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Stage Specific Inhibition of Osteoblast Lineage Differentiation by FGF2 and Noggin

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Abstract Fibroblast growth factor 2 (FGF2) and noggin are two unrelated ligands of two distinctly different signaling pathways that have a similar inhibitory effect on osteoblast differentiation. Because of their differences, we postulated that they probably acted at a different stage within the osteoprogenitor differentiation pathway. This study was performed on primary murine bone cell cultures under conditions where alkaline phosphatase (AP) and type I collagen expression (Col1a1) were observed by day 7 (preosteoblast stage), followed by bone syaloprotein (BSP) at day 11 (early osteoblast) and osteocalcin (OC) by day 15–18 (mature osteoblast stage). FGF2 completely inhibited expression of AP and the mRNA transcript for Col1a1, while noggin showed only a partial inhibition of these markers of preosteoblast differentiation. However, the markers of differentiated osteoblasts (BSP and OC) were completely inhibited in both the FGF2 and noggin treated cultures, suggesting that noggin acts at later point in the osteoprogenitor differentiation pathway than FGF2. To further verify that the inhibition was occurring at a different stage of osteoblasts development, primary cultures derived from transgenic mice harboring segments of the collagen promoter driving green fluorescent protein (GFP) that activate at different levels of osteoblast differentiation were analyzed. Consistent with the endogenous markers, pOBCol3.6GFP and pOBCOL2.3GFP transgene activity was completely inhibited by continuous addition of FGF2, while noggin showed partial inhibition of pOBCol3.6GFP and complete inhibition of the pOBCol2.3GFP transgene. Upon removal of either agent, endogenous and GFP markers of osteoblast differentiation reappeared although at a different temporal pattern. This work demonstrates that FGF2 and noggin can reversibly modulate osteoblast lineage differentiation at different maturational stages. These agents may be useful to enrich for and maintain a population of osteoprogenitor cells at a defined stage of differentiation. J. Cell. Biochem. 88: 1168–1176, 2003.

Key words: FGF2; noggin; osteoblast differentiation; Col1a1 promoter; GFP

The feasibility of somatic gene therapy in treatment of genetic or degenerative skeletal disorders requires a strategy that will integrate a number of molecular and cellular manipulations. This includes the isolation and expansion of mesenchymal stem cells susceptible to genetic manipulation, the ability to express corrected genes in a differentiation appropriate manner and an effective way of inhibiting the expression of defective genes. Moreover, these procedures need to be performed while the osteoprogenitor cells are maintained in an undifferentiated stage that still retains a prolonged replication potential and the ability to differentiate when placed in the appropriate environment.

Marrow stromal cells (MSC) isolated from adult bone marrow can be induced to differentiate into a variety of mesenchymal tissues such as bone, cartilage, fat, muscle, fibrous tissue and stromal bone marrow components that are supportive of hematopoiesis [Owen, 1988; Friedenstein, 1995; Aubin, 1998b; Bianco et al., 1999; Dennis et al., 1999; Pittenger et al., 1999]. Under osteoblast inductive conditions (addition of dexamethasone, bone morphogenic proteins, ascorbic acid, β-glycerol phosphate) the lineage undergoes a series of maturational steps characterized by subsequential expression of bone related genes like type I collagen (Col1a1),

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alkaline phosphatase (AP), osteopontin (OP), bonesialoprotein (BSP), osteocalcin (OC), and other markers of osteoblast lineage [Aubin and Turksen, 1996; Aubin, 1998a; Kalajzic et al., 2002]. While markers of differentiated osteoblasts are well defined, markers of uncommitted and undifferentiated osteoprogenitors have yet to be established. STRO-1 and activated leucocyte cell adhesion molecule (ALCAM) [Gronthos et al., 1994; Bowen et al., 1997; Bruder et al., 1997], although not specific, can assess proportion of undifferentiated cells in the culture. These markers can be used to enrich population containing progenitor cells, which will be useful for initiating a cell population for a somatic gene therapy protocol. However, there is the additional problem of maintaining this population in an expanded but undifferentiated state, a concept that is not addressed in large proportion of studies presenting gene therapy treatments. The long-term success of any somatic gene therapy protocol will be difficult to interpret if the level of differentiation of the transplanted cell is not clearly characterized.

The biological effects of growth factors on the proliferation and differentiation of cells within the osteoblast lineage have been extensively studied. Basic fibroblast growth factor FGF2 has been reported to induce proliferation of cells within the mesenchymal lineages, while the reports of the effect on differentiation showed substantial variability dependent on the timing of the treatment, dose and the species used [Pitaru et al., 1993; Hanada et al., 1997; Martin et al., 1997; Debiais et al., 1998; Scutt and Bertram, 1999; Walsh et al., 2000; Zhang et al., 2002]. The osteoprogenitor lineage is capable of regulating it's own progression toward the differentiated stage by balanced production of BMP's and their antagonists noggin and gremlin [Gazzerro et al., 1998; Pereira et al., 2000].

In this study, we have examined the effects of FGF2 and noggin on the differentiation of osteoprogenitor cells in a primary marrow stromal cell culture model. In conjunction with the expression of markers of osteoblastic differentiation, promoter-GFP markers that activate at distinct stages of osteoblasts differentiation were used in these analyses. We have determined stage specific effects of FGF2 and noggin on osteoblastic differentiation. Our results show that FGF2 inhibits osteoblast lineage differentiation at the stage of undifferentiated osteoprogenitor while noggin prevents differentiation at the preosteoblastic level. In addition we demonstrate reversibility of the inhibition as the cells resume the process of osteoblastic differentiation after the termination of treatment.

MATERIALS AND METHODS

Cell Culture

Primary marrow stromal cell culture (MSC). Young adult (2-months old) mice were sacrificed by CO_2 inhalation. After the attached muscle was dissected the epiphyseal growth plate was removed from the femurs and tibiae. The marrow was collected by flushing culture media (αMEM supplemented with 100 U/ml streptomycin, 100 μg/ml penicillin, and 10% FCS) through the marrow space with a 25-gauge needle. The bone marrow clumps were resuspended using a 18-gauge needle followed by filtration through a 70 μm cell strainer (Falcon no. 2350). Approximately 20×10^6 cells per well were plated in 6 well Falcon tissue culture plates. On day 4, half of media containing nonadherent cells was removed and replaced with fresh media. Medium was completely changed on day 7 to αMEM supplemented with 50 μg/ml ascorbic acid, dexamethasone (10^{-8} M), and β-glycerophosphate (8 mM). Medium was changed every 2 days for the duration of the experiment. During the course of the study, the serum LOT that is consistently used through a series of experiments was changed. The new serum LOT was significantly more osteogenic as judged by a higher number of ALP positive colonies in control cultures (experiments shown in Figs. 3 and 4 vs. experiments presented in Figs. 1 and 2). However, the effects of FGF2 and noggin were consistent in cultures with either serum.

Treatment with noggin and FGF2. Cells were exposed to recombinant human FGF2 or recombinant human noggin 10–12 h after plating. Concentrations used in this study were 10^{-9} M for FGF2 and 0.25–0.5 μg/ml of noggin (Regeneron pharmaceuticals, Tarrytown, NY). Continuous treatment was obtained by supplementing the regular changes of media with FGF2 or noggin. In order to test the reversibility of differentiation inhibition, effectors were removed on day nine of culture and cells were grown under the control osteoblast inductive conditions (dexamethason 10^{-8} M, ascorbic acid 50 μg/ml and β-glycerolphosphate at 10 mM final concentration) until day 21.

Transgenic Mice and Analysis of Transgene Expression

Transgenic mice. pOBCol3.6GFP contains 3.6 kb of 5′ flanking sequence and 114 bp of the rat Col1a1 first exon ligated to the GFP and pOBCol2.3GFP contains a Hind III deletion of the 5′ flanking sequence to −2.3 kb. Generation and characterization of these transgenic mice has been previously described [Kalajzic et al., 2002].

Analysis of transgene expression. GFP expression in cell culture was visualized using an Olympus IX50 inverted microscope equipped with an IX-FLA inverted reflected light fluorescence (Olympus America, Inc., Melville, NY). A specific excitation wavelength was obtained using filters for GFPtpz (exciter: D500/20; dichroic: 525DCLP; emitter: D550/40) and GFPemd (exciter: D470/40; dichroic: 495LP; emitter: D525/50) and recorded with a SPOT-camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Phase contrast images were converted to grayscale.

Distribution of GFP expression in cell culture was observed with a FluorImager SI (Molecular Dynamics, Sunnyvale, CA) using a 515 nm emission spectrum at PMT settings of 800. Images were processed with ImageQuaNT software, and fluorescence is detected as dark signal on the white background.

Analysis of Osteoblastic Differentiation

Alkaline phosphatase histochemistry. Alkaline phosphatase histochemistry was performed for 20 min at room temperature according to manufacturer instructions, (SIGMA kit no. 86-R). During the incubation period, cells were protected from direct light exposure and drying. In order to assess the total number of colonies, same wells were counterstained with haematoxylin according to the manufacturer instructions.

Mineralization. Mineralization was assessed by von Kossa method. Briefly, cells were fixed for 10 min in 2% paraformaldehyde in 0.1 M cacodylic buffer. The plates were incubated with 5% silver nitrate solution for 30 min under a bright light, washed with water, treated with a 5% sodium thiosulfate solution for 2–3 min followed by washing with water and air drying.

RNA extraction and Northern blot analysis. Total RNA was isolated from the primary cultures using TRIzol Reagent (GibcoBRL) according to manufacturer's instructions. In addition, the isopropanol precipitate was redisolved in 300 μl of GTC buffer (4.5 M guanidinium isothiocyanate, 1 mM β-MSH, 15 mM Sodium *N*-lauryl sarcosine, 10 mM Na citrate, pH 7.0) followed by 300 μl isopropanol. RNA was separated on a 2.2 M formaldehyde, 1% agarose gel and transferred onto nylon membrane (Maximum Strength Nytran, Schleicher & Schuell). The membranes were probed with cDNA fragments for alcam [Bowen et al., 1997], rat COL1A1 (pα1R2), mouse type III collagen [Metsaranta et al., 1991], mouse OC fragment, mouse bone sialoprotein [Young et al., 1994], and mouse osteopontin [Craig et al., 1988]. Probes were radiolabeled using the random primer method using (α^{32}P) dCTP (New England Nuclear; 3,000 Ci/mM) obtaining approximately 1×10^9 cpm/μg. Filters were hybridized with 3×10^6 cpm/μl at 42°C in 50% formamide, 5 × SSPE (1 × SSPE = 0.149 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 1.2 × Denhardt's, 0.5% sodium dodecyl sulfate [Sambrook et al., 1989]. Filter were washed with once in 6 × SSPE and 0.5% SDS for 10 min at room temperature, once in 0.1 × SSPE and 0.1% SDS for 10 min at 37°C and once in 0.1 × SSPE and 0.1% SDS for 10 min at 65°C.

In vivo analysis of osteoblastic differentiation. In vitro expanded and passaged marrow stromal cells derived from pOBCol2.3GFP transgenic mice were cultured for 3 weeks in the presence of 10^{-9} M FGF2. Their osteogenic potential was examined after they have been subcutaneously implanted in C.B-17/IcrHsd scid bg imunodeficient mice (Harlan, Indianapolis, IN). Briefly, cells were trypsinized in 0.25% trypsin, 1 mM EDTA, followed by centrifugation and cell pellet was resuspended in tendon rat type I collagen gel solution (Becton Dickinson Labware, Bedford, MA) and allowed to polymerize at 37°C in 96 well plate [Rutherford et al., 2002]. Two hundred microliters of collagen gel with MSC were surgically implanted under the skin of animals. Five weeks later mice were euthanatized and formed tissues were collected. Tissue was fixed for 48 h in 4% paraformaldehyde, decalcified in 15% EDTA for 24 h, and embedded in paraffin (paraplast X-TRA tissue embedding medium, Fisher, Pittsburg) at 56°C. Five mirometers thick sections were deparaffinized and examined by fluorescent microscopy using FITC/Texas Red fluorescent cube of Zeiss Axiovert 200 M microscope and photographed by Axiocam digital

camera. Same sections were stained with hematoxylin and eosin and imaged.

RESULTS

Inhibitiory Effect of FGF2 on Osteblastic Differentiation

Differentiation of osteoprogenitor cells in primary MSC cultures was characterized by the expression of number of osteoblastic markers. ALP positive colonies and type I collagen expression is evident by day 7–9 of culture followed by BSP and osteocalcin expression as well as mineralization of bone nodules at later time points. Continuous treatment with FGF2 completely inhibited expression of preosteoblastic markers (ALP, Col1a1, Col3a1) at all time points and prevented the culture to express any of the other markers of mature osteoblast stage (BSP, OC) (Fig. 1A,B). However cells did proliferate under the FGF2 treatment and generated larger colonies with cells exhibiting fibroblastic phenotype (data not shown). Differentiation of osteoprogenitor population was inhibited and population of undifferentiated cell was generated as detected by increase in expression levels of activated leucocyte cell adhesion molecule (ALCAM) a marker of mesenchymal stem cell population and a strong increase in osteopontin expression which exhibits high levels of expression in undifferentiated primary MSC cultures (Fig. 1B).

Inhibitiory Effect of Noggin on Osteblastic Differentiation

Continuous treatment with noggin partially reduced the ALP expression (Fig. 2A) and completely inhibited the expression of late markers of osteoblast differentiation (BSP and OC). Expression of Col1a1 and Col3a1 was diminished only at later time points (day 15, 18) (Fig. 2B). ALCAM expression was unchanged and the osteopontin was expressed at equal level in control and treated cells. This suggested that the cells treated with noggin are blocked at level of osteoblastic differentiation where the preosteoblastic markers were acquired, but a full osteoblast differentiation cannot be achieved without the presence of high BMP levels, that are blocked by noggin treatment.

Analysis of Stage Specific Inhibition of Osteoblast Differentiation Using Transgenic Markers of Osteoblast Lineage

MSC derived from pOBCol3.6GFP transgenic mice (Fig. 3A) have low level of transgene expression from day 5–9 in cells that exhibit fibroblastic shape and form colonies (Fig. 3A, day 9). In untreated cells this expression shows an additional increase with the appearance of nodule formation and mineralization. GFP can be detected in nonmineralized and mineralized regions of the control culture. FGF treated cells did not show any detectable transgene expression at all time points. Noggin treatment inhibited the pOBCol3.6GFP expression levels that are associated with secondary increase related to mature osteoblast formation. Low level of pOBCol3.6GFP expression was not affected with noggin treatment.

In control cells the 2.3 promoter activates in differentiated osteoblast (Fig. 3A, day 15). In contrast to pOBCol3.6GFP transgene, pOB-

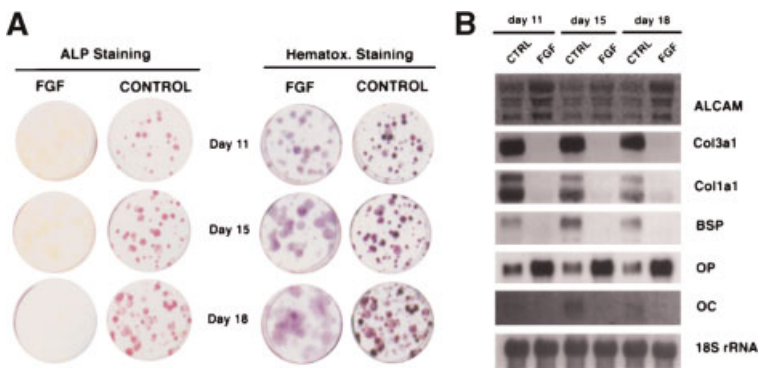

Fig. 1. Effects of continuous treatment with FGF2 on osteoblastic differentiation. Primary marrow stromal cell cultures were continuously treated with FGF2 at 10^{-9} M concentration and analyzed for the expression of bone marker genes by histochemical and mRNA analysis. **A**: Complete inhibition of alkaline phosphatase expression was detected at all three time points (day 11, 15, and 18) while, hematoxylin counterstaining revealed the presence of large colonies in the cultures treated with FGF2. **B**: Northern blot analysis of mRNA extracted from different time points of primary marrow stromal cell culture. Complete inhibition of markers of osteoblastic differentiation is observed in cells treated with FGF2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

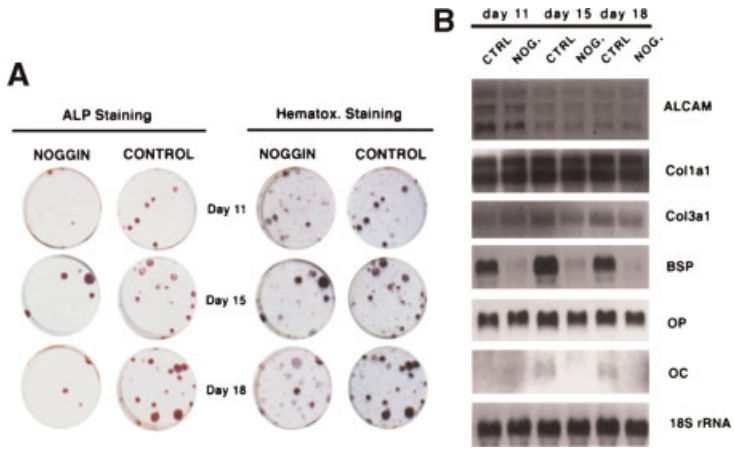

Fig. 2. Effects of continuous treatment with noggin on osteoblastic differentiation. Primary MSC culture were continuously treated with 0.5 μg/ml of noggin, and osteoblast differentiation was monitored by staining for ALP and hematoxylin and by assessing mRNA bone markers levels using northern blot analysis. **A**: ALP and hematoxyilin staining of cultures treated with noggin. A decrease in the number of ALP positive colonies but not a complete inhibiton of ALP expression was observed. **B**: Northern blot analysis of mRNA derived from noggin treated primary MSC cultures. Noggin completely inhibited BSP and OC mRNA levels while decrease in type I collagen expression can be detected only at later time points. Noggin addition did not change a characteristic decrease in ALCAM during osteoblastic progression. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Col2.3GFP expression was completely inhibited by both FGF2 and noggin. FGF2 and noggin prevented cells from differentiation into a mature osteoblast. A complete image of GFP expression in whole plate is shown in Figure 3B.

Ability of FGF2 and Noggin Treated Cells to Resume Osteoblastic Differentiation

To test the reversibility of the inhibitory effects on differentiation, cultures were treated with FGF2 and noggin for 9 days and subsequently grown in control media. Expression of bone markers in the recovering cultures was compared to continuously treated and control cultures. The effects of continuous FGF2 and noggin on osteoblastic differentiation are shown in Figure 4A,B. The strong level of osteocalcin expression in control cultures on day 21 was completely inhibited by noggin. Noggin did not affect Col1a1 expression in cultures at the pre-osteoblastic level (day 9) but did have a strong inhibitory effect once the culture contained a large proportion of mature osteoblasts (day 21). FGF2 treatment showed a complete inhibition of all bone markers at all time points.

Following termination of treatment with noggin, cells regained strong expression of Col1a1, ALP, and BSP and a low, but detectable osteocalcin level (Fig. 4A,B). Noggin treated and recovered cells show evidence of mineralization and therefore these cells can attain a stage of fully differentiated osteoblast once the treatment terminates. Following FGF2 withdrawal cells gained expression of alkaline phosphatase and a low levels of type I collagen expression suggesting that these cells can progress towards osteoblasts lineage (Fig. 4A,B). However, a full osteoblastic differentiation can be detected only after the cells treated with FGF2 are implanted subcutaneously in SCID/beige mice. A fully developed ossicle containing cortical, trabecular bone and bone marrow is detected 4 weeks after the implantation of FGF2 treated cells (Fig. 4E).

Following termination of treatment pOBCol3.6GFP expression can be detected in both FGF2 and noggin treated/recovered cultures. Weak expression is detected after FGF2 withdrawal while high level of GFP expression is evident after treatment with noggin was terminated (Fig. 4C). pOBCol2.3GFP activity was present after the treatment with noggin was discontinued, but only a few cells exhibited 2.3 promoter activity after FGF2 withdrawal (Fig. 4D) but was strongly active in the osteoblasts and osteocytes of the implanted ossicle. Our results indicate that osteoblast differentiation can be inhibited at different maturational stages and that treated cells still maintain their ability to progress towards a differentiated osteoblast once treatment is terminated.

DISCUSSION

Our study suggests that it is possible to achieve stage-specific inhibition of osteoprogenitor

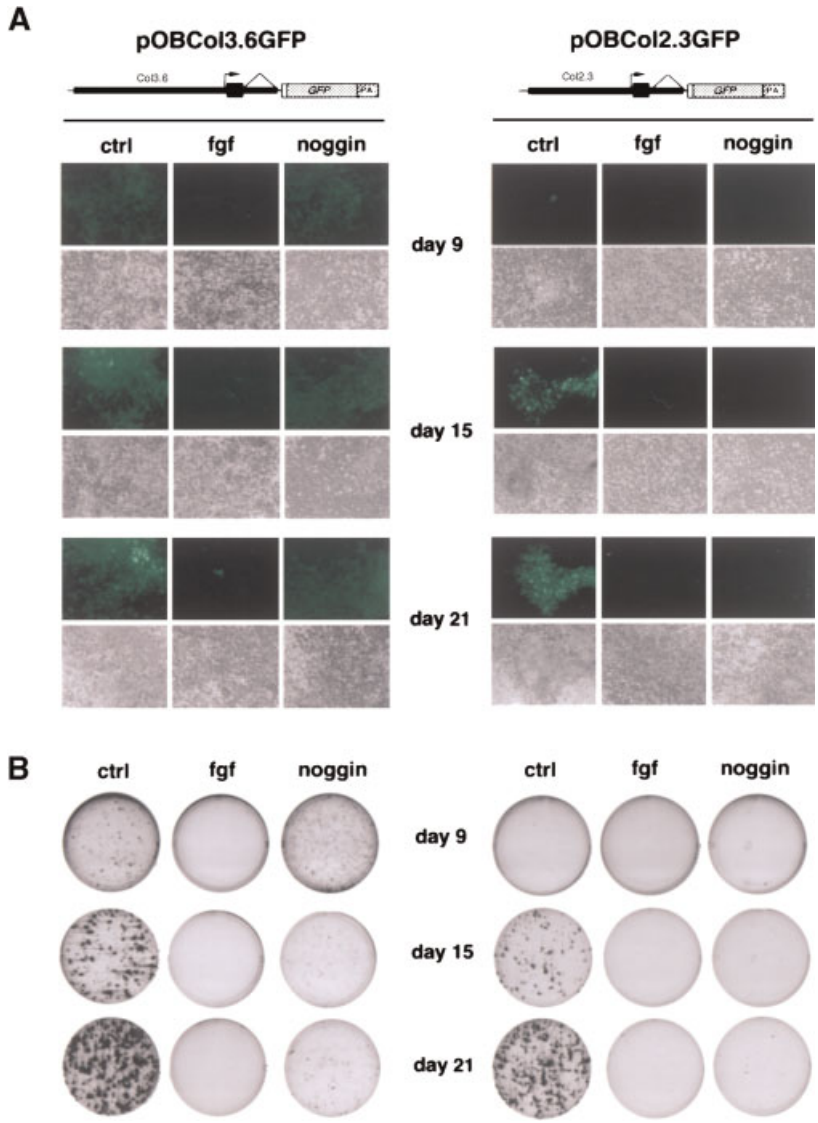

Fig. 3. Effects of FGF2 and noggin on pOBCol3.6GFP and pOBCol2.3GFP activity. **A**: MSC derived from pOBCol3.6GFP and pOBCol2.3GFP transgenic mice were continuously treated with FGF2 and noggin. Fluorescent and phase contrast images were taken on days 9, 15, and 21. Treatment with FGF2 completely inhibited promoter activity in both transgenes while noggin showed only a complete inhibition of pOBCol2.3GFP expression. Low level of pOBCol3.6GFP expression localized to forming colonies (day 9) was not inhibited by noggin treatment. However, the high level of 3.6 promoter activity that activates with mineralization is completely abolished and mineralization can not be detected (day 15, 21). **B**: Fluorimiger analysis of sister plates imaged on the same time points. This technique detects fluorescence in a live cultures and fluorescent signal can be visualized as dark spots on the whole tissue culture well. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

differentiation using FGF2 and noggin in primary MSC cultures. Cells continuously treated with FGF2 exhibited fibroblastic morphology (data not shown) and did not show any expression of markers characteristic for preosteoblast (Col1a1−, ALP−) and osteoblasts stage (BSP−, OC−). Interestingly, these cells expressed high levels of mesenchymal stem cell markers ALCAM and osteopontin that are regularly strongly expressed in first days of untreated MSC cultures when only undifferentiated stage cells of the lineage is present (ALCAM+, OP+). Therefore our experiments indicated that the osteoblastic differentiation was inhibited at the level prior to preosteoblastic stage (Fig. 5). Another intriguing observation is that this progenitor population of cells continue to expand in the presence of continuous FGF-2 and that these cells maintain their ability to progress to osteoblast lineage once FGF2 is

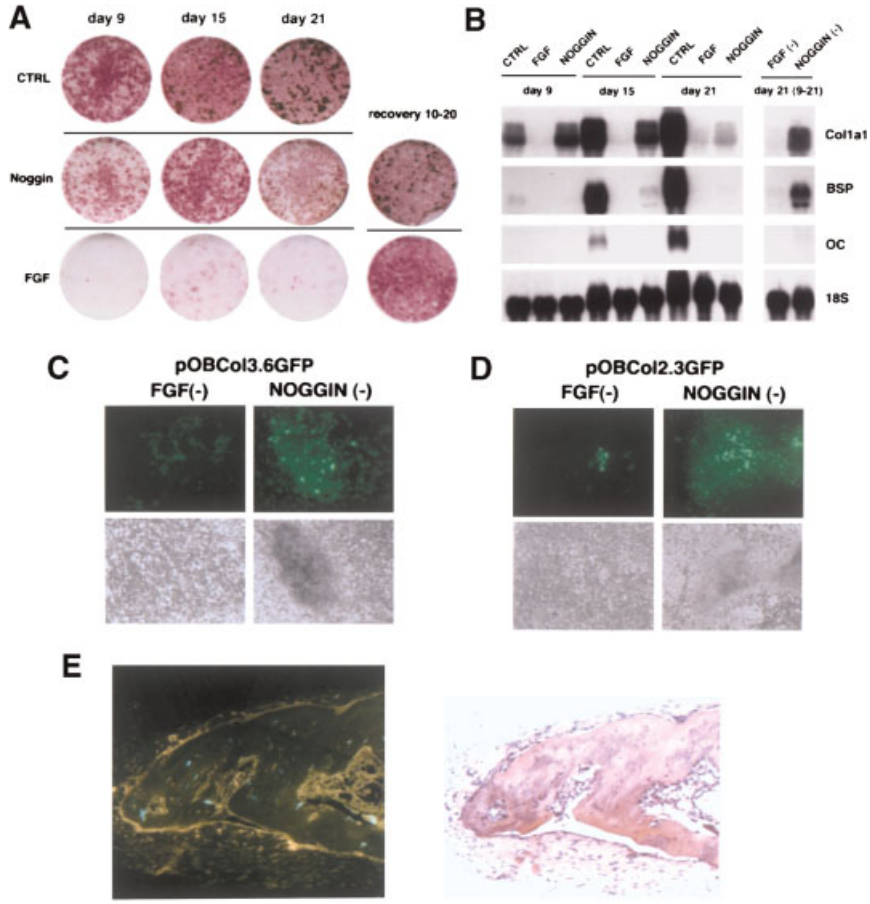

Fig. 4. Effects of FGF2 and noggin on osteoblastic differentiation: treatment and recovery. **A**: Primary MSCs were treated with FGF2 at concentration 10^{-9} M and noggin 0.25 μg/ml and markers of osteoblastic differentiation were measured on day 9, 15, 21 of treatment. Recovery group was treated until day 9 and after which the treatment was terminated and ability to resume osteoblastic differentiation was assessed. A: ALP staining and mineralization by von Kosa method. ALP positive staining was detected in FGF treated/recovered group while mineralization was not detected. Noggin treated/recovered group showed a strong ALP staining and a resumption of strong mineralization. **B**: Northern blot analysis of mRNA extracts showed a significant recovery of BSP and Col1a1 expression and low levels of OC mRNA in noggin treated/recovered group, while the FGF treated/recovered cells shows only a low levels of Col1a1 and BSP mRNA. **C**, **D**: GFP expression after termination of FGF and noggin treatment analyzed by fluorescent microscopy (as a control continuously treated cells are shown in Fig. 3A). **E**: Four-week-old heterotopic bone implant stained imaged under fluorescent light (**left panel**) and same position imaged after with hematoxylin-eosin staining (**right panel**). Note: pOBCol2.3GFP expression is detected in cells embedded within the bone matrix. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

removed. This was detected as the presence of diffuse and expanded staining for ALP after the FGF2 treatment has been terminated. Furthermore the ability of FGF expanded population of cells to achieve a stage of fully differentiated osteoblast was confirmed after examination of heterotopic bone implants generated by FGF treated cells. Similarly, a study analyzing effects of FGF2 on human MSC proliferation and differentiation indicated an increase in bone markers after the termination of treatment. Also, in vivo data is consistent with our observation that FGF can expand population of undifferentiated osteoprogenitors yielding an increased bone formation that occurs after in vivo implantation [Martin et al., 1997]. In vivo anabolic effect of FGF2 has been described in growing rats where basic FGF stimulated endosteal and endochondral bone formation [Nagai et al., 1995].

In contrast to prior observations that FGF2 induces osteoblastic differentiation in vitro we have evidenced that continuous treatment with FGF completely inhibits MSC in entering the osteoblast lineage pathway. It is likely that short treatment with FGF2 can expand the population of osteoprogenitors due to its proliferative effects. Therefore, after the induction

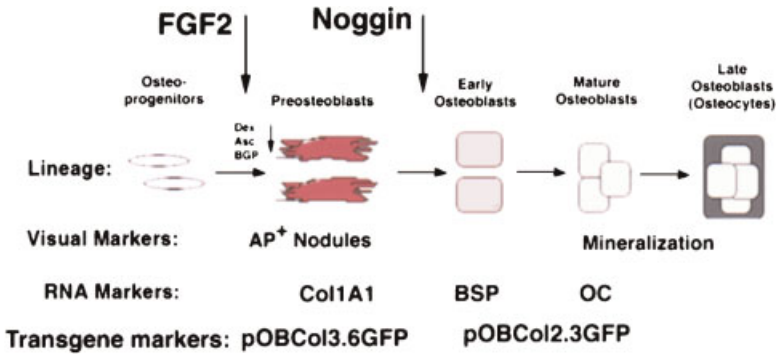

Fig. 5. Diagram of differentiation stage specific effect of FGF2 and noggin. FGF2 prevents the expression of preosteoblastic markers and keeps the lineage at the progenitor stage (ALP−, Col1a1−, pOBCol3.6GFP−). Noggin allows osteoprogenitors to enter the osteoblast lineage pathway characterized by ALP and Col1a1 expression but inhibits the differentiation to mature osteoblasts (BSP−, OC−). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of differentiation following termination of treatment, increased mineralization and expression of bone markers at later time points can be observed [Zhang et al., 2002].

Similarly to FGF2, cultures treated with the BMP inhibitor noggin show a complete inhibition of BSP and OC, however, in contrast to FGF2 only a partial inhibition of AP and Col1a1 at later stages of cultures. This indicates that noggin inhibits osteblastic differentiation at the stage of preosteoblast (ALP +, Col1a1 +, BSP−, OC−) a later stage then the one where FGF2 showed its inhibitory effect (Fig. 5).

It has been reported that cells of osteoblast lineage produce BMPs and that BMPs regulate the expression of themselves (data not shown) and the expression of their inhibitors (gremlin and noggin) [Gazzerro et al., 1998; Pereira et al., 2000]. Published data indicate that BMPs are necessary for induction of differentiation of osteoprogenitors into osteoblast lineage and that noggin treatment inhibits the expression of both osteocalcin and alkaline phosphatase (AP) [Abe et al., 2000]. Abe et al. [2000] presented data that that BMP-2 and -4 are expressed in the bone marrow where they participate in maintenance of the continuous supply of osteoblasts, and that BMP induced osteoblastic differentiation is a requirement for the osteoclast development. Clearly, it is difficult to compare these results to our data due to differences in basal conditions, cell density and noggin concentrations. An explanation for these differences could be in osteoblast inductive conditions that we have used in our culture system. Therefore noggin treatment in this case would exhibit the full effect only on maturation of preosteoblasts into fully differentiated osteoblasts. In accordance with our data is a study in which noggin was overexpressed by osteoblast lineage cells using osteocalcin promoter. Maturation of osteoblast was interrupted resulting in a phenotype of woven bone and fractures possibly due to the block in the maturation of osteoblasts or their dysfunctional performance [Devlin et al., 2000].

We utilized the stage specific inhibitory effects of FGF and noggin to further validate the utility of the Col1a1-GFP transgenic mice as a visual marker of osteoblastic differentiation in cell culture. Previously we have demonstrated that the pOBCol3.6GFP transgene activates at the stage of preosteoblastic differentiation while the pOBCol2.3GFP activates in mature osteoblasts [Kalajzic et al., 2002]. In the present study, FGF2 inhibited the expression of both transgenes, while noggin selectively inhibited the pOBCol2.3GFP expression indicating that noggin acts later in the lineage. These are the predicted responses of the transgenes as markers of an acquired level of osteoblastic differentiation. Noggin appears to have it most potent affect on cells between preosteoblastic and early osteoblast level of differentiation. Development of marker of differentiation prior to expression of pOBCol3.6GFP will be required to define the position within the lineage that is most affected by FGF2.

The expansion of osteoprogenitor cell is a primary interest of the somatic gene therapy. FGF2 or noggin treatment of progenitor cell cultures will provide an expanded population of cells at a defined level of differentiation, a requirement to judge the effectiveness of retroviral

transduction protocols and for transplantation studies in diseases of the skeletal system.

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