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A new function of Nell-1 protein in repressing adipogenic differentiation

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ABSTRACT

A theoretical inverse relationship has long been postulated for osteogenic and adipogenic differentiation (bone versus adipose tissue differentiation). This inverse relationship in theory at least partially underlies the clinical entity of osteoporosis, in which marrow mesenchymal stem cells (MSCs) have a predilection for adipose differentiation that increases with age. In the present study, we assayed the potential anti-adipogenic effects of Nell-1 protein (an osteoinductive molecule). Using 3T3-L1 (a human preadipocyte cell line) cells and human adipose-derived stromal cells (ASCs), we observed that adenoviral delivered (Ad)-Nell-1 or recombinant NELL-1 protein significantly reduced adipose differentiation across all markers examined (Oil red O staining, adipogenic gene expression [*Pparg*, *Lpl*, *Ap2*]). In a prospective fashion, Hedgehog signaling was assayed as potentially downstream of Nell-1 signaling in regulating osteogenic over adipogenic differentiation. In comparison to Ad-LacZ control, Ad-Nell-1 increased expression of hedgehog signaling markers (*Ihh*, *Gli1*, *Ptc1*). These studies suggest that Nell-1 is a potent anti-adipogenic agent. Moreover, Nell-1 signaling may inhibit adipogenic differentiation via a Hedgehog dependent mechanism.

1. Introduction

Numerous studies have proposed an inverse relationship between the differentiation of multipotent mesenchymal stem cells (MSCs) toward osteoblastic and adipocytic cell fates [1–4]. A number of signaling cascades have been implicated in this cell fate decision, including bone morphogenetic protein (BMP), Hedgehog, and Wnt (Wingless Protein) signaling, to name only a few [5–10]. In cases such as Hedgehog and Canonical (or β-catenin dependent) Wnt Signaling, a general enhancement of osteogenic over adipocytic differentiation has been repeatedly observed – or a 'shift' in lineage differentiation [8–14]. Thus, such concepts as 'loss of bone is a gain in fat' is a commonly held conception [1].

The growth factor Nell-1 has long been recognized to have osteoinductive properties. Nell-1 was first discovered as overexpressed in prematurely fusing (ossifying) calvarial sutures of human patients [15]. Nell-1 overexpressing mice demonstrate calvarial bone overgrowth and inappropriate cranial suture fusion [16]. Conversely, animals deficient in functional Nell-1 demonstrate major skeletal defects, including defects in the cranial, axial and appendicular skeleton [17,18]. Moreover, Nell-1 has been observed to induce bone formation both in small and large mammalian models [19–23], and *in vitro* across numerous cell types [16,24,25]. The effects of Nell-1 on adipogenic differentiation have yet to be systematically evaluated. However, recent evidence has found that Nell-1 can reverse the pro-adipogenic effects of high-dose BMP2 (bone morphogenetic protein2) both *in vitro* and *in vivo* (data in submission).

In this present study, we expose an immortalized pre-adipocyte cell line 3T3-L1 and human primary adipose-derived stromal cells (hASCs) to enhanced Nell-1 signaling via an adenoviral vector. As Nell-1 signaling is a potent pro-osteogenic inducer, we hypothesized that Nell-1 would negatively effect adipogenesis. We then in a candidate fashion examined Nell-1 mediated changes to the Hedgehog signaling pathway, to potentially explain the molecular mechanisms whereby Nell-1 may deleteriously impact the process of fat formation.

2. Materials and methods

2.1. Isolation of cells

3T3-L1 cells were a kind gift of the Tontonoz Laboratory, University of California, Los Angeles. ASCs were isolated from human adult lipoaspirate. No individually identifiable information about

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the donor was received, therefore IRB approval was not requested. Fat tissues were obtained from $N = 3$ healthy patients. After standard liposuction procedures, the lipoaspirate (100 ml) was washed with an equal volume of PBS and centrifuged at 1800 RPM for 10 min. The tissue fraction on the top layer was collected and transferred into a new tube. An equal volume of digestion solution (RPMI, 3.5% BSA, 10 μg/ml DNAse, 1 mg/ml Collagenase II) was added and transferred into a shaking incubator for 30–45 min at 37 °C 250 RPM. Following the digestion, the solution was filtered using a 70 μm cell strainer, and centrifuged at 1800 RPM for 10 min. The fatty top layer containing the adipocytes was discarded and the pellet resuspended in PBS 5 mM EDTA. After two washes in PBS 5 mM EDTA, the pellet was resuspended in red blood cell lysis solution for 10 min at RT. After adding 3 volumes of PBS 5 mM EDTA and centrifuging at 1500 RPM for 5 min, the pellet was resuspended in PBS 5 mM EDTA prior to counting the number of viable stromal vascular fraction (SVF) cells via Trypan blue staining. Cells were expanded in 100 mm dishes thereafter.

2.2. In vitro adipogenic differentiation

For adipogenic differentiation, 3T3-L1 or hASCs were seeded in 12-well plates at a density of 50,000 cells per well. All assays were performed in triplicate wells. After attachment, cells were treated with adipogenic differentiation media (ADM). For 3T3-L1 cells, ADM consisted of Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, 1 μM dexamethasone, 0.5 mM IBMX, 5 μg/mL insulin, 1% penicillin/streptomycin. For hASCs, propriety ADM was purchased (Stem Cell Technologies, Inc.). Cells were maintained for 3 days in ADM, and thereafter refreshed with DMEM, 10% FBS, and insulin only for 3T3-L1 cells, or propriety ADM for hASCs. All experiments were performed in biological triplicate (cells derived from 3 separate patients), and each assay was performed in triplicate wells ($N = 3$ wells per cell type per experiment, $N = 3 \times 3 = 9$ samples per condition). Cell medium was supplemented in all cases with recombinant human Nell-1 protein at various dosages, or adenoviral delivered Nell-1 (Ad-Nell-1). Control virus was used at equal concentrations (Ad-LacZ). Ad-Nell-1 and Ad-LacZ were used as previously published, at a concentration of 20 and 40 pfu/cell [16].

2.3. Adipogenic assessments

Assessments of adipogenic differentiation were performed as previously described [10,26,27]. Oil red O staining was performed using a 0.2% solution in 60% isopropanol/40% deionized water. Real time PCR was performed as previously described, performed in triplicate wells per RNA isolate [28]. Specific mouse and human genes and primer sequences are listed in Table 1.

2.4. Bromodeoxyuridine incorporation

Proliferation was assessed by bromodeoxyuridine (BrdU) incorporation assays as previously described [29]. Cells were grown in 96 well plates seeded at a density of 1000 cells/well. After 2, 4 and 6 days growth, BrdU labeling was performed for a period of 8 h. Next, BrdU incorporation was quantified using a photometric ELISA (Roche Applied Science, Indianapolis, Ind.). $N = 6$ per treatment group, experiments were performed on three separate occasions.

2.5. Osteogenic differentiation and assessments

Osteogenic differentiation and assessments were performed using hASCs as previously described. To assess early to intermediate osteogenesis, alkaline phosphatase staining and quantification was performed as previously described; quantification was in each case normalized to total protein content in sister wells [30]. To assess bone nodule formation Alizarin red staining and quantification was performed as previously described; quantification was performed by CPC leaching and photometric quantification, normalized to total protein content [31].

2.6. Statistical analysis

Means and standard deviations were calculated from numerical data, as presented in the text, figures and figure legends. In figures, bar graphs represent means, whereas error bars represent one standard deviation. Statistical analysis was performed using the appropriate ANOVA when more than two groups were compared, followed by a post hoc Student's *t*-test to directly compare two groups. The exact statistical analysis for each dataset is described in the figure legends. Inequality of standard deviations was excluded by employing the Levene's test. $^{*}P \leqslant 0.01$ was considered to be significant.

3. Results

3.1. Nell-1 effects on 3T3-L1 adipogenic differentiation

3T3-L1 preadipocytes are one of the most commonly studied adipoprogenitor cell lines, and represent a unipotent cell type. 3T3-L1 cells were exposed to standard adipogenic differentiation media with adenoviral (Ad)-Nell-1 or Ad-LacZ as a control. After seven days, while Ad-LacZ treated cells showed robust lipid accumulation (Fig. 1A), Ad-Nell-1 showed only sparse lipid droplets (Fig. 1B). Two viral titers were assessed, with both showing the same trend. Real time quantitative PCR demonstrated an over 500% increase in *Nell-1* expression with Ad-Nell-1 treatments with both viral titers (Fig. S1). Oil red O staining results were quantified by leaching with isopropanol and photometric quantification, normalized to total protein content of sister wells (Fig. 1C). Quantification demonstrated an approximate 80% reduction in Oil red O staining intensity. Importantly Trypan blue staining showed no increase in cytotoxicity with Ad-Nell-1 treatment (data not shown).

Table 1
Primer sequences.

Gene name	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
Ap2, mouse	CCAACTCTTTTGTGCCAGAGA	GGCTACATTGGTGTTGAGCTTTT
Gapdh, mouse	CTTTTGCGAAGTGCAGGAAAAT	TGTTGACTGAGTCTTCTGGACAA
Gli1, mouse	GAGCCCTTCTTTAGGATTCCCA	ACCCCGAGTAGAGTCATGTGG
Gli3, mouse	CACAGCTCTACGGCGACTG	CGGCCTTCTCGCTGACATC
Ihh, mouse	CTCAGACCGTGACCGAAATAAG	CCTTGGACTCGTAATACACCCAG
Lpl, mouse	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAATA
Pparg, mouse	CACTCCTCGCCCTATTGGC	CCCTCCTGCTTGGACACAAAG
Ptc1, mouse	GCCACAGCCCCTAACAAAAAT	ACCCACAATCAACTCCTCCTG

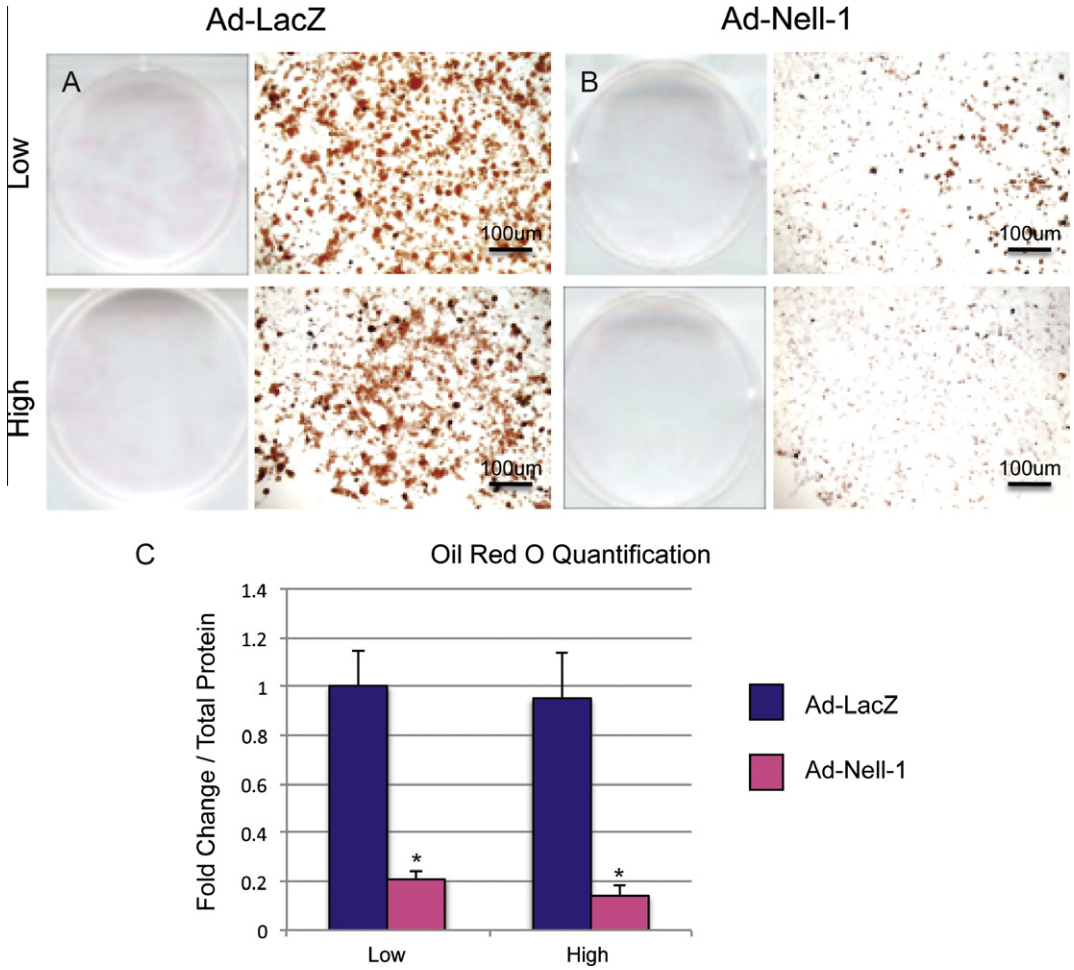

Fig. 1. Adipogenic differentiation of 3T3-L1 cells. (A and B) Oil red O staining after seven days adipogenic differentiation under control conditions (Ad-LacZ) or Nell-1 overexpressing conditions (Ad-Nell-1). (C) Quantification of Oil red O by isopropanol leaching and photometric quantification, normalized to average protein content in sister wells. Ad-Nell-1 and Ad-LacZ were used as previously published, at a concentration of 20 and 40 pfu/cell [16]. All experiments will performed in triplicate wells on three separate occasions. $^{*}P < 0.01$.

3.2. Nell-1 effects on 3T3-L1 adipogenic gene expression

To confirm the anti-adipogenic effects of Nell-1 signaling on 3T3-L1 pre-adipocytes, cells were cultured in the presence of Nell-1 overexpressing adenovirus (Ad-Nell-1) or control virus (Ad-LacZ); RNA was harvested at stratified timepoints post induction (two, four and six days). As expected, all markers gradually increased overtime in adipogenic medium under control conditions (including *Peroxisome proliferating factor gamma* [*Ppar*], *Lipoprotein lipase* [*Lpl*] and *Adipocyte protein 2* [*Ap2*]) (Fig. 2, blue bars). Significantly Ad-Nell-1 addition to adipogenic medium resulted in a significant reduction in all markers (Fig. 2, red bars). These data confirmed the significant anti-adipogenic effects of Nell-1 signaling in 3T3-L1 cells.

3.3. Nell-1 effects on 3T3-L1 proliferation

Theoretically a reduction in the proliferation of pre-adipocytes by Nell-1 signaling could result in a reduction in Oil red O staining (see again Fig. 1). To confirm or reject this hypothesis, bromodeoxyuridine (BrdU) incorporation assays were performed in the presence of control (Ad-LacZ) or Nell-1 overexpressing adenovirus (Ad-Nell-1) (Fig. S2). Interestingly, no significant change in BrdU incorporation was observed (Fig. S2, compare red and blue bars). Thus, we reasoned that Nell-1 signaling likely inhibits adipoprogenitor cell differentiation rather than pre-adipocyte expansion *in vitro*.

3.4. Nell-1 effects on human ASC differentiation

Mesenchymal stem cells (MSCs) clearly retain important biological differences from 3T3-L1 pre-adipocytes. We next inquired as to whether Nell-1 signaling would have the same negative effects on adipogenic differentiation a human MSC population: adipose-derived stromal cells or human (h) ASCs. We opted to utilize recombinant protein instead as a more defined and quantitative delivery of Nell-1 alternative source. Recombinant human (rh) NELL-1 from CHO cells was supplemented to standard adipogenic or osteogenic ASC differentiation medium (Fig. 3). As expected, rhNELL-1 had a negative effect on adipogenic differentiation, as demonstrated by Oil red O staining of lipid accumulation (ORO, appearing red) (Fig. 3A). In stark contrast, rhNELL-1 increased markers of osteogenic differentiation by biochemical staining, including alkaline phosphatase (ALP, an intermediate marker of osteodifferentiation appearing purple) and Alizarin red (AR, a stain for bone nodule formation appearing red) (Fig. 3B and C). Quantification of each stain confirmed a significant upregulation of osteogenic differentiation and converse and negative effect on adipocytic differentiation (Fig. 3D). Thus, Nell-1 signaling inhibited adipogenic differentiation in both a unipotent pre-adipocyte cell line, and a primary MSC.

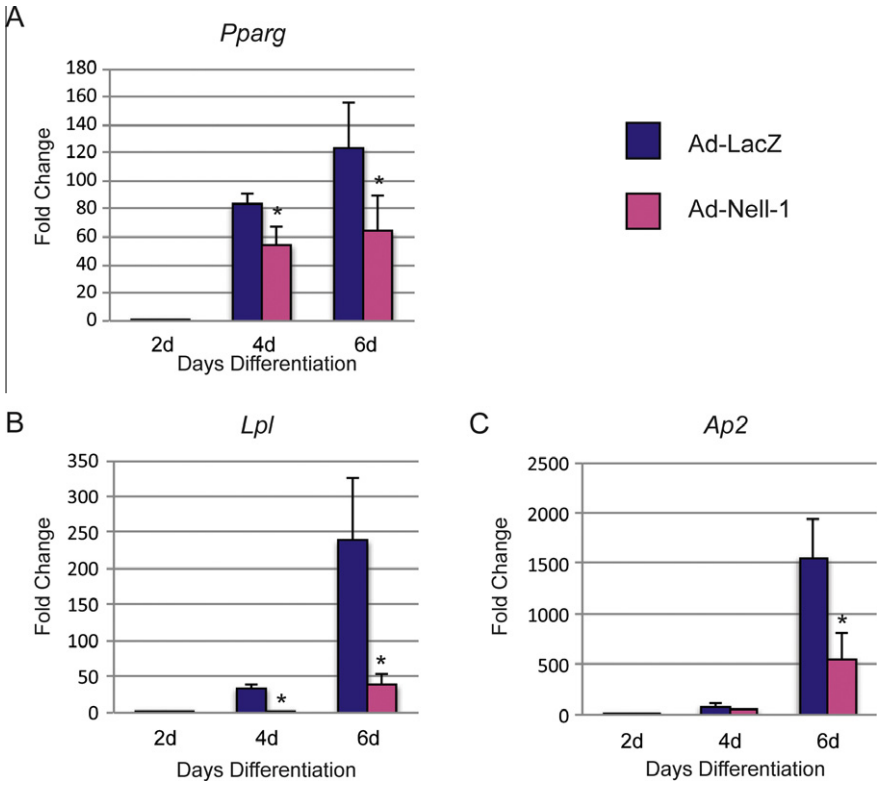

Fig. 2. Adipogenic gene expression in 3T3-L1 cells. Specific gene expression was assessed at 2, 4 and 6 days after induction of adipogenesis in 3T3-L1 cells with or without control virus (Ad-LacZ, blue bars) or Nell-1 overexpressing virus (Ad-Nell-1, red bars) via quantitative real-time PCR. (A) *Peroxisome proliferating factor gamma* (*Pparg*) expression. (B) *Lipoprotein lipase* (*Lpl*) expression. (C) *Adipocyte protein 2* (*Ap2*) expression. Ad-Nell-1 and Ad-LacZ were used as previously published, at a concentration of 20 pfu/cell [16]. $^{*}P < 0.01$. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

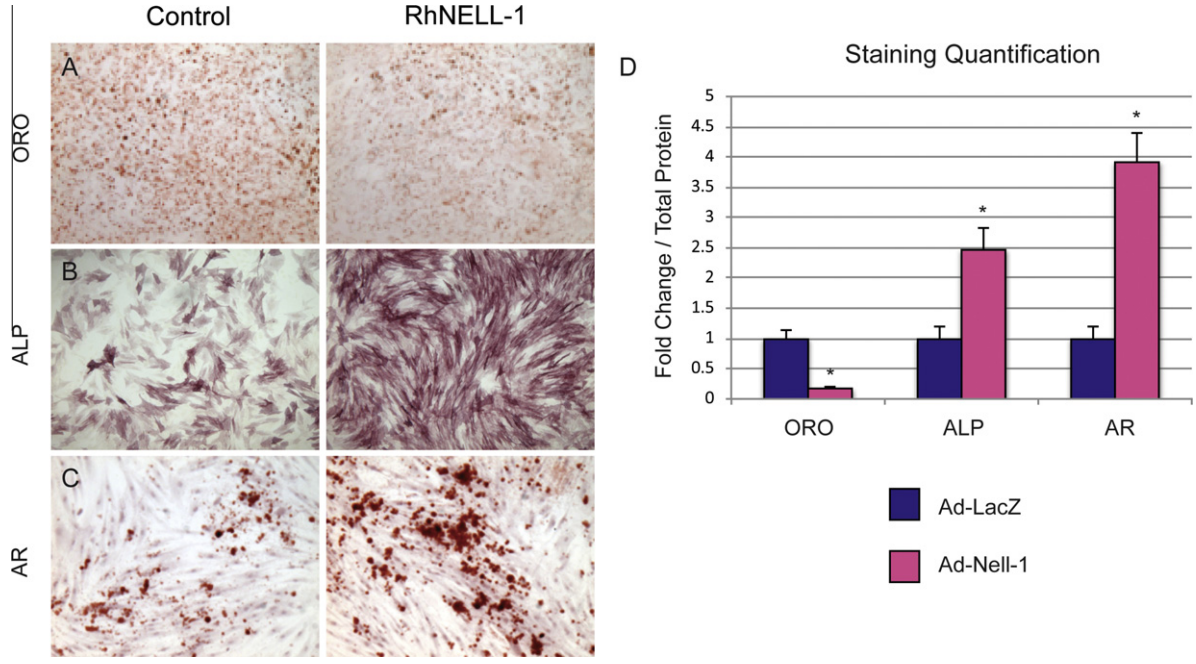

Fig. 3. Osteogenic and adipogenic differentiation of primary human ASCs. Recombinant human (rh) NELL-1 was supplemented to standard osteogenic or adipogenic differentiation mediums (300 ng/ml). (A) Oil red O (ORO) staining was performed after 9 days differentiation. (B) Alkaline phosphatase (ALP) staining was performed after 5 days differentiation. (C) Alizarin red (AR) staining was performed after 10 days differentiation. (D) Quantification of ALP, AR and ORO staining. In the case of ALP, enzymatic activity was assessed, normalized to total protein content. In the case of AR, CPC leaching was performed, normalized to total protein content. In the case of ORO, isopropanol leaching was performed, normalized to total protein content. $^{*}P < 0.01$.

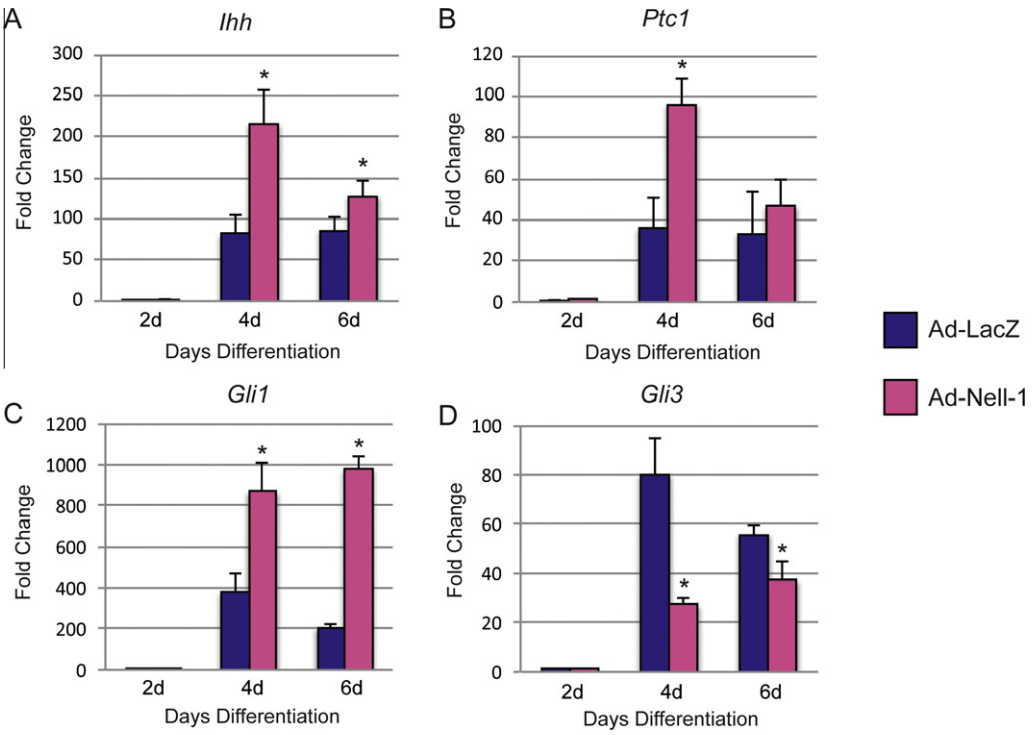

Fig. 4. Hedgehog signaling gene expression in 3T3-L1 cells. After 2, 4 and 6 days in adipogenic differentiation medium, RNA expression of common Hedgehog signaling genes was assessed by quantitative real-time PCR. (A) *Indian Hedgehog* (*Ihh*) gene expression. (B) *Patched1*, a marker of Hedgehog signaling activity (*Ptc1*). (C) *Gli1*, a transcription factor and marker of Hedgehog signaling activity. (D) *Gli3*, a transcription factor and negative regulator of Hedgehog signaling. Ad-Nell-1 and Ad-LacZ were used as previously published, at a concentration of 20 pfu/cell [16]. $^{*}P < 0.01$.

3.5. Nell-1 effects on Hedgehog signaling

Hedgehog signaling has been known to positively regulate osteogenic differentiation at the expense of adipogenic differentiation in multiple cell types, including ASCs [10,32–34]. We therefore next inquired as to the effects of Nell-1 on hedgehog signaling. 3T3-L1 cells were cultured in the presence of control virus (Ad-LacZ) or Nell-1 overexpression virus (Ad-Nell-1) (Fig. 4). *Indian Hedgehog* (*Ihh*) ligand showed upregulation after both 4 and 6 days differentiation (Fig. 4A). Likewise two markers of hedgehog signaling activation showed similar upregulations, *Patched1* (*Ptc1*) and *Gli1* (Fig. 4B and C). Conversely, *Gli3*, generally considered a negative regulator of Hedgehog pathway activity, showed a reduction in transcript abundance (Fig. 4D). Thus and in summary Nell-1 appears to reduce adipogenic differentiation of 3T3-L1 cells with concomitant induction of Hedgehog ligand expression and Hedgehog signaling activity.

4. Discussion

Adipocytes and osteoblasts arise from a common mesenchymal progenitor cells, and a theoretical inverse relationship exists between these two cell types (Fig. S3). Numerous bipotent or multipotent cell types exist for future tissue engineering applications, including traditional bone marrow MSCs (BMSCs), adipose-derived stromal cells (ASCs) and umbilical cord-derived (UC-) MSCs. Accumulating data suggests that this bipartite destiny of MSCs (bone versus fat) can be shifted given appropriate cytokine stimulation [1]. Here, we sought to examine whether Nell-1 would be an appropriate signal to shift the balance from adipocytic to osteogenic differentiation.

In fact we found this to be the case: namely that Nell-1 definitively represses adipogenic differentiation *in vitro*. First, Nell-1 represses adipogenesis in a unipotent cell (a pre-adipocyte – or 3T3-L1 cell). Secondarily, Nell-1 likewise serves to repress adipogenic differentiation in a multipotent ASC. This suggests the effect of Nell-1 on adipogenesis are direct repression of lipogenesis, rather than an indirect repression via enhancement of the converse lineage (osteogenic) differentiation.

No doubt, Nell-1 in one of many cytokines known to 'shift' this MSC differentiation away from fat and towards bone. One particular cytokine pathway of interest is Hedgehog signaling, which in multiple cell types including ASCs has been shown to cause a similar 'shift' in differentiation [10,32–34]. Three mammalian Hedgehog ligands exist, including Sonic Hedghog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) – and as Dhh appears limited to the mammalian testes, both Shh and Ihh have been studied in greater detail. Both ligands appear to have a similar and equivalent pro-osteogenic effect (for example the Ihh null has severe skeletal defects) – however Shh has been studied in greater detail *in vitro*, where the N-terminal domain is responsible for signal propagation [34–36]. In our previous manuscript, we detailed the extent to which Shh-N positively regulated osteogenesis and inhibited adipogenesis, both *in vitro* and *in vivo* in mouse ASCs [10].

Given the striking similarities in effect between Shh and Nell-1, we sought here to define the extent to which Nell-1 may positively regulate hedgehog signaling. In fact, a positive relationship was observed. These studies suggest highly that at least some portion of the differentiation effects of Nell-1 are via activation and/or potentiation of Hedegehog signaling. Whether Nell-1 directly increases Hedgehog ligand (*Shh* or *Ihh*) expression, directly increases *Gli* transcription or Gli activation, or inactivates a Hedgehog repressor (*Ptc1*, *Gli3*, *Rab23* among numerous others) remains as yet unknown. Certainly there exist other potentially signaling networks that may work to transduce a Nell-1 signal into a 'shift' in lineage differentiation. For example, MAPK signaling in known to modulate both osteogenic and adipogenic differentiation and has been previously shown to be up-regulated in certain contexts by

Nell-1 [37]. In addition, Canonical Wnt signaling is well known to overall produce a similar 'shift' in differentiation and again has been shown to be up-regulated both *in vitro* and *in vivo* by Nell-1 protein (in submission). These studies suggest the multifactorial and interwoven nature of cell fate decisions, and suggest Nell-1 to be integral to the balance of osteo- and adipogenic MSC differentiation.

In summary, in addition to the well-recognized role of Nell-1 in promotion of osteogenic differentiation and bone formation, Nell-1 significantly represses adipogenic differentiation. This anti-adipogenic effect of Nell-1 may be through potentiation of Hedgehog signaling among other signaling cascades. These studies suggest the future therapeutic utility of Nell-1 for either skeletal or soft-tissue regenerative medicine.

Conflict of interest

Drs. X.Z., K.T., and C.S. are inventors of Nell-1 related patents. Drs. X.Z., K.T., and C.S. are founders of Bone Biologics Inc. which sublicenses Nell-1 patents from the UC Regents.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.111.

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