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The Role of Noggin in Human Mesenchymal Stem Cell Differentiation

Leonard Rifas*

Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri

Abstract Noggin is a secreted protein that inhibits the binding of BMPs to their cognate receptor. Its role in human mesenchymal stem cell differentiation has not been well studied. Here we studied the effect of noggin on human mesenchymal stem cell differentiation induced by inflammatory cytokines (activated T cell conditioned medium (ACTTCM) or the combination of four T cell cytokines, TNF-α, TGF-β, IFN-γ, and IL-17 (TTII)], BMPs or dexamthasone (DEX). HMSC treated with TTII alone rapidly induced alkaline phosphatase (AlkP) activity. Inclusion of noggin resulted in an additive effect. Noggin acted additively with DEX to induce a significantly higher level of AlkP induction than either noggin or DEX alone. Noggin was examined for its ability to inhibit mineralization in long-term cultures of HMSC stimulated with BMP-2, BMP-6, BMP-7, DEX, or TTII. Surprisingly, noggin alone induced mineralization while it did not inhibit mineralization induced by TTII or BMP-2, BMP-6 or BMP-7. Interestingly, when HMSC were treated with both noggin and DEX they acted synergistically to induce mineralization nearly threefold over DEX alone and 30-fold over noggin alone. RT-PCR analysis showed that T cell cytokines-induced noggin, Runx2, BMP-2, and osteocalcin gene expression, while noggin alone induced BMP-2 and osteocalcin gene expression, but not Runx2, although it increased the expression of ActRII, a receptor for BMP-2. These results suggest that in HMSC, the anabolic action of inflammation on bone formation occurs through the induction of noggin, which then induces BMP-2 receptor and BMP-2 leading to the activation of the differentiation process. J. Cell. Biochem. 100: 824–834, 2007. © 2006 Wiley-Liss, Inc.

Key words: alkaline phosphatase; bone morphogenetic proteins; differentiation; gene expression; inflammation; mineralization; osteoblasts; T cells

Bone formation results from the action of multiple signals on mesenchymal stem cells that drive their differentiation into osteoblasts [Reddi, 2001]. The most potent signal comes from the bone morphogenetic proteins (BMPs) which belong to the TGF-β super family [Ducy and Karsenty, 2000]. Among BMPs, BMP-2/4, BMP-6, and BMP-7, have potent osteogenic activity as characterized by their ability to induce alkaline phosphatase, collagen type I, osteocalcin, and bone matrix mineralization [Friedman et al., 2006]. The BMPs exert their signaling activity by binding to type II (BMPR-II and ActR-II and ActR-IIB) and type I (BMPR-IA or ALK-3, BMPRIB or ALK-6, and ActR-IA or ALK-2) serine/threonine kinase receptors (reviewed in Cao and Chen, 2005). It is not clear at this time whether BMPs are involved in bone remodeling in vivo although they participate in the process of fracture repair [Yoshimura et al., 2001].

Opposing the BMPs are a class of antagonists. These include noggin, chordin, gremlin, follistatin, and the DAN/Cerebrus family [Chen et al., 2004]. Noggin has been extensively studied for its ability to bind to BMP-2, -4, and -7 in a manner that inhibits the BMPs from binding to their receptor [Zimmerman et al., 1996]. This action of noggin has been most extensively studied in the mouse model where bone development can be examined in the context of transgenic mice overexpressing either a BMP or noggin or both [Glaser et al., 2003; Wu et al., 2003; Wijgerde et al., 2005]. Such studies have determined that noggin does play a pivotal role in the development of bone by regulating the action of BMPs in a spatial/temporal manner [Nakamura et al., 2003; Peng

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*Correspondence to: Leonard Rifas, Department of Pediatrics, Washington University School of Medicine, Campus Box 8208, 660 South Euclid Avenue, St. Louis, MO 63110. E-mail: L.Rifas@charter.net

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et al., 2005] as well as in fracture healing by regulating the development of the cartilaginous collar and subsequent bone repair [Yoshimura et al., 2001]. As a result of these studies, it has become the paradigm that noggin is an antagonist of BMP-induced osteogenesis. However, the role of noggin in human osteoblast differentiation induced by BMPs has not been well studied. Herein, we have examined the role of noggin in human mesenchymal stem cell differentiation induced by BMPs, dexamethasone, or inflammatory cytokines. We now report that the role of noggin appears to be anabolic in that it induces human mesenchymal stem cells into osteoblasts.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Recombinant Cytokines

Recombinant human TNF-α, TGF-β_1, IFN-γ, IL-17, and BMP-6 and recombinant mouse noggin were obtained from R&D Systems (Minneapolis, MN). Recombinant human BMP-2 was a kind gift of Genetics Institute, Cambridge, MA. Recombinant human BMP-7 was kindly provided by Dr. Keith Hruska, Washington University School of Medicine, St. Louis, MO. Recombinant human noggin was obtained from Peprotech (Rocky Hill, NJ).

Isolation of T Cells

Peripheral blood mononuclear cells (PBMC) were obtained in the form of buffy coats from the American Red Cross, St. Louis, MO. CD4 + T cells were isolated as previously described [Rifas, 2006]. The use of human buffy coats was approved by the Washington University Institutional Review Board.

T Cell Cultures

T cells were cultured and activated as previously described [Rifas and Arackal, 2003; Rifas et al., 2003; Rifas, 2006]. After a 72 h incubation period activated T cell conditioned medium (ACTTCM) was harvested and frozen at -80°C until used in the experimental protocols.

Preparation HMSC

Human rib specimens from three donors were obtained from the Missouri Transplantation Services, St. Louis, MO, as donor tissue. Bone marrow preparations derived from iliac crest, from one donor, was purchased from Stem Cells Technologies (Vancouver, CA) and one donor from Cambrex BioScience (Walkersville, MD). HMSC were prepared as previously described [Rifas et al., 1995] and used at passages two to three.

Cytokine Stimulation of HMSC

Cells were seeded into 6- or 12-well tissue culture plates at 2×10^4 cells/cm^2 in Dulbecco's Modified Eagle's Medium (DMEM, low glucose formulation, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Hyclone) and incubated for 4 days. The confluent cells were then treated as described below.

Alkaline Phosphatase Cytochemistry

Cells were fixed for alkaline phosphatase cytochemical staining as previously described [Prabhakar et al., 1998]. Briefly, alkaline phosphatase, cells were cultured in DMEM, 10% FBS (Hyclone), 10 mM β-glycerophosphate, and 100 μM ascorbic acid 2-phosphate (differentiation medium) and treated as indicated. Medium was changed every 3–4 days, with addition of test agents, for 3 days. The cell layers were then rinsed twice with PBS and fixed in 50% ethanol for 10 min. The cell layers were rehydrated with MilliQ water for 5 min prior to staining for alkaline phosphatase (Sigma kit 105-L).

Alkaline Phosphatase Assay

HMSC were cultured for 6–7 days in differentiation medium in the absence or presence of activated T cell conditioned medium (ACTTCM), TTII (TNF-α, 120 pg/ml; TGF-β, 300 pg/ml; IFN-γ, 6 ng/ml; and IL-17, 2 ng/ml), human or mouse noggin (200 ng/ml), or dexamethasone (DEX, 100 nM) or a combination of TTII and noggin or dexamethasone and noggin as described in the figure legends. Cell layers were assayed for alkaline phosphatase activity as previously described [Rifas et al., 2003].

Alizarin Red Staining

Cells were incubated in differentiation medium and treated with TTII, BMP-2, BMP-6, or BMP-7 at 100 ng/ml, human or mouse noggin (200 ng/ml), or DEX (100 nM) for 10 days or 3 weeks as noted in the figure legends. Medium

was changed every 3–4 days and test agents readded each time. For noggin experiments with BMPs, TTII, or DEX, the noggin and test agents were pre-incubated together in medium for 1 h prior to addition to cells. After the appropriate incubation time, cell layers were fixed and stained with a 1% solution of Alizarin red, pH 4.1, for 10 min, followed by three rinses with MilliQ water then a 10 min wash with PBS, as previously described [Stanford et al., 1995] with some modification. Quantitation of Alizarin red staining was performed as previously described [Rifas, 2006].

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

HMSC were seeded into 100 mm culture dishes, grown to confluence, and incubated for 24 h with either medium alone, noggin, Dex, TTII, or a combination of DEX or TTII with noggin. Media were aspirated and total RNA extracted using a Qiagen RNeasy mini kit (Qiagen, Inc., Valencia, CA). The isolated RNAs were treated on column with a DNase digestion kit (Qiagen) according to the manufacturer's instructions to eliminate genomic DNA contamination. RT-PCR was performed using a one-step RT-PCR Kit (Qiagen). Amplification was performed on 100 ng total RNA per sample using the following primers: ACTRII (sense) 5′-GCTGACTTTGGCTTGGCTGTTC-3′ (antisense) 5′-TCC TGGGCTTAGATGCTTGACTC-3′ (541nt) [de la Pena et al., 2005]; BMP-2 (sense) 5′-TCAAGCCAAACACA AACAGC-3′ (antisense) 5′-ACGTCTGAACAATGGCATGA-3′ (200nt) [Bunger et al., 2003]; BMP-6 (sense) 5′-CTCAACCGCAAGAGCCTTCTGG-3′ (antisense) 5′-TCTTTGTGGTGTCG CTGACGAG-3′ (167nt); BMPRIA (sense) 5′-AAGGTGACAGTACACAGGAAAC-3′ (antisense) 5′-TGGACAGTGCCCTGAGCAATA-3′ (240nt) [de la Pena et al., 2005]; DLX-5 (sense) 5′-TTG CCCGAGTCTTCAGCTAC-3′ (antisense) 5′-TCTTTCTCTGGCTGGTTGGT-3′(254nt) [Zuk et al., 2002]; Noggin (sense) 5′-ACTAGAGCTCGCAGCACTATCTCCACATCCGC-3′ (antisense) 5′-ACT TAGAAGCTTCTAGCACGAGCACTTGCACTCGG-3′ (641nt) [Sutherland et al., 2004]; Osteocalcin (sense) 5′-ATGAGAGCCCTCACACTCCTC-3′ (antisense) 5′-GCCGTAGA AGCGCCGATAGGC-3′ (297nt) [Hay et al., 2000]; Osterix (sense) 5′-CAAGCACTAAT GGGCTCCTTTCAC-3′ (antisense) 5′-TGGGCAGACAGTCAGAAGAGCTGT-3′ (165nt); Runx-2 (sense) 5′-CCGCACGACAACCGCACCAT-3′ (antisense) 5′-CGCTCCGGCCC ACAAATCTC-3′ (289nt) [Veis et al., 2000]: β-actin (sense) 5′-TGTGCCCATCTACGA GGGGTATGC-3′ (antisense) 5′-GGTACATGGTGGTGCCGCCAGACA-3′ (439nt) [Bunger et al., 2003]; GAPDH (sense) 5′-GCTTGTCATCAATGGAAATCCCATCACCAT-3′ (anti-sense) 5′-CTTGAGGCTGTTGTCATACTTCTCATGGTT-3′ (250nt).

Statistical Analysis

Group mean values were compared by ANOVA using Fisher's least significant difference post-hoc test or Student's *t*-test as appropriate. A *P*-value less than 0.05 was considered significant.

RESULTS

Noggin Induces HMSC Differentiation into the Osteoblast Phenotype

We have previously demonstrated that T cell cytokines induce BMP-2 in HMSC [Rifas, 2006]. Since noggin inhibits the action of BMPs [Zimmerman et al., 1996], we examined whether this BMP antagonist would effectively inhibit induction of HMSC into the osteoblast phenotype when stimulated with T cell cytokines or dexamethasone (Fig. 1). Our first series of experiments were designed to test whether human noggin would inhibit T cell cytokine induction of HMSC into the osteoblast phenotype (Fig. 1A). Stimulation of HMSC for 3 days with a dose curve of noggin alone or a dose curve of noggin in the absence or presence of TTII resulted in the induction of alkaline phosphatase. The results showed that noggin effectively induced AlkP rapidly at a dose as low as 50 ng/ml and was additive with TTII over the entire dose range. Since this result was totally unexpected, we then examined the effect of the highest does of noggin (200 ng/ml) in the rest of the experiments. Stimulation with either ACTTCM or TTII for 7 days (Fig. 1B) resulted in a significant increase in alkaline phosphatase as expected [Rifas, 2006]. However, surprisingly, when HMSC were stimulated with a combination of ACTTCM or TTII and human noggin, a significant additive stimulation, rather than an inhibition, of alkaline phosphatase was found. We then asked whether noggin would alter the effect of DEX, a very potent differentiation-inducing agent [Cheng et al., 1994]

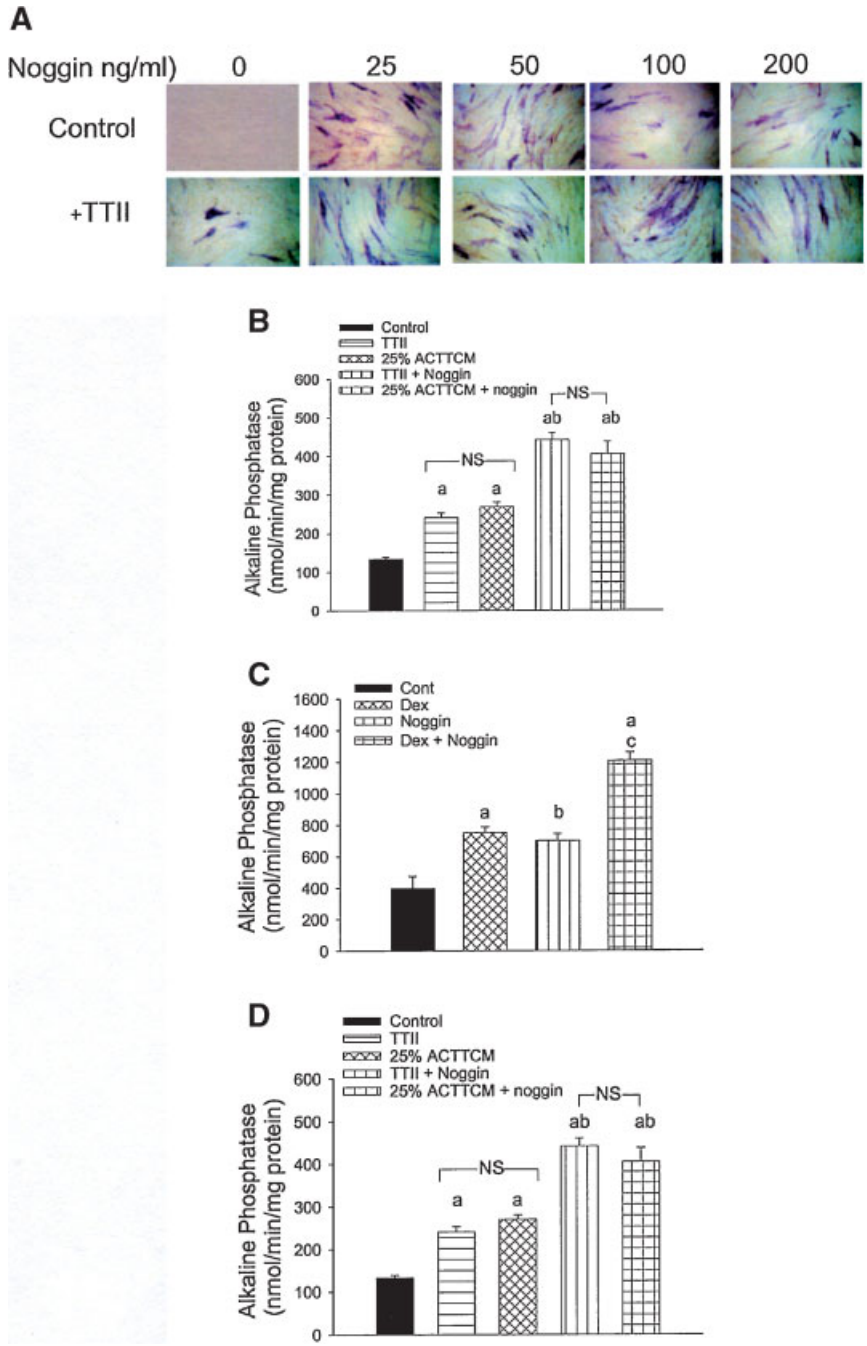

Fig. 1. The effect of noggin on HMSC differentiation into the osteoblast phenotype. In all the experiments, cells were incubated in differentiation medium with the agents and for the times indicated then either stained for AlkP or lysed and assayed for AlkP as a measure of osteoblast commitment as described in Material and Methods. **A**: Effect of human noggin alone and in combination with TTII on HMSC differentiation. Cells were treated for 3 days with a dose curve of human noggin (0–200 ng/ml) or a dose curve of noggin in the presence of TTII, then stained for alkaline phosphatase as described in Materials and Methods. **B**: Cells were treated with medium alone (control), 25% ACTTCM or TTII alone, or in the presence of 200 ng/ml human noggin for 7 days. a = $P < 0.001$ verses control; b = $P < 0.001$ verses either TTII or ACTTCM alone or control. **C**: Cells were treated with medium alone (control), Dexamethasone (DEX, 100 nM), human noggin, 200 ng/ml, or DEX plus human noggin (200 ng/ml) for 6 days. a, Noggin versus cont, $P = 0.002$; b, Dex versus noggin, NS; c, DEX + noggin versus noggin, $P < 0.001$. NS = not significant. **D**: Cells were treated with medium alone (control) or TTII, 200 ng/ml mouse noggin, 200 ng/ml human noggin, or a combination of TTII with either noggin for 6 days. a = $P < 0.001$ versus control or TTII + noggin versus TTII alone. b = $P < 0.01$ versus control. c = $P < 0.001$ versus noggin alone or TTII alone. NS, not significantly different. In all experiments, data represent mean ± SEM of three independent determinations. Significance determined by ANOVA with comparison made using the Fishers least significant difference post-hoc test.

(Fig. 1C). Dexamethasone significantly induced alkaline phosphatase within 6 days as expected, and again, noggin alone significantly induced alkaline phosphatase twofold over control. But more interestingly, the combination of noggin and DEX resulted in an approximately twofold increase above either noggin or DEX alone suggesting an additive effect. To insure that this effect was not due to the preparation of human noggin used in the above experiments, HMSC were treated for 6 days with either TTII or mouse or human noggin or a combination of TTII with mouse or human noggin and alkaline phosphatase activity measured (Fig. 1D). When HMSC were treated with either human or mouse noggin individually, both noggins significantly stimulated alkaline phosphatase activity more than twofold over control. When either mouse or human noggin was tested in combination with TTII, the combinations induced a twofold increase in alkaline phosphatase over TTII alone. These four experiments demonstrated that noggin not only does not inhibit HMSC differentiation, but in fact can induce differentiation alone and act in concert with T cell cytokines or DEX.

Induction of Mineralization by Noggin and BMPs

Since noggin-induced alkaline phosphatase in HMSC, but has been reported to either inhibit BMP induction of murine stromal cell differentiation [Gazzerro et al., 2003], or have no effect on osteoblast differentiation [Nifuji et al., 2004], we examined the effect of noggin and BMPs on mineralization in HMSC (Fig. 2). All the BMPs tested (BMP-2, BMP-6, and BMP-7) induced mineralization in HMSC within 10 days. Noggin alone potently induced mineralization in HMSC confirming its ability to induce the full mineralizing osteoblast phenotype in HMSC. Unexpectedly, noggin did not inhibit the effect of BMP induction of the osteoblast phenotype. Interestingly, the combination of BMP-2 and noggin showed a significant additive effect on HMSC mineralization. These data clearly demonstrate that noggin acts anabolically in human HMSC and does not inhibit BMP signaling.

Noggin has Different Effects on the Induction of Mineralization by TTII or DEX

Having established the effect of noggin on mineralization and that noggin did not inhibit

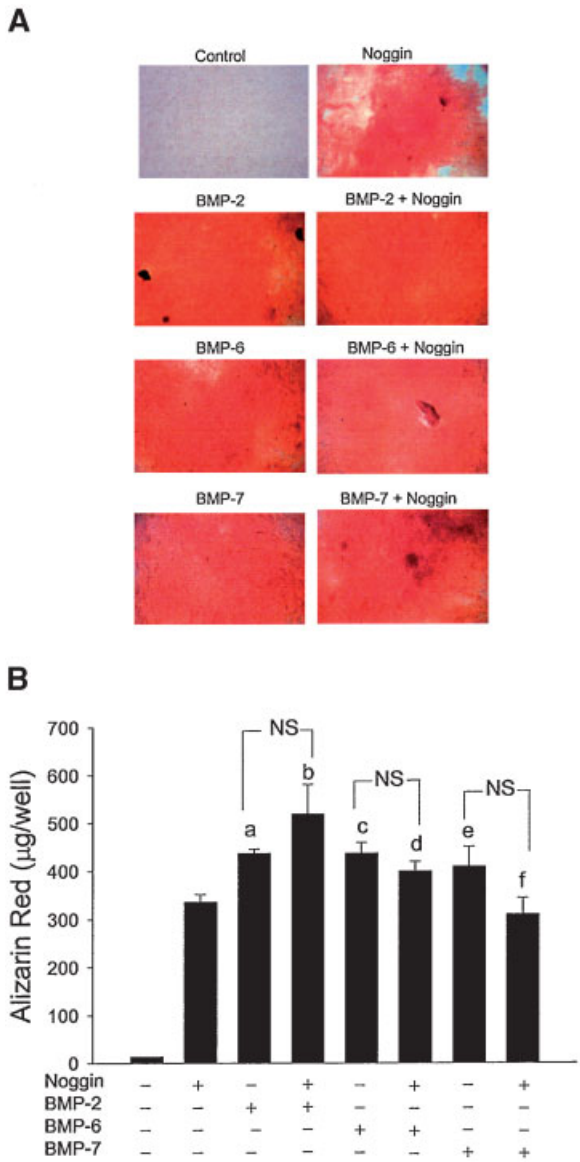

Fig. 2. Noggin induces mineralization, but does not effect BMP induction of mineralization, in HMSC. **A**: Cells were grown to confluence then treated for 10 days with BMP-2 (100 ng/ml), BMP-6 (100 ng/ml), or BMP-7 (100 ng/ml) in the absence or presence of recombinant mouse noggin (200 ng/ml). For the combination assay, the medium was prepared and both BMP and noggin added and allowed to incubate together for an hour prior to addition to the cells. After 10 days the cell layers were fixed and stained with Alizarin Red as described in Material and Methods. **B**: Quantitation of alizarin red staining in cultures. Data represent the mean ± SEM of two independent experiments. Significance determined by ANOVA with comparison made using Fisher's least significant difference post-hoc test. a = $P < 0.001$ versus control; b = $P < 0.001$ versus noggin alone; c–f, $P < 0.001$ versus control, P = NS versus noggin; NS = not significantly different.

BMPs, we examined the effect of Noggin alone or noggin in combination with TTII or DEX. Noggin alone again induced extracellular matrix mineralization (Fig. 3). Both TTII- and DEX-induced HMSC mineralization. Interestingly, when HMSC were treated with both TTII and noggin, noggin did not inhibit TTII-induced mineralization and in fact showed a slightly additive effect with TTII. Surprisingly, the presence of noggin with DEX resulted in an approximately threefold increase in mineralization over that induced by DEX alone and a 30-fold increase over that of noggin alone suggesting that noggin and Dex act synergistically to induce the mineralizing osteoblast phenotype. These data also demonstrate that noggin functions somewhat differently with T cell cytokines and DEX but supports the notion that noggin is an effective anabolic agent.

TTII Induces Noggin Gene Expression

Since we found that noggin plays a pivotal role in HMSC differentiation, we determined whether TTII would induce the expression of noggin. Within 4 h after stimulation with TTII, noggin gene levels were found to be nearly sevenfold over control (Fig. 4).

Effect of Noggin, TTII, and DEX on Osteogenic Genes

We next examined the effect of TTII, DEX, noggin alone, or a combination of TTII or DEX with noggin, on several genes that are specific

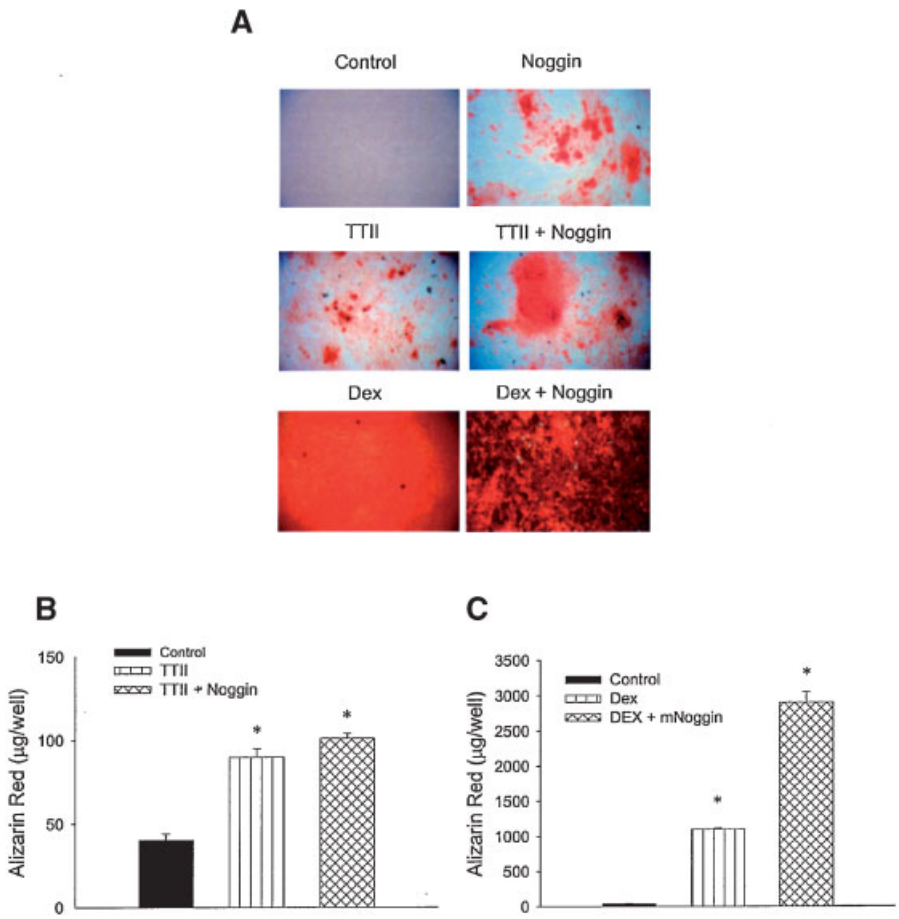

Fig. 3. Noggin enhances mineralization in HMSC induced by DEX and TTII. HMSC were treated for 21 days with TTII, noggin (200 ng/ml), Dex (100 nM), or a combination of TTII and noggin (200 ng/ml), or DEX and noggin in mineralization medium as described in Materials and Methods. **A**: Alizarin red staining of the cell layers. **B**: Quantitation of the Alizarin Red staining of cells treated with TTII, noggin, or TTII plus noggin as shown in A. a, $P < 0.001$ versus control; b, NS versus noggin; c, $P = 0.001$ versus noggin. **C**: Quantitation of the Alizarin Red staining of cells treated with DEX, noggin, or DEX plus noggin as shown in A. a, $P < 0.001$ versus control; b, $P < 0.001$ versus noggin; c, $P < 0.001$ versus noggin. Significance determined using Student's *t*-test. Data represent the mean ± SEM. Representative of two independent experiments.

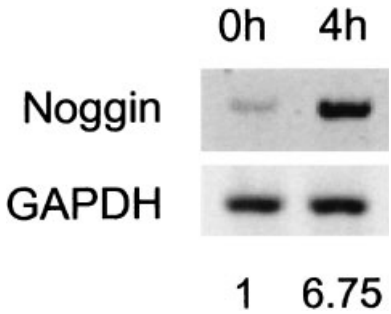

Fig. 4. TTII rapidly induces Noggin expression in HMSC. HMSC were incubated with medium alone (control) or TTII for 4 h then total RNA isolated and subjected to RT-PCR. The numbers below the figures represent fold change from control normalized to GAPDH. Representative of two independent experiments.

for osteoblast differentiation. We also examined noggin expression as well (Fig. 5).

Within 24 h Runx2 expression was upregulated 2.6-fold by DEX and 1.8-fold by TTII. Noggin slightly reduced upregulation of Runx2 expression by DEX while slightly increasing induction by TTII. Noggin had no direct effect on Runx2.

Osterix expression was unchanged by noggin but was upregulated 30% by DEX. Noggin inhibited the induction of osterix by DEX to a level below that of control. TTII slightly inhibited osterix expression and noggin did not change the effect. None of the treatments regulated DLX5.

Interestingly, noggin was a major stimulator of osteocalcin gene expression, inducing it to nearly fivefold over control. Dex and TTII were less effective than noggin alone with twofold increases. Surprisingly, noggin inhibited DEX stimulation of osteocalcin down to control levels while it had no effect on the elevated induction by TTII.

Noggin stimulated its own gene expression approximately fivefold. But interestingly, DEX induced a 12-fold increase in noggin gene expression and TTI induced a eightfold increase. When noggin was added simultaneously with DEX, noggin reduced the DEX induction of noggin by nearly half while not affecting TTII induction of noggin to the same extent. This data suggests that noggin may be self-regulating, feeding back to control is own expression.

Noggin-induced BMP-2 gene expression greater than twofold as did DEX but noggin had no effect on BMP-6 gene expression, which was only induced by DEX. And lastly, examination of two receptors that regulate BMP signaling in HMSC, BMPRIA, and ActRII, showed that BMPRIA was not regulated but noggin induced a nearly twofold increase in ActRII.

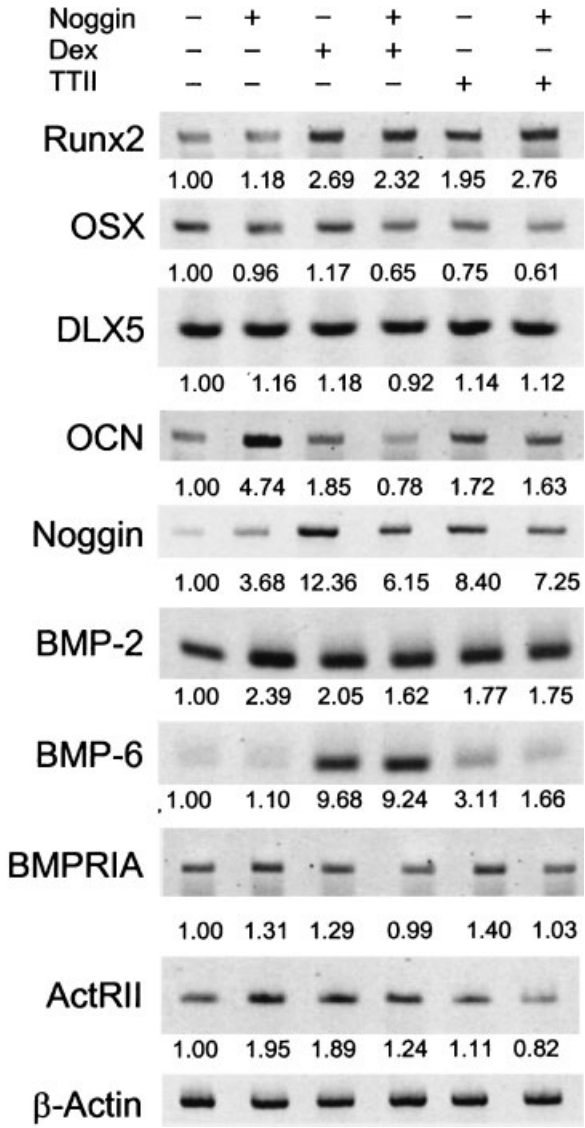

Fig. 5. Effect of TTII, dexamethasone, and noggin on osteoblast specific gene expression in HMSC. HMSC were incubated in differentiation medium and cells treated for 24 h with medium alone (control), dexamethasone (DEX,100 nM), TTII, noggin (200 ng/ml), or a combination of TTII and noggin, or DEX and noggin. RT-PCR was carried out with 100 ng total RNA. All genes were normalized to β-actin. The numbers below the bands represent fold change from control. Representative of three independent experiments.

DISCUSSION

This study is the first to report an anabolic effect of noggin in human mesenchymal stem cells. We have previously demonstrated that

four cytokines released by activated T cells, TNF-α, TGF-β, IFN-γ, and IL-17 (TTII), synergize to induce BMP-2 in HMSC and induce alkaline phosphatase and extracellular matrix mineralization [Rifas, 2006]. In order to explore more fully the role of BMP-2 induced by TTII, we examined whether noggin, a major antagonist of BMP-2 binding to its receptor [Zimmerman et al., 1996], would abrogate the effect of T cell induction of HMSC differentiation. Surprisingly, we found that noggin not only did not inhibit T cell induction of HMSC into the osteoblast phenotype, as judged by stimulation of alkaline phosphatase activity, it could, in fact, increase the effect of TTII.

Noggin has been described as an inhibitor of BMP-2, BMP-4, and BMP-7 by binding to specific regions of these molecules that recognize their cognate receptors [Zimmerman et al., 1996]. BMP-6 is antagonized by sclerostin [Winkler et al., 2003], another member of the BMP antagonist family [Sutherland et al., 2004], and, interestingly, noggin can bind sclerostin, resulting in a mutual attenuation of the activity of each BMP antagonist [Winkler et al., 2004]. The binding of noggin to BMPs occurs in a stoichiometric fashion on a per molar basis [Zimmerman et al., 1996] and is very tight. Because of this property, in our experiments, noggin was always preincubated with all the test agents studied prior to addition to the cells. Despite this precaution, the presence of noggin did not inhibit the action of the tested BMPs suggesting that another unknown mechanism is at play. One possibility is that noggin may have inhibited sclerostin, allowing for the action of BMP-2 in the TTII stimulated cultures and both BMP-2 and BMP-6 in the DEX-induced HMSC. However, we did not find sclersotin expression by RT-PCR (data not shown) and we have previously determined that neither BMP-6 nor BMP-7 protein is induced by T cell cytokines in this system [Rifas, 2006]. Although our data confirm induction of BMP-6 gene expression by DEX as previously reported [Liu et al., 2004], the lack of sclerostin expression coupled with the high noggin expression suggests that mutual inhibition of the inhibitors is not the mechanism. Thus, the mechanism of HMSC differentiation by DEX may be the high induction of BMP-6, while under inflammatory conditions either BMP-2 or noggin or both, may play significant roles depending on the circumstances.

One of the earliest events in HMSC commitment to the osteoblast phenotype is the expression of alkaline phosphatase activity [Rifas et al., 1994]. We examined the effects of TTII, DEX, or noggin alone or noggin in combination with TTII or DEX on alkaline phosphatase activity in HMSC. To our surprise, noggin did not inhibit either TTII or DEX induction of alkaline phosphatase, but rather acted in an additive manner to significantly increase their effects. Furthermore, and even more surprisingly, noggin alone was able to induce HMSC into a committed osteoblast lineage. These data are opposite to that seen in the murine system where overexpression of noggin in vivo [Gazzerro et al., 2003; Wu et al., 2003] or unknown levels of noggin secreted by cultured overexpressing mesenchymal stem cells in the presence of BMPs [Gazzerro et al., 2003] results in the suppression of BMP induction of the osteoblast phenotype. Furthermore, our data does not support the notion that noggin has no effect on osteoblast differentiation as previously reported in C1 mesodermal cells and embryonic murine skeletal cells [Nifuji et al., 2004]. These contradictions may reflect differences in the role of noggin between mouse and human. With regard to normal bone formation, noggin has been described to be essential in the process of skeletal development [Wijgerde et al., 2005] where it may balance out the effect of endogenous BMP signaling [Abe et al., 2000; Yoshimura et al., 2001; Nakamura et al., 2003; Peng et al., 2005]. Our data suggests that this may be the case in HMSC where noggin may either balance the effect of BMPs or act, in a novel manner, in their absence as an alternative ligand for BMP receptors to induce differentiation. Alternatively, the data suggests that noggin may dimerize with low levels of BMP-2, acting as a chaperone to enhance BMP signaling to induce differentiation. Further studies will be needed to define the mechanism.

Of interest is the finding that noggin did not inhibit mineralization induced by any of the BMPs or TTII, an inducer of BMP-2. This data is opposite to that seen in the murine system where noggin is a potent inhibitor of BMP-induced differentiation of stromal cells [Gazzerro et al., 2003]. Although we found that noggin acted in concert with TTII or DEX to induce alkaline phosphatase rapidly, after long term incubation, mineralization by TTII or BMPs was unaffected by noggin while noggin

synergized with DEX to increase mineralization. DEX is a potent inhibitor of mineralization in MC3T3-E1 mouse pre-osteoblasts due to its inhibition of autologous BMP-2 [Luppen et al., 2003]. However, human mesenchymal stem cells do not produce significant levels of autologous BMP-2 unless stimulated with a specific agent [Rifas, 2006], demonstrating a basic difference between mouse and human pre-osteoblastic cells. However, of particular interest is that during the process of mineralization, noggin synergizes with DEX while not that of TTII, suggesting that the mechanism by which BMPs and TTII induce mineralization is similar and distinct from that by DEX. Thus, the induction of BMP-2 by T cell cytokines appears to be a key pathway for inflammatory-induced bone formation [Rifas, 2006] which may be orchestrated by noggin.

In order to more fully understand the mechanism by which noggin may effect HMSC differentiation, we examined the expression of several osteoblast related genes in response to noggin itself and in response to inflammatory cytokines or DEX at the 24 h time point. We have found several key points. First, with respect to the action of the inflammatory cytokine cocktail, we found that T cells can induce noggin (as early as 4 h which maintains for at least 24 h), Runx2, BMP-2, and osteocalcin. Second, Noggin alone can induce BMP-2 and osteocalcin, but not Runx2, but increases the expression of ActRII, a receptor for BMP-2. Third, neither T cell cytokines nor noggin regulates BMP-6 gene expression while DEX induces Runx2, BMP-2, BMP-6, and noggin and exogenous noggin enhances the induction of alkaline phosphatase and mineralization. Thus, there seems to be a spatial/temporal activation of osteoblast differentiation by T cell cytokines which involves the early activation of noggin which may then stimulate BMP-2 followed by Runx2 then osteocalcin. The fact that noggin does not enhance the effect of TTII-induced noggin suggests that the T cell cytokines activate noggin which then induces its own gene expression. This data, taken together with our previous reports on T cell induction of the osteoblast phenotype [Rifas et al., 2003; Rifas, 2006] in which we found that BMP-2 expression is maximal at 16 h and Runx2 at 24 h, suggests that noggin may act as an early response gene.

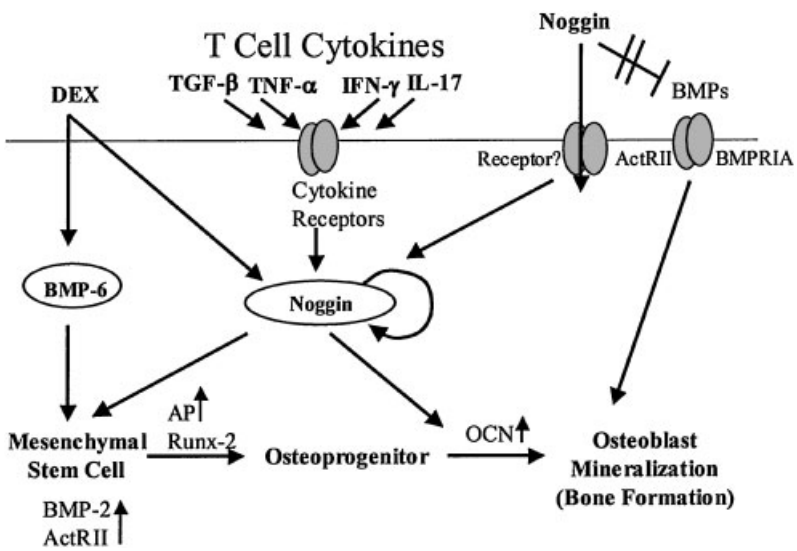

Fig. 6. Schematic of the regulation of HMSC differentiation by T cell cytokines, dexamethasone, and noggin. T cell cytokines can induce noggin, which in turn induces the upregulation of ACTRII and BMP-2 in the mesenchymal stem cell. Once BMP-2 binds to its receptors it induces alkaline phosphatase and Runx-2. The downstream effect of the initial Runx-2 activation would be induction of osteocalcin as the cell differentiates into a mineralizing osteoblast. Noggin may bind to either BMP receptors or perhaps an unknown receptor and can induce itself and may autoregulate its own expression. Dexamethasone induces BMP-2, BMP-6, Runx-2, and noggin in HMSC with the end result a full mineralizing osteoblast phenotype. The presence of noggin with DEX results in a higher level of alkaline phosphatase activity and mineralization. Although noggin can act additively with T cell cytokines and DEX to stimulate high levels of alkaline phosphatase, it does not act in concert with BMPs to induce a higher level of mineralization. Noggin does not inhibit the effect of BMPs or T cell cytokines on induction of the mineralizing osteoblast phenotype suggesting that the mechanism of T cell cytokine induction of the osteoblast phenotype is through noggin induction of BMP-2.

With respect to glucocorticoids, our data also points out that noggin appears to act synergistically with DEX to induce osteoblast differentiation as well, although a different mechanism may be involved since DEX stimulates BMP-6 gene expression while noggin does not. Nonetheless, the fact that DEX is a potent stimulator of noggin further suggests that noggin may be crucial in the induction of osteoblast differentiation by glucocorticoids as well.

Finally, the expression of osterix in the human has been reported to occur only in fetal osteoblasts and chondrocytes, but not in the adult cell types [Milona et al., 2003; Gao et al., 2004]. The lack of an effect of any of the agents tested on osterix suggests that in the human system, osterix may not regulate adult osteoblast differentiation. Similarly, BMP-2 has been reported to be a regulator of both osterix and DLX5 in the murine system [Lee et al., 2003]. Our data does not support this notion in HMSC, again pointing out a species difference with respect to the process of differentiation.

We have now demonstrated for the first time that in the human system, noggin plays a key role in the process of human mesenchymal stem cell differentiation into the osteoblast phenotype. The pathways that may be involved in this process is summarized in Figure 6. These data form a new paradigm with respect to the mechanism of BMP regulation of human bone formation and may help define the process by which inflammation regulates osteoblasts differentiation.

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