INTRODUCTION:
Human adipose-derived stromal cells (hASCs) can differentiate into both osteoblasts and adipocytes, a characteristic which lends to their potential use in therapeutic bone and soft tissue regeneration treatments. However, a current hindrance in the use of ASCs is the lack of an effective growth factor regime that can efficiently induce hASC osteogenic differentiation for bone formation. This study sought to determine a combination of cytokines that can optimally induce hASCs to undergo osteogenesis, while at the same time impeding adipogenesis. Previous studies have implicated several signaling pathways involved in the determination of hASCs into either osteogenic or adipogenic lineages. Among these pathways are the osteogenic Nell-1 pathway and the Hedgehog signaling pathway, which has previously been demonstrated to be both osteoinductive and anti-adipogenic properties. Sonic Hedgehog (Shh) was thus used in this study to examine whether the equilibrium between osteogenesis and adipogenesis in hASCs can be affected (as has been previously documented in mouse ASCs). Likewise, another novel cytokine, Nell-1, was also used to investigate the potential additive effects of Shh-N + Nell-1 on hASC differentiation. We hypothesized that the monotherapy of Nell-1 or Shh-N could shift the balance of hASC differentiation toward osteogenesis, away from adipogenesis, and furthermore, that exposure to both Shh-N and Nell-1 could provide a highly effective combination for osteoinduction of hASCs in vitro.

METHODS:
ASCs were isolated from human liposapirate of 3 separate patient samples after standard protocol. Passage 3 or less cells were used for all assays. To test whether Hedgehog or Nell-1 signaling could influence cell differentiation, hASCs were cultured at a density of 35,000 cells / well in 12-well dishes with standard osteogenic or adipogenic differentiation medium. To this, either vehicle control, N-terminal Sonic Hedgehog (Shh-N) or recombinant human Nell-1 protein (rhNell-1) was added alone or in combination. Assessments were accomplished using alkaline phosphatase (ALP) staining and quantification as a marker of early differentiation and Alizarin red (AR) staining and quantification for bone nodule formation. Additional evaluation of adipogenic differentiation was done using Oil Red O (ORO) staining for lipid droplet accumulation. Quantifications of AR stains were achieved using a photometric approach alongside CPC leaching whereas ALP activity was measured by recording the amount of p-nitrophenol formed from the enzymatic hydrolysis of p-nitrophenylphosphate. In addition to cell staining assays, we also utilized qRT-PCR to quantify markers of osteogenesis. RNA isolation was first performed with the RNeasy Mini Kit (Qiagen Sciences). Secondly, TaqMan Reverse Transcription Reagents (Applied Biosystems) was used to reverse transcribe 1 mg of isolated RNA. Lastly, qRT-PCR was achieved by using the Applied Biosystems Prism 7900HT Sequence Detection System and Power SYBR Green Master Mix (Applied Biosystems). From this, levels of expression of RUNX2, a marker of osteoblast differentiation, and PPARG, a marker of adipogenesis, were quantified. Finally, proliferation assays were also carried out through bromodeoxyuridine (BrdU) incorporation. 1,000 cells per well were cultured in 96 well plates and at time points of 2, 4 and 6 days growth, samples were exposed to and labeled by BrdU for 8 hours. BrdU was subsequently quantified using photometric ELISA.

RESULTS:
Both Shh-N and Nell-1 increased staining intensity for both ALP and AR as expected from previous studies demonstrating their pro-osteogenic effects (Fig. 1). Moreover, the combination of Shh-N+Nell-1 showed an additive increase in staining intensity for both markers (Fig. 1). In contrast, both cytokines when supplemented to adipogenic medium reduced adipogenic differentiation, as demonstrated by ORO staining (data not shown). Again, an additive effect was observed with Shh-N+Nell-1 reducing adipogenesis to a greater degree than either alone (not shown).

Quantitative RT-PCR results confirmed the results of biochemical stainings (Fig. 2). The master osteogenic transcription factor RUNX2 showed an additive increase with the combination of Shh-N + Nell-1 (Fig. 2A). Conversely, the primary adipocyte transcription factor PPARG showed significant decrease in expression for samples treated with either Shh-N or Nell-1, and an even greater repression when the two cytokines were combined (Fig. 2B). Proliferation assays (BrdU incorporation) found that Shh-N but not rhNell-1 resulted in a slight increase in cell division (Fig. 2C). Therefore, reductions in adipogenesis cannot be explained by reductions in cell number by either cytokine.

DISCUSSION:
In this study, we utilized monotherapies of either Shh-N or Nell-1 as well as a combination treatment consisting of both cytokines to determine if these agents can be used as viable inducers of hASC osteoblast. Passage 3. Our results reveal that monotherapies of Shh-N and Nell-1 drive hASCs toward osteoblastic cell fates at the cost of adipocytic differentiation. More importantly, simultaneous use of both Shh-N and Nell-1 shows an additive increase in osteogenic differentiation. Overall, these results demonstrate that Shh-N and Nell-1 can act additively as an effective growth factor combination for hASC osteodifferentiation.

SIGNIFICANCE:
This study presents a promising step forward in the development of an optimal growth factor regime to be used to induce therapeutically sufficient levels of osteogenesis in hASCs. By establishing more optimal regimes, the goal of being able to utilize advantageous osteogenic differentiated hASCs for bone tissue engineering becomes closer in sight.