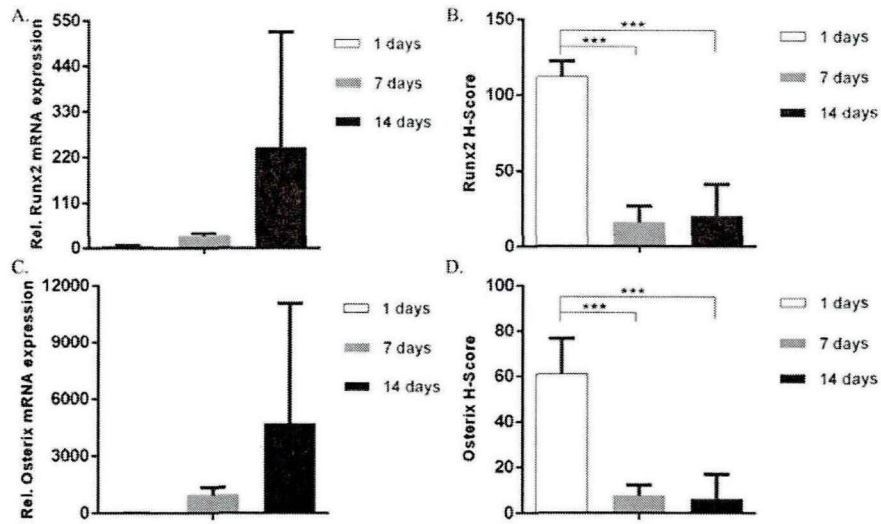


Figure 3. Expression of Runx2 and osterix mRNA and protein in the mouse fracture model

A. Runx2 mRNA expression at days 1, 7 and 14. B. Runx2 protein expression at days 1, 7 and 14. C. Osterix mRNA expression at days 1, 7 and 14. D. Osterix protein expression at days 1, 7 and 14. *** $P < 0.001$.

Table

Table 1. Primers used for reverse transcription-quantitative PCR.

Name	Sequence
NPY	5'-CTCGTGTGTTTGGGCATTC-3'
NM_023456	3'-TAGTGTCGCAGAGCGGAGTA-5'
NPY1R	5'-TATACCACTCTTCTCTTGGTGCTG-3'
NC_000004.12	3'-CTGGAAGTTTTTGTTCAGGAACCCA-5'
Runx2	5'-GACGAGGCAAGAGTTTCACC-3'
NM_009820	3'-GGACCGTCCACTGTCACTTT-5'
Osterix	5'-GATGGCGTCCTCTCTGCTTG-3'
NM_130458.3	3'-TCTTTGTGCCTCCTTTCCCC-5'
GAPDH	5'-TCCACCACCCTGTTGCTGTA-3'
NM_002046.3	3'-ACCACAGTCCATGCCATCAC-5'

References

- [1] S.C. Baraban, Neuropeptide Y and limbic seizures, *Rev Neurosci*, 9 (1998) 117-128.
- [2] J. Lay, S.E. Carbone, J.J. DiCello, N.W. Bunnett, M. Canals, D.P. Poole, Distribution and trafficking of the μ -opioid receptor in enteric neurons of the guinea pig, *American journal of physiology. Gastrointestinal and liver physiology*, 311 (2016) G252-266.
- [3] L.E. Kuo, J.B. Kitlinska, J.U. Tilan, L. Li, S.B. Baker, M.D. Johnson, E.W. Lee, M.S. Burnett, S.T. Fricke, R. Kvetnansky, H. Herzog, Z. Zukowska, Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome, *Nat Med*, 13 (2007) 803-811.
- [4] P.A. Baldock, S.J. Allison, P. Lundberg, N.J. Lee, K. Slack, E.J. Lin, R.F. Enriquez, M.M. McDonald, L. Zhang, M.J. During, D.G. Little, J.A. Eisman, E.M. Gardiner, E. Yulyaningsih, S. Lin, A. Sainsbury, H. Herzog, Novel role of Y1 receptors in the coordinated regulation of bone and energy homeostasis, *J Biol Chem*, 282 (2007) 19092-19102.
- [5] K. Yang, H. Guan, E. Arany, D.J. Hill, X. Cao, Neuropeptide Y is produced in visceral adipose tissue and promotes proliferation of adipocyte precursor cells via the Y1 receptor, *FASEB J*, 22 (2008) 2452-2464.
- [6] B. Zhang, X. Zhang, J. Xiao, X. Zhou, Y. Chen, C. Gao, Neuropeptide Y upregulates Runx2 and osterix and enhances osteogenesis in mouse MC3T3-E1 cells via an autocrine mechanism, *Molecular medicine reports*, 22 (2020) 4376-4382.
- [7] E.C. Khor, P. Baldock, The NPY system and its neural and neuroendocrine regulation of bone, *Curr Osteoporos Rep*, 10 (2012) 160-168.
- [8] H. Horsnell, P.A. Baldock, Osteoblastic Actions of the Neuropeptide Y System to Regulate Bone and Energy Homeostasis, *Curr Osteoporos Rep*, 14 (2016) 26-31.
- [9] T.K. McIntosh, D. Ferriero, Changes in neuropeptide Y after experimental traumatic brain injury in the rat, *J Cereb Blood Flow Metab*, 12 (1992) 697-702.
- [10] W. Huang, Z. Li, Z. Li, R. Yang, Does traumatic brain injury result in accelerated

- mandibular fracture healing?, *J Oral Maxillofac Surg*, 70 (2012) 2135-2142.
- [11] J. Morley, S. Marsh, E. Drakoulakis, H.C. Pape, P.V. Giannoudis, Does traumatic brain injury result in accelerated fracture healing?, *Injury*, 36 (2005) 363-368.
- [12] P. Dong, X. Gu, G. Zhu, M. Li, B. Ma, Y. Zi, Melatonin Induces Osteoblastic Differentiation of Mesenchymal Stem Cells and Promotes Fracture Healing in a Rat Model of Femoral Fracture via Neuropeptide Y/Neuropeptide Y Receptor Y1 Signaling, *Pharmacology*, 102 (2018) 272-280.
- [13] J. Shang, B. Ma, G. Zhu, P. Dong, C. Wang, X. Gu, Y. Zi, Role of Zip1 in the regulation of NPY expression by MLT to promote fracture healing in rats, *European journal of histochemistry : EJH*, 64 (2020).
- [14] M. Haffner-Luntzer, F. Müller-Graf, R. Matthys, A. Abaei, R. Jonas, F. Gebhard, V. Rasche, A. Ignatius, In Vivo Evaluation of Fracture Callus Development During Bone Healing in Mice Using an MRI-compatible Osteosynthesis Device for the Mouse Femur, *Journal of visualized experiments : JoVE*, (2017).
- [15] K.J. Livak, T.D. Schmittgen, Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method, *Methods*, 25 (2001) 402-408.
- [16] G.L. Brattbauer, The peroxidase-antiperoxidase (PAP) method, *Methods Mol Biol*, 34 (1994) 165-173.
- [17] M.E. Dombrowski, A.S. Olsen, N. Vaudreuil, B.K. Couch, Q. Dong, M. Tucci, J.Y. Lee, N.V. Vo, G. Sowa, Rabbit Annulus Fibrosus Cells Express Neuropeptide Y, Which Is Influenced by Mechanical and Inflammatory Stress, *Neurospine*, 17 (2020) 69-76.
- [18] Y.C. Shi, P.A. Baldock, Central and peripheral mechanisms of the NPY system in the regulation of bone and adipose tissue, *Bone*, 50 (2012) 430-436.
- [19] B.K. Taylor, W. Fu, K.E. Kuphal, C.O. Stiller, M.K. Winter, W. Chen, G.F. Corder, J.H. Urban, K.E. McCarson, J.C. Marvizon, Inflammation enhances Y1 receptor signaling, neuropeptide Y-mediated inhibition of hyperalgesia, and substance P release from primary afferent neurons, *Neuroscience*, 256 (2014) 178-194.
- [20] Z.S. Xiao, A.B. Hjelmeland, L.D. Quarles, Selective deficiency of the

"bone-related" Runx2-II unexpectedly preserves osteoblast-mediated skeletogenesis, *J Biol Chem*, 279 (2004) 20307-20313.

[21] G.S. Stein, J.B. Lian, A.J. van Wijnen, J.L. Stein, M. Montecino, A. Javed, S.K. Zaidi, D.W. Young, J.Y. Choi, S.M. Pockwinse, Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression, *Oncogene*, 23 (2004) 4315-4329.

[22] D. Fan, Z. Chen, D. Wang, Z. Guo, Q. Qiang, Y. Shang, Osterix is a key target for mechanical signals in human thoracic ligament flavum cells, *J Cell Physiol*, 211 (2007) 577-584.

[23] Z. Maruyama, T. Komori, [Function of Osterix in osteoblasts], *Nihon Rinsho*, 65 Suppl 9 (2007) 81-83.

[24] K. Nakashima, X. Zhou, G. Kunkel, Z. Zhang, J.M. Deng, R.R. Behringer, B. de Crombrughe, The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation, *Cell*, 108 (2002) 17-29.

[25] J. Caetano-Lopes, H. Canhão, J.E. Fonseca, Osteoblasts and bone formation, *Acta reumatologica portuguesa*, 32 (2007) 103-110.

[26] K. Tatemoto, M. Carlquist, V. Mutt, Neuropeptide Y--a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide, *Nature*, 296 (1982) 659-660.

[27] J.M. Allen, T.E. Adrian, K. Tatemoto, J.M. Polak, J. Hughes, S.R. Bloom, Two novel related peptides, neuropeptide Y (NPY) and peptide YY (PYY) inhibit the contraction of the electrically stimulated mouse vas deferens, *Neuropeptides*, 3 (1982) 71-77.

[28] S. Lin, D. Boey, H. Herzog, NPY and Y receptors: lessons from transgenic and knockout models, *Neuropeptides*, 38 (2004) 189-200.

[29] P.A. Baldock, N.J. Lee, F. Driessler, S. Lin, S. Allison, B. Stehrer, E.J. Lin, L. Zhang, R.F. Enriquez, I.P. Wong, M.M. McDonald, M. During, D.D. Pierroz, K. Slack, Y.C. Shi, E. Yulyaningsih, A. Aljanova, D.G. Little, S.L. Ferrari, A. Sainsbury, J.A. Eisman, H. Herzog, Neuropeptide Y knockout mice reveal a central role of NPY in

the coordination of bone mass to body weight, *PLoS One*, 4 (2009) e8415.

[30] I. Matic, B.G. Matthews, T. Kizivat, J.C. Igwe, I. Marijanovic, S.T. Ruohonen, E. Savontaus, D.J. Adams, I. Kalajzic, Bone-specific overexpression of NPY modulates osteogenesis, *J Musculoskelet Neuronal Interact*, 12 (2012) 209-218.

[31] K. Tatemoto, Neuropeptide Y: complete amino acid sequence of the brain peptide, *Proc Natl Acad Sci U S A*, 79 (1982) 5485-5489.

[32] S. Grässel, D. Muschter, Do Neuroendocrine Peptides and Their Receptors Qualify as Novel Therapeutic Targets in Osteoarthritis?, *Int J Mol Sci*, 19 (2018).

[33] L. Li, M. Sapkota, M. Gao, H. Choi, Y. Soh, Macrolactin F inhibits RANKL-mediated osteoclastogenesis by suppressing Akt, MAPK and NFATc1 pathways and promotes osteoblastogenesis through a BMP-2/smad/Akt/Runx2 signaling pathway, *Eur J Pharmacol*, 815 (2017) 202-209.

[34] J.S. Moon, S.H. Kim, S.H. Oh, Y.W. Jeong, J.H. Kang, J.C. Park, H.J. Son, S. Bae, B.I. Park, M.S. Kim, J.T. Koh, H.M. Ko, Relaxin augments BMP-2-induced osteoblast differentiation and bone formation, *J Bone Miner Res*, 29 (2014) 1586-1596.

[35] J. Chen, G. He, Y. Wang, D. Cai, MicroRNA-223 promotes osteoblast differentiation of MC3T3-E1 cells by targeting histone deacetylase 2, *International Journal of Molecular Medicine*, (2018).

[36] X. Zhang, M. Yang, L. Lin, P. Chen, K.T. Ma, C.Y. Zhou, Y.F. Ao, Runx2 overexpression enhances osteoblastic differentiation and mineralization in adipose--derived stem cells in vitro and in vivo, *Calcif Tissue Int*, 79 (2006) 169-178.

[37] H. Luo, H. Gao, F. Liu, B. Qiu, Regulation of Runx2 by microRNA-9 and microRNA-10 modulates the osteogenic differentiation of mesenchymal stem cells, *Int J Mol Med*, 39 (2017) 1046-1052.

[38] J. Han, J. Zuo, D. Zhu, C. Gao, The correlation between SNPs within the gene of adrenergic receptor and neuropeptide Y and risk of cervical vertigo, *J Clin Lab Anal*, 32 (2018) e22366.

[39] Y. Li, Z. Liu, Y. Tang, W. Feng, C. Zhao, J. Liao, C. Zhang, H. Chen, Y. Ren, S.

- Dong, Y. Liu, N. Hu, W. Huang, Schnurri-3 regulates BMP9-induced osteogenic differentiation and angiogenesis of human amniotic mesenchymal stem cells through Runx2 and VEGF, *Cell Death Dis*, 11 (2020) 72.
- [40] T. Komori, Molecular Mechanism of Runx2-Dependent Bone Development, *Mol Cells*, 43 (2020) 168-175.
- [41] T. Shimizu, Participation of Runx2 in mandibular condylar cartilage development, *Eur J Med Res*, 11 (2006) 455-461.
- [42] S. Qu, J. Wu, Q. Bao, B. Yao, R. Duan, X. Chen, L. Li, H. Yuan, Y. Jin, C. Ma, Osterix promotes the migration and angiogenesis of breast cancer by upregulation of S100A4 expression, *J Cell Mol Med*, 23 (2019) 1116-1127.
- [43] J. Hecht, V. Seitz, M. Urban, F. Wagner, P.N. Robinson, A. Stiege, C. Dieterich, U. Kornak, U. Wilkening, N. Brieske, C. Zwingman, A. Kidess, S. Stricker, S. Mundlos, Detection of novel skeletogenesis target genes by comprehensive analysis of a Runx2(-/-) mouse model, *Gene Expr Patterns*, 7 (2007) 102-112.
- [44] A. Ho-Shui-Ling, J. Bolander, L.E. Rustom, A.W. Johnson, F.P. Luyten, C. Picart, Bone regeneration strategies: Engineered scaffolds, bioactive molecules and stem cells current stage and future perspectives, *Biomaterials*, 180 (2018) 143-162.
- [45] C. Gao, S. Peng, P. Feng, C. Shuai, Bone biomaterials and interactions with stem cells, *Bone Res*, 5 (2017) 17059.
- [46] C. Shuai, Y. Xu, P. Feng, G. Wang, S. Xiong, S. Peng, Antibacterial polymer scaffold based on mesoporous bioactive glass loaded with in situ grown silver, *Chemical Engineering Journal*, 374 (2019) 304-315.
- [47] Y. Yang, J. Zan, W. Yang, F. Qi, C. He, S. Huang, S. Peng, C. Shuai, Metal organic frameworks as a compatible reinforcement in a biopolymer bone scaffold, *Materials Chemistry Frontiers*, 4 (2020) 973-984.