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Comparison of Proliferation and Differentiation of Calvarial Osteoblast Cultures Derived from Msx2 Deficient and Wild Type Mice

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A B S T R A C T

We analyzed proliferation and differentiation of calvarial osteoblasts derived from Msx2 deficient in comparison with wild type mice. Calvarial osteoblast cultures from five to eight days old Msx2 deficient, heterozygous and wild type mice were studied for difference in proliferation and differentiation. Proliferation rate was assessed by counting cell number, BrdU and Calcein AM labeling. Differentiation was assessed by Von Kossa and alkaline phosphatase staining, northern blot hybridization with bone differentiation markers, infection of cell cultures with retrovirus expressing GFP under the control of type I collagen promoter fragment. At day six, cell number in cell culture derived from Msx2 deficient mice was 20% lower then in culture from wild type mice. There were 16.8% BrdU labeled cells in cell culture from Msx2 deficient mice, 20.9% in culture from heterozygous mice and 21.6% in culture from wild type mice. Cell cultures from Msx2 deficient mice showed lower intensity of fluorescence when marked with Calcein AM then cultures from wild type mice. Von Kossa staining showed increased mineralization and northern blot analysis showed increased levels of bone differentiation markers in cell cultures derived from Msx2 deficient mice. GFP came on earlier in Msx2 deficient cultures after infection with Col2.3 GFP retrovirus. We conclude that calvarial osteoblasts derived from Msx2 deficient mice have a lower rate of proliferation and demonstrate increased osteoblastic differentiation when compared to osteoblasts derived from wild type mice.

Key words: *differentiation, Msx2 deficient mice, osteoblast, proliferation*

Introduction

Msx2 is a member of highly conserved Msx homeobox gene family[1]. The vertebrate Msx gene family is related to the *Drosophila msh* gene and includes Msx1, 2 and 3. Msx1 and Msx2 function as transcriptional regulators that control cellular proliferation and differentiation during embryonic development[2]. They are expressed in numerous tissues at many stages of development while *Msx3* is primarily expressed in the central nervous system. Strong expression of Msx2 is found at the extreme ends of the osteogenic fronts of calvarial sutures. Msx2 deficient mice[3] were viable but exhibited pleiotropic defects of skeletal and ectodermal organs and appendages, while heterozygous mice were unaffected. The calvarium of adult Msx2 deficient mice contained a large, midline foramen spanning the frontal bones, and the interparietal and supraoccipital bones were small and abnormally shaped. Osteoprogenitors in Msx2 deficient osteogenic fronts were reduced by 46% at birth. Msx2 expression in these cells[4,5] suggests that Msx2 is directly involved in calvarial bone growth. Msx2 deficient mice also have defects in limb endochondral bone formation[6], as well as defects of tooth[7], hair follicle[8], mammary gland[3] and cerebellum development[9].

Gain-of-function mutation of Msx2 causes Boston-type craniosynostosis, a condition caused by premature closure of cranial sutures[10]. However, the molecular basis of the relationship between this mutation and the disease has not been identified yet.

Msx2, homeodomain protein, is a transcription factor expressed very early in primary osteoblastic culture and inhibits collagen type I synthesis in ROS 17/2.8 cells[5]. It also inhibits osteocalcin gene promoter in MC3T3E1 cells[11].

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Msx1 and 2 do not require homeodomain DNA-binding sites for transcriptional repression[12]. Msx1 acts as transcriptional factor by binding to TAAT binding protein[13] while Msx2 inhibition of transcription is not quite understood yet. Both *Msx* genes control osteogenesis by regulating *Runx2* expression[2].

In our previous studies Msx2 was overexpressed in primary chicken calvarial osteoblast culture. Osteoblast differentiation of those cells was inhibited while antisense Msx2 expression inhibited proliferation and caused premature differentiation[14]. These results suggested that one of the biological roles of Msx2 was to inhibit differentiation and stimulate proliferation in preosteoblastic cells in the osteogenic front of the sutures, therefore playing an important role in the development of the skull.

To confirm these findings and our hypothesis, we extended studies into a mammalian system using Msx2 deficient mice as a model to compare proliferation and differentiation of calvarial osteoblast cultures derived from Msx2 deficient and wild type mice. We assessed and compared differentiation and proliferation rates of calvarial osteoblast cells derived from wild type, heterozygous and homozygous Msx2 deficient mice. Proliferation rate was assessed by counting cell number, BrdU and CalceinAM labeling. Differentiation assessment was performed by Von Kossa staining, hybridization of total cell RNA to collagen and osteocalcin probes and infection of the cells with Col2.3 GFP retrovirus, that expresses GFP as osteoblasts differentiate[15]. Based on those experiments we suggest that calvarial osteoblasts derived from Msx2 deficient mice have a lower rate of proliferation and demonstrate increased osteoblastic differentiation than cells derived from wild type mice. Heterozygous mice did not present significant differences in proliferation and differentiation when compared to wild type mice.

Materials and Methods

Mice genotyping

Msx2 deficient mice were generated as described in Satokata et al.[3,16]. Msx2 deficient mice were maintained on liquid diet due to abnormal dentition and were bred in C57BL/6J background. Animals were maintained in the Center for Laboratory Animal Care at University of Connecticut Health Center (UCHC) and procedures approved by UCHC Animal Care Committee. Male and female heterozygous animals were bred to produce offspring for experiments. After birth mice were notched and their tails were clipped for genotyping[3]. Msx2 deficient, heterozygous and wild type mice were genotyped by PCR using the primers Sxg2 (neo; 5'-TCTGGACGAAGAGCATCAGG--3'), Sxg4 (5'-CCCTCTCTGTCCTCTAGGAC-3') and Sxg5 (5'-GCCTGAGGGCAGCATAGGCT-3'). The wild type (359 bp) and mutant (650 bp) allele PCR products were amplified using Sxg4 and Sxg5, and Sxg2 and Sxg5, respectively, in separate reactions using two stage PCR program: 94°C for 1 min; 62°C for 1.5 min; 72°C for 2 min (3 cycles), 94°C for 1 min; 60°C for 2 min; 72°C for 2 min (30 cycles), 72°C for 5 min.

Primary mouse calvarial osteoblast culture

Calvariae were obtained, free of sutures and adherent tissue, from 5–8 days old mice, placed into cold PBS with penicillin/streptomycin (Life Technologies, Rockville, MD, USA) and placed on 4°C for 48–36 hours until genotyping was performed. According to genotyping results calvariae were divided into three groups: wild type, heterozygous and homozygous, that are processed separately as described in Dodig et al.[15,17]. Calvarial cells were isolated by four sequential 15-minute digestions in 0.05% trypsin (Life Technologies, Rockville, MD, USA) and 0.1% collagenase P (Roche Molecular Biochemicals, Indianapolis, IN, USA) at 37°C on a rocking platform (85 rpm). Fractions 2–4 were collected, resuspended in media, counted and plated at 150000 cells per 35 mm dish. Cells were grown in DMEM (Life Technologies, Rockville, MD, USA) with 10% fetal bovine serum (Life Technologies, Rockville, MD, USA) until confluence and then differentiation media containing αMEM (Life Technologies, Rockville, MD, USA), 10% fetal bovine serum, 50 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and 5 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA) was used to maintain the cells.

Assessment of differentiation

Alkaline Phosphatase staining – plates were rinsed with PBS, fixed with citrate-acetone formaldehyde fixative, and stained for enzyme activity using 0.25% AS-BI phosphate alkaline solution and fast red violet LB base (Sigma-Aldrich, St. Louis, MO, USA, procedure No.86). Plates were scanned using Umax Astra 4000U scanner (Umax Technologies, Dallas, TX, USA).

Von Kossa staining – Briefly after fixation, cells were exposed for 45 minutes to bright light in a 5% solution of AgNO3 (Sigma-Aldrich, St. Louis, MO, USA). The reaction was stopped with Na-thiosulphate[18], cells were washed and the cultures photographed. Plates were scanned using Umax Astra 4000U scanner.

Northern Blot analysis – RNA was isolated using TRIZOL reagent (Life Technologies, Rockville, MD, USA)[19]. 10 μg of total RNA was separated on 1% agarose (BMA, Miami, FL, USA) 1.1M formaldehyde gel and transferred to nylon membrane (Schleicher and Schuell, Keene, NH, USA). Membranes were hybridized[20] with mouse type I collagen and osteocalcin [^{32}P] CTP-labeled probes. Hybridization was at 42°C. Signal was scanned by 8-bit scanner Umax Astra 3400 and signal density was assessed by embedded algorithms of ImageTool 3.0 software (UTHSCSA, San Antonio, Texas, USA).

Assessment of proliferation

Cell counting – cell number was determined after trypsinizing the cells from the plate by counting using hemacytometer for three independent samplings per plate.

BrdU staining – at day 6 of the culture cells were incubated in 10 μM BrdU (Sigma-Aldrich, St. Louis, MO, USA) on 37°C for 90 min[21]. After incubation cells were fixed in ethanol, permeabilized and denatured with 2 N

HCl on 37°C for 1h and washed with 0.1 M borate buffer (pH 8.5). Cells were incubated for 1h with monoclonal anti-BrdU antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:1000, followed with biotinylated secondary antibody diluted 1:200 for 1h (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Avidin/biotin-HRP reagent was added afterwards and positive cells were visualized by adding DAB (Vectastain DAB kit, Vector Laboratories, Burlingame, CA, USA). Cells were counterstained with hematoxylin and counted under the microscope. Difference between cultures was analyzed with Statistica7 software (StatSoft, USA) and probability of error (P) was determined by t-test.

Calcein-AM – at days 1, 3 and 6 of the culture, cells were washed with PBS and treated with 2 μM CALCEIN--AM (Molecular Probes, Eugene, OR, USA) at 37°C for 20 min. Cells were washed with PBS again, fed with fresh media and examined under Zeiss Axiovert 200 invert microscope (Zeiss, Göttingen, Germany). Dishes were scanned under fluorescent optics using a 5x objective. Signal density was assessed by embedded algorithms of ImageTool 3.0 software (UTHSCSA, San Antonio, Texas, USA).

Results

Comparison of differentiation

Differentiation of the culture derived from wild type and Msx2 deficient mice was compared by observation of type I collagen and osteocalcin mRNA, alkaline phophatase staining, Von Kossa staining for minerals and activity of osteoblast specific 2.3 kb fragment of collagen promoter.

Mouse calvarial culture was observed at four different time points: day 4, 9, 14 and 20 (Figure 1A). Difference in differentiation became apparent in Von Kossa stained plates at day 14 and remained at day 20. Cultures derived from Msx2 deficient mice were more mineralized at later time points than culture derived from wild type animals. Cultures from heterozygous animals showed an intermediate phenotype. Northern blot analysis of the culture derived from Msx2 deficient mice showed upregulation of collagen (Figure 1B, C) and osteocalcin (Figure 1B, D) at later time points.

Calvarial cultures from wild type and Msx2 deficient mice were infected with ROSA Col2.3GFPemd virus. Col2.3 is a 2.3 kb fragment of the rat Col1a1 promoter that is active at a specific stage of osteoblast differentiation. There were more cells positive for GFPemd in the culture derived from Msx2 deficient than wild type mice (Figure 2), while culture from heterozygous mice resembled culture from wild type mice (data not shown).

Comparison of proliferation

Proliferation of the cell cultures from wild type, heterozygous and Msx2 deficient mice were compared by BrdU and Calcein-AM staining.

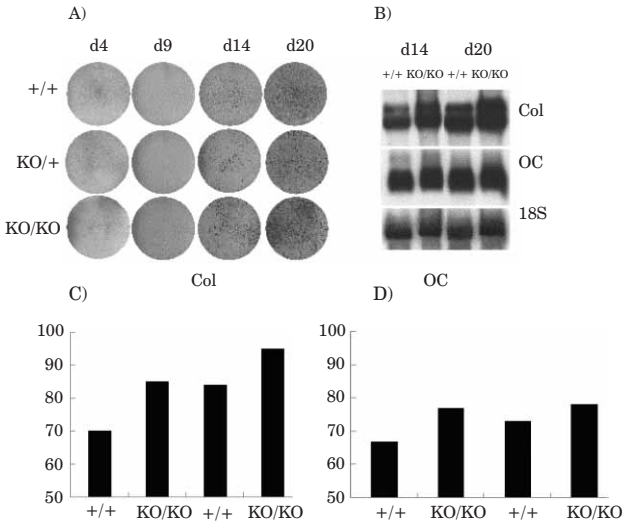

Fig. 1. Comparison of differentiation by alkaline phosphatase, Von Kossa staining and Northern blot analysis. Alkaline phosphatase, Von Kossa staining (A) and northern blot analysis (B) of collagen type I (Col) and osteocalcin (OC) of representatives of three separate cultures (+/+ – wild type, KO/+ – heterozygous and KO/KO – homozygous for Msx2 deficiency) show increased differentiation in cultures derived from Msx2 deficient mice; increase in signal density of Col (C) and OC (D) mRNA signals in cell culture from Msx2 deficient mice.

At day 6, cell cultures from wild type, heterozygous and Msx2 deficient mice were treated with BrdU in order to determine number of cells in S-phase of the cell cycle, which reflects the proliferation rate of the cultures. Culture from wild type mice had 21.6%, culture from heterozygous mice had 20.9% and culture from Msx2 deficient mice had 16.8% BrdU positive cells (Figure 3). Proliferation rates of cultures derived from wild type and Msx2 deficient mice presented statistically significant difference with $p<0.05$.

Cultures derived from wild type and Msx2 deficient mice were incubated with Calcein-AM at days 1, 3 and 6 in order to visualize cell number difference. The culture from wild type mice has more cells than the culture from Msx2 deficient mice and difference became greater with

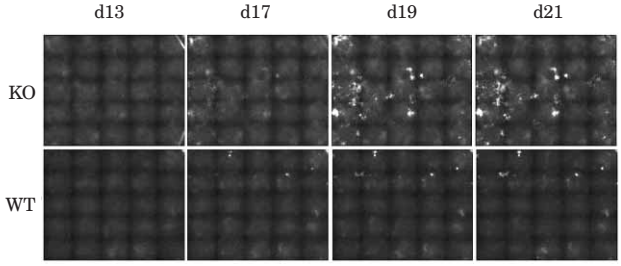

Fig. 2. Comparison of differentiation by infection with ROSA Col2.3 GFPemd. Culture derived from Msx2 deficient mice (KO) showed earlier and stronger activation (days 19 and 20) of osteoblast specific Col2.3 promoter then culture from wild type mice (WT). 30 mm plates were scanned using 5x magnification.

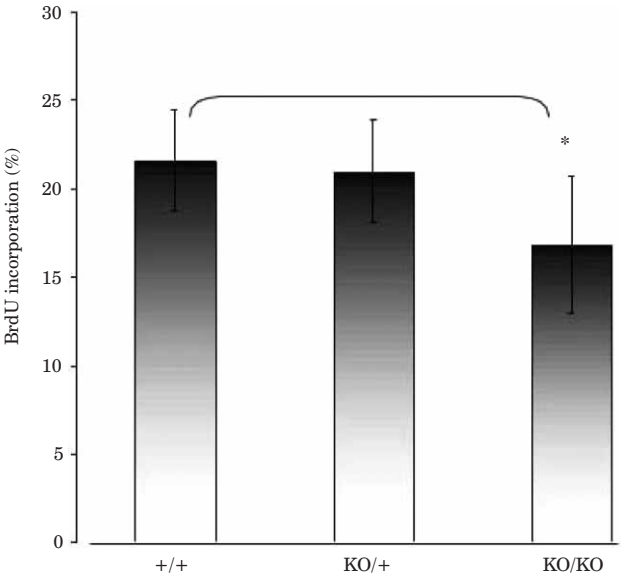

*Fig. 3. Comparison of cell number by BrdU staining. BrdU incorporation in 6 days old cells from culture derived from wild type mice (+/+) was 21.6%, from culture derived from heterozygous mice (KO/+) was 20.9% and from culture derived from knock-out mice (KO/KO) was 16.8%. Difference between cultures from wild type and Msx2-deficient mice was statistically significant (*p<0.05).*

time (Figure 4A, B). Culture from heterozygous mice did not differ from culture derived from wild type mice (data not shown).

Disscusion

In these studies we examined the role of Msx2 in the development of skull. In order to assess the function of Msx2 in this process, we compared differentiation and proliferation rates of primary calvarial osteoblast cultures from Msx2 deficient mice, mice heterozygous for Msx2 deficiency and wild type mice. We found that osteoblastic differentiation was induced in Msx2 deficient cultures. Furthermore, the proliferation of Msx2 deficient cultures was decreased. Those findings support the role of Msx2 in inhibiting differentiation and stimulating proliferation.

Msx2 is expressed primarily in cells at the extreme end of the osteogenic fronts of the cranial sutures and in the cells between the sutures. There is decreased or absent expression in the cells flanking the front[5]. Those results suggest that Msx2 could play the role in maintaining the committed osteoblasts of osteogenic front in undifferentiated stage as well as stimulating their proliferation, enabling the growth of cranial bones.

Calvarial cultures from Msx2 deficient mice showed decreased proliferation and increased differentiation. Differentiation was assessed by using well-known markers of osteoblast differentiation as alkaline phosphatase, collagen type I, osteocalcin, mineralization level, and a retroviral vector expressing GFP under the control of osteoblast specific 2.3 kb fragment of collagen type I promoter[22,23].

Boston type craniosynostosis is the human disease caused by gain-of-function mutation in MSX2 gene[6]. Transgenic mouse model with Msx2 overexpression has enhanced growth at the osteogenic fronts as well as increased BrdU labeling when compared to normal mice[4,24]. It is hypothesized that increased growth and proliferation of the cells of osteogenic ridges causes that neighboring calvarial bones to meet prematurely and in humans that process could downregulate Msx2 expression, allowing osteoblastic differentiation and premature closure of the suture[5].

Adult Msx2 deficient mice have large, midline foramen spanning the frontal bones of calvaria[24]. Skeletal staining and alkaline phosphatase histochemistry indicate delay in bone growth and ossification. BrdU labeling of Msx2 deficient osteoprogenitors at osteogenic fronts is reduced[3]. In that case, Msx2 absence is responsible for decreased proliferation and inability of osteogenic ridges to expand and proper suture closer is absent. Significantly, the entire calvaria is missing in $Msx1^{-/-};Msx2^{-/-}$ mutant, suggesting that Msx1 and Msx2 function together to regulate osteogenesis during calvaria development[3]. They have critical and functionally redundant roles in regulating calvarial morphogenesis[2].

Another model for analysis of Msx2 function in osteoblast differentiation is Msx2 retroviral overexpression[5]

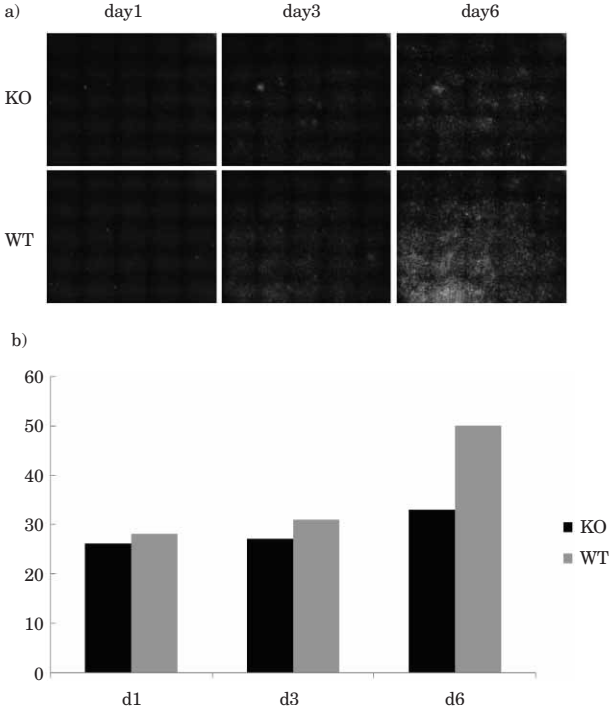

Fig. 4. Comparison of cell number by calcein-AM staining. Culture from Msx2 deficient mice (KO) at days 3 and 6 had lower cell number than culture from wild type mice (WT). 30 mm plates were scanned using 5x magnification.

in primary culture of chick calvarial osteoblast cells that prevents osteoblast differentiation. Expression of antisense Msx2 mRNA decreases proliferation and accelerates their differentiation.

Those findings are consistent with our results, confirming the hypothesized role of Msx2 as a factor inhibiting differentiation and stimulating proliferation in regulation of calvarial growth and proper closure of sutures.

Conclusions

Calvarial osteoblasts from Msx2 deficient mice display decreased proliferation and increased differentiation. Transduction of osteoblastic cells with ROSACol2.3GFPemd and assessment of GFP induction tempo and level can be an effective method to measure osteoblastic lineage progression. Our results support our original hypothesis that Msx2 affects calvarial development by delaying osteoblastic differentiation in the osteogenic fronts of the sutures.

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USPOREDBA PROLIFERACIJE I DIFERENCIJACIJE KULTURE OSTEOBLASTA IZ TJEMENIH KOSTIJU MSX2 KNOCK-OUT I DIVLJEG TIPA MIŠA

SAŽETAK

Analizirani i uspoređivani su proliferacija i diferencijacija osteoblasta porijeklom iz Msx2 knock-out miša i divljeg tipa. Uspostavljene su kulture osteoblasta iz tjemenih kostiju pet do osam dana starih miševa Msx2 knock-out homozigota, heterozigota te divljeg tipa. Brzina proliferacije je određena brojanjem stanica, te BrdU i Calcein AM bojanjem. Tempo diferencijacije je određen bojanjem stanica na alkalnu fosfatazu, Von Kossa bojanjem na minerale, Northern hibridizacijom biljega koštane diferencijacije, te infekcijom stanične kulture retrovirusom koji sadrži GFP pod kontrolom promotorskog fragmenta kolagena tipa I. Šesti dan, broj stanica u staničnoj kulturi Msx2 knock-out miševa je bio 20% niži nego u kulturi iz divljeg tipa. U staničnoj kulturi Msx2 knock-out miševa, BrdU označenih stanica je bilo 16,8% u kulturi iz heterozigota 20,9%, a u kulturi iz divljeg tipa 21,6%. Prilikom Calcein AM označavanja, stanična

kultura iz Msx2 knock-out miševa je imala slabiji intenzitet fluorescencije nego stanična kultura iz divljeg tipa miševa. Von Kossa bojanje je pokazalo pojačanu mineralizaciju u kulturi Msx2 knock-out miševa, dok je Northern hibridizacija pokazala i pojačanu ekspresiju biljega koštane diferencijacije. GFP se pojavio ranije u kulturi Msx2 knock-out miševa nakon infekcije s Col2.3 GFP retrovirusom. Zaključujemo da osteoblasti tjemenih kostiju iz Msx2 knock-out miša sporije proliferiraju te brže diferenciraju nego osteoblasti iz miša divljeg tipa.