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Commentary

Exogenous nitric oxide enhances calcification in embryonic stem cell-derived osteogenic cultures

D.D. Ehnes [a,1], R.M. Geransar [b,1], D.E. Rancourt [b], N.I. zur Nieden [a,b,*]

[a] University of California Riverside, Department of Cell Biology & Neuroscience and Stem Cell Center, College of Natural and Agricultural Sciences, 1113 Biological Sciences Building, Riverside, CA 92521, USA

[b] Institute of Maternal & Child Health, University of Calgary, Heritage Medical Research Building, 3330 Hospital Drive NW, Calgary, Alta., Canada T2N 4N1

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ABSTRACT

While the involvement of nitric oxide in bone formation, homeostasis and healing has been extensively characterized, its role in directing pluripotent stem cells to the osteogenic lineage has not been described. Yet, the identification of chemical inducers that improve differentiation output to a particular lineage is highly valuable to the development of such cells for the cell-based treatment of osteo-degenerative diseases. This study aimed at investigating the instructive role of nitric oxide (NO) and its synthesizing enzymes on embryonic stem cell (ESC) osteogenic differentiation. Our findings showed that NO levels may support osteogenesis, but that the effect of nitric oxide on osteoblast differentiation may be specific to a particular time phase during the development of osteoblasts *in vitro*. Endogenously, nitric oxide was specifically secreted by osteogenic cultures during the calcification period. Simultaneously, messenger RNAs for both the endothelial and inducible nitric oxide synthase isoforms (eNOS and iNOS) were upregulated during this late phase development. However, the specific eNOS inhibitor L-N^5-(1-Iminoethyl)ornithine dihydrochloride attenuated calcification more so than the specific iNOS inhibitor diphenyleneiodonium. Exogenous stage-specific supplementation of culture medium with the NO donor S-nitroso-N-acetyl-penicillamine increased the percentage of cells differentiating into osteoblasts and enhanced calcification. Our results point to a primary role for eNOS as a pro-osteogenic trigger in ESC differentiation and expand on the variety of supplements that may be used to direct ESC fate to the osteogenic lineage, which will be important in the development of cell-based therapies for osteo-degenerative diseases.

1. Introduction

Osteoblasts derived from pluripotent stem cells are a potentially promising candidate for the stem cell-assisted therapy of degenerative bone and joint disorders or skeletal injuries (zur Nieden, 2011; McClelland Descalzo et al., 2014). However, before pluripotent stem cells may be applied in human therapies, more research is needed to understand the exact mechanisms whereby such cells differentiate into specialized cells. In particular, the study of signaling molecules, such as nitric oxide (NO), that may play a role in differentiation is a growing area of research that may facilitate the development of homogenous tissue cultures for application in stem cell therapy.

In the past few years, our understanding of osteogenesis from embryonic stem cells (ESCs) has made tremendous progress. Differentiation occurs in several distinct temporal phases: first, naive ESCs transition into a primed state equivalent to an epiblast stem cell (Martello and Smith, 2014), then, cells exit from pluripotency to gastrulate and undergo a primitive streak-like state while simultaneously inducing the neural crest. All of these events are important for future osteogenesis, as bone can arise from both the mesoderm or the neural crest (reviewed in Keller and zur Nieden (2011)). Regardless of the embryonic origin, the crucial step in osteogenic differentiation is the commitment of mesenchymal cells, which subsequently turn into osteoprogenitors. Each of these phases is characterized by the expression of a number of genes and distinct morphological features, and the end result is the maturation of osteoblasts and the calcification of the extracellular matrix (Davis and zur Nieden, 2008).

Now that a number of groups have demonstrated differentiation of ESCs and induced pluripotent stem cells into skeletal cell types (Buttery et al., 2001; Phillips et al., 2001; zur Nieden et al., 2003; Sottile et al., 2003; Li and Niyibizi, 2012; Egusa et al., 2014;

* Corresponding author at: University of California Riverside, 1113 Biological Sciences Building, Department of Cell Biology and Neuroscience, Riverside, CA 92521, USA. Tel.: +1 951 827 3818; fax: +1 951 827 4037.
E-mail address: nicole.zurnieden@ucr.edu (N.I. zur Nieden).
[1] Authors contributed equally as first authors.

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Kanke et al., 2014; Trettner et al., 2014), research has shifted towards the identification of factors that can improve the output of these cell types, which remains insufficient in basic osteogenic medium. For example, we have recently demonstrated that the yield of chondrocytes within differentiated cultures can be increased to 57% simply through the continuous supplementation of culture with bone morphogenetic protein-2 (BMP-2) (zur Nieden et al., 2005). Additionally, we and others have used Affymetrix gene microarrays to identify pathways associated with osteogenic lineage specification (Bourne et al., 2004; zur Nieden et al., 2007). Our analysis confirmed a number of signaling cues known to be involved in osteogenesis, such as BMP-2 and retinoic acid, among others. Manipulation of these factors has led to significant enhancement in osteogenic differentiation from 49% to over 90% and correlates with the regulation of nuclear beta-catenin activity (zur Nieden et al., 2007). There appears to be substantial crosstalk between identified signaling pathways: for example, addition of basic fibroblast growth factor to BMP-2 induced cultures significantly up-regulated osteocalcin (*Ocn*) and Core binding factor alpha 1 (*Cbfa1*) mRNAs, two factors uniquely associated with bone tissue (Rose et al., 2013).

Based on our desire to further characterize the involvement of signaling pathways during ESC osteogenic differentiation, we have determined that increased levels of NO are generated during early ESC commitment (Ding et al., 2012). Concomitant with NO up-regulation, expression of *T-brachyury* was enhanced, a gene which is predominantly expressed in the primitive streak *in vivo*. As a known transcriptional target of beta-catenin (Arnold et al., 2000), *T-Brachyury* has a conserved role in mesoderm differentiation in all vertebrates. Consistent with these results, nuclear activity of beta-catenin was also responsive to NO levels in ESC-derived primitive streak-like cells, indicating that NO may be involved in early commitment at a stage of differentiation that precedes mesenchymal and osteoblast specification.

While the described role for NO in early differentiation has only recently been reported, both the inducible nitric oxide synthase (iNOS) and the constitutively active eNOS isozymes that catalyze the production of NO from L-arginine have long been confirmed to play a role during the later stages of osteoblast differentiation *in vivo*. For instance, evidence from $eNOS^{-/-}$ gene knock-out mice has shown that exogenous supplementation with a NO donor can restore *Cbfa1* and *OCN* expression (Afzal et al., 2004). Similarly, rapid induction of *iNOS* mRNA expression occurs during pathogenic responses and results in release of high concentrations of NO that are thought to promote bone resorption and bone formation (Aguirre et al., 2001; Armour et al., 2001; Lin et al., 2003), and may play a regulatory role in post-fracture healing (Namkung-Matthai et al., 2000). *In vitro*, a limited number of studies report that increased NO production is associated with the proliferation and differentiation of mesenchymal stem cells and calvarial osteoblasts (Sosroseno et al., 2008; Orciani et al., 2009). Exogenous NO augments proliferation in human osteoblast cells and fetal calvarial osteoblasts (Lin et al., 2008; Sugiatno et al., 2009). Based on this past literature, we aimed to determine whether appreciable differences in maturation of osteoprogenitors derived from ESCs may result from augmentation of the effect of NO and NOS enzymes.

2. Methods

2.1. Tissue culture

Murine ESCs (D3, American Type Culture Collection) were routinely passaged every two days into high glucose Dulbecco's Modified Eagle Medium, 15% fetal bovine serum (FBS, selected batch), 0.1 mM β-mercaptoethanol, 1% non-essential amino acids, 50 U/ml penicillin and 50 μg/ml streptomycin (all Invitrogen). Differentiation was induced in medium without Leukemia Inhibitory Factor (LIF, EMD Millipore) through embryoid body (EB) formation as described (zur Nieden et al., 2003; Trettner et al., 2011). Five day-old EBs were dispersed into a single cell suspension and plated into osteogenic induction medium containing 10 mM β-glycerophosphate (Sigma-Aldrich), 50 μg/ml ascorbic acid (Sigma-Aldrich), and 5×10^{-8} M 1α,25-$(OH)_2$ vitamin D_3 (VD_3, EMD Millipore) (zur Nieden et al., 2003) at a density of 50,000 cells/cm^2.

2.2. NOS inhibition/NO supplementation

NOS inhibitors or NO donors were added to cell medium to final concentrations as described below, in accordance with their IC_{50} values. Chemical modulators of NO included 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), a stable radical scavenger for NO [100 μM], and the exogenous NO donor S-nitroso-N-acetylpenicillamine (SNAP) [100 μM]. The flavoprotein binder diphenyleneiodonium (DPI) was used as a specific iNOS inhibitor [50 nM] and L-N^5-(1-Iminoethyl)ornithine, dihydrochloride (L-NIO) as a specific inhibitor of eNOS [1 μM] as we have done before (Ding et al., 2012). All substances were dissolved in DMSO. Appropriate solvent controls were run alongside all experiments.

2.3. NO content

Extracellular NO production was measured using a Griess-Diazo spectrophotometric-based quantitative assay. ESCs were cultivated in phenol red-free medium containing the appropriate concentrations of the respective chemicals 24 h prior to the assay. Cell culture medium was harvested and equal volumes reacted with Griess reagent (Sigma-Aldrich) at room temperature for 15 min. Changes in absorbance were recorded at 540 nm with a Bio-Rad Microplate Manager 5.2. Triplicates of each biological sample ($n=3$) were measured for each time-point. As a qualitative measure of intracellular NO content, cells were incubated with diaminofluorescein (DAF-2DA, Sigma-Aldrich, 4 mM) for 30 min at 37 °C in phenol-red free medium containing NO modulators and cultures were photographed.

2.4. Gene expression analysis

Cellular RNA lysates were purified using a QIAshredder Mini Spin Column prior to RNA isolation with a Qiagen RNeasy Mid Kit. RNA was quantified using Ribogreen dye (Molecular Probes) as described (zur Nieden et al., 2007). Reverse transcription was carried out using SuperScript II Reverse Transcriptase (Invitrogen) in a MJ Research PTC-200 Peltier Thermal Cycler with random hexamers at 65 °C for 5 min, 25 °C for 15 min, 42 °C for 50 min and a final step at 70 °C for 15 min. PCR reactions were carried out from one tenth of the cDNA in cycles of 45 s at the respective primer annealing temperature and extension at 94 °C for 45 s. Reactions contained Taq DNA polymerase (Thermo), deoxynucleotides (0.2 mM each), $MgCl_2$ (1.5 mM) and primer mix (0.5 μM) in a reaction volume of 25 μl. PCR products were semi-quantitatively analyzed using electrophoresis of 2%- or 3% agarose gels. For quantitative PCR, the reaction composition and cycle conditions were modified as described (zur Nieden et al., 2007). Dissociation curves were generated post-run for analysis of amplicon species and *n*-fold gene regulation calculated according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001) with *Gapdh* as the housekeeper gene. All primer sequences have been described previously (zur Nieden et al., 2003, 2005, 2007; Ehnes et al., 2015).

2.5. Lowry protein assay

Cells were rinsed twice in phosphate buffered saline (PBS) and lysed in radio immunoprecipitation assay (RIPA) buffer with the