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Expression of Runx2 transcription factor in non-skeletal tissues, sperm and brain

Jae-Hwan Jeong¹, Jung-Sook Jin¹, Hyun-Nam Kim¹, Sang-Min Kang¹, Julie C. Liu⁴, Christopher J. Lengner^{4,+}, Florian Otto², Stefan Mundlos³, Janet L. Stein⁴, Andre J. van Wijnen⁴, Jane B. Lian⁴, Gary S. Stein⁴, and Je-Yong Choi^{1,*}

¹ Department of Biochemistry and Cell Biology, School of Medicine, Cell and Matrix Research Institute, Skeletal Diseases Genome Research Center, Kyungpook National University, Daegu 700-422, Republic of Korea

² Division of Hematology/Oncology, Medical Center, University of Freiburg, 79106 Freiburg, Germany

³ Max Planck Institute for Molecular Genetics, Ihnestr. 73, 14195 Berlin, Germany

⁴ Department of Cell Biology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655, USA

Abstract

Runx2 is a master transcription factor for chondrocyte and osteoblast differentiation and bone formation. However expression of Runx2 (by RT-PCR), has been reported in non-skeletal tissues such as breast, T cells and testis. To better define Runx2 activity in non-skeletal tissues, we examined transgenic (Tg) mice expressing *LacZ* gene under control of 3.0 kb (3 Kb Tg) or 1.0 kb (1 Kb Tg) of the Runx2 distal (P1) promoter, Runx2 *LacZ* knock-in (Runx2^{+/LacZ}) and Runx2/P1 *LacZ* knock-in (Runx2/P1^{+/LacZ}). In the Runx2 3 Kb Tg mouse, β -galactosidase (β -gal) expression appeared in various non-skeletal tissues including testis, skin, adrenal gland and brain. β -gal expression from both 3 Kb and 1 Kb Tg, reflecting activity of the Runx2 promoter, was readily detectable in seminiferous tubules of the testis and the epididymis. At the single cell level, β -gal was detected in spermatids and mature sperms not in sertoli or Leydig cells. We also detected a positive signal from the Runx2^{+/LacZ} and Runx2/P1^{+/LacZ} mice. Indeed, Runx2 expression was observed in isolated mature sperms, which was confirmed by RT-PCR and western blot analysis. Runx2, however, was not related to sex determination and sperm motility. Runx2 mediated β -gal activity is also found robustly in the hippocampus and frontal lobe of the brain in Runx2^{+/LacZ}. Collectively, these results indicate that Runx2 is expressed in several non-skeletal tissues particularly sperms of testis and hippocampus of brain. It suggests that Runx2 may play an important role in male reproductive organ testis and brain.

Keywords

Runx2; transgenic mice; β -galactosidase; sperm; brain

*Corresponding author: Je-Yong Choi, D.D.S., Ph.D., 101 Dong-In Dong, Jung-Gu, Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Daegu 700-422, Republic of Korea, Tel) +82-53-420-4823, Fax) +82-53-422-1466, E-mail) jechoi@knu.ac.kr.

+Current Address: White Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142 USA

INTRODUCTION

Runx2 is known as an essential transcription factor in bone formation (Stein et al, 2004). Ablation of Runx2 or Runx2 C-terminus truncation causes a complete lack of bone formation in the mouse (Choi et al, 2001; Komori et al, 1997; Otto et al, 1997). Disruption of even one copy of the Runx2 gene results in the human cleidocranial dysplasia (CCD; MIM 119600) (Kim et al, 2006a; Lee et al, 1997; Mundlos et al, 1997), with similar CCD phenotypic features also observed in Runx2 heterozygous mice (Otto et al, 1997).

Runx2-deficient mice display a lack of both intramembranous and endochondral ossification and show an absence of osteoblast differentiation, as well as retardation of chondrocyte differentiation (Choi et al, 2001; Komori et al, 1997; Otto et al, 1997). A large number of *in vitro* studies have shown that Runx2 is a positive regulator for the expression of bone matrix genes, including type I collagen, osteopontin, bone sialoprotein, osteocalcin, matrix metalloproteinase 13, Indian hedgehog and fibronectin (Komori, 2006; Lian et al, 2004; Otto et al, 2003). Furthermore, Runx2 is essential for the recruitment and commitment of preosteoprogenitor cells into osteoblasts from mesenchymal stem cells (Komori, 2008).

The Runx2 gene locus has been identified in chromosome 17 and chromosome 6p21 in mice and human, respectively (Mundlos et al, 1997; Otto et al, 1997). Gene structure between mouse and human is very similar (Terry et al, 2004). Expression of Runx2 is regulated by two distinct promoters that generate two different N-terminal isoforms as type I and type II Runx2. The distal P1 promoter produces type II Runx2 (MASNS type, Runx2 p57/P1) and the proximal P2 promoter give rise to type I (MRIPV type, Runx2 p56/P2) (van Wijnen et al, 2004; Xiao et al, 2001). While type I Runx2 was first cloned as a T-cell specific factor (Ogawa et al, 1993; Satake et al, 1995), type II Runx2 was originally cloned as a bone-specific factor (Banerjee et al, 1997; Ducy et al, 1997; Mundlos et al, 1997; Stewart et al, 1997). P1 promoter becomes highly expressed during osteoblast differentiation and is stimulated by several bone forming homeodomain and Hox proteins (Msx2, Dlx3, Dlx5, Hoxa10) (Lee et al, 2005). Usage of the two Runx2 promoters has been reported during embryogenesis and calvarial development (Park et al, 2001; Smith et al, 2005), and both are responsive to the osteogenic BMP signal (Banerjee et al, 2001; Lee et al, 2000; Prince et al, 2001). Surprisingly, however, selective deletion of type I Runx2 in mouse results in formation of mineralized tissues and postnatal survival (Xiao et al, 2004). Hence, both promoters and Runx2 isoforms are contributing to the bone formation.

Although Runx2 has been shown to be an essential transcription factor in skeletal development, a few studies have reported that Runx2 is also expressed in non-skeletal tissues such as breast (Otto et al, 1997; Shore, 2005), T cells (Satake et al, 1995), and testis (Lengner et al, 2002; Ogawa et al, 2000). These studies may predict that Runx2 may have a significant function in non-skeletal tissues. However, the expression of Runx2 at the single cell level has not been well examined in these non-skeletal tissues. Because the lethality at birth prevents any analysis of pathology of the tissues during postnatal development of the mature organs, we have addressed the cell type tissue specificity of Runx2 expression. Our studies used *in vivo* mouse genetic approaches with the two different transgenic mice expressing *LacZ* under control of 3.0 Kb (3 Kb Tg) or 1.0 Kb (1 Kb Tg) of the Runx2 distal (P1) promoter and two different Runx2 *LacZ* knock in mice (Runx2^{+/LacZ}), a Runx2 types independent Runx2 *LacZ* mice (Otto et al, 1997), and type II Runx2 trackable Runx2 P1 *LacZ* knock-in mouse (Runx2/P1^{+/LacZ}) lines (Liu et al, 2007).

MATERIALS AND METHODS

Animals and generation of transgenic mice

Animal care and experiments were carried out in accordance with institutional animal regulations. All animals were maintained on a 12 h light:12 h darkness cycle at 22–25°C in specific pathogen free (SPF) conditions and fed with standard rodent chow and water *ad libitum*. The 3 Kb transgenic (Tg) and Runx2^{+/LacZ} mice were established in previous studies (Lengner et al, 2002; Otto et al, 1997). Mouse tail genomic DNA was isolated by phenol-chloroform extraction following cell lysis with Triton X-100 and digested with proteinase K. For some experiments, DNA was isolated from the whole tissues of paraffin block using QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Genotyping of Runx2^{+/LacZ} and Runx2 Δ C mice were performed as previously described method by using the Runx2 Δ C and Runx2^{+/LacZ} mice specific primers; 5'-

ACTCTAGAAAATTATAGCGAACCCCAAG-3' and 5'-

GATCTAGAGCCTTGATGAGTGACATAGGT-3' (Runx2 Δ C) and 5'-

GCATCGAGCTGGGTAATAA GCGTTGGCAAT-3' and 5'-

GACACCAGACCAACTGGTAATGGTAGCGAC-3' (Runx2^{+/LacZ}), respectively (Choi et al, 2001; Otto et al, 1997). Runx2/P1^{+/LacZ} mice were genotyped by using two sets of specific primers; 5'-CCAACTTAATCGCCTTGCAGC-3' and 5'-

CGTGGGAACAAACGGCGGATT-3' for the targeted allele and 5'-

TCTGGAAAAAAGGAGGGACTATG-3' and 5'-GGTGCTCGGATCCCAAAAGAA-3' for the wild type allele.

For making a 1 Kb Runx2-P1 Tg mouse, 1 Kb Runx2 distal promoter with *LacZ* gene vector was constructed after deletion of 3 Kb Tg vector with *Nco*I. After restriction digestion with *Not*I and *Xho*I, 1 Kg Tg vector was purified using S&S ELUTIP minicolumns (Schleicher & Schuell bioscience, Dassel, Germany) and was dialyzed 4 times against 1 L of injection buffer (10mM Tris-HCl pH 7.4, 0.1mM EDTA) for overnight using Slide-A-Lyzer dialysis cassette (10,000MWCO) (Pierce, Rockford, IL). After dialysis, DNA was diluted with injection buffer to final concentration of 4ng DNA/ μ l and was filtered using Ultrafree-MC 0.22 μ m GV DURAPORE (Millipore, Carrigtwohill, Ireland). Trimmed and clean 1 Kb Tg vector (4ng/ μ l) was injected into the pronuclei of fertilized eggs from C57BL/6 mice. For genotyping, 1 Kb Tg mice were identified by PCR with specific primers (upper; 5'-GGAAGCCACAGTGGTAGGCAGTC-3', lower; 5'-ACCTGCGTTTCACCCTGCCATAAAGA-3'). All mice were maintained on a C57BL/6 background.

The β -galactosidase (β -gal) staining

The β -gal staining was carried out as described (Joyner, 2000). Whole tissues were fixed for 30 min in 4% paraformaldehyde in 0.1 M Potassium phosphate buffer pH7.4 containing 2 mM MgCl₂, 5 mM ethyleneglycoltetraacetic acid (EGTA). Then, whole tissues were washed three times with each 30 min using solution A contained 0.1 M potassium phosphate buffer pH 7.4, 2 mM MgCl₂, 5 mM ethyleneglycoltetraacetic acid (EGTA), 0.01% Na deoxycholate, 0.02% Nonidet P-40. Then, tissues were added to the X-gal staining solution which contained 0.5 mg/mL X-gal, 10 mM K₃Fe(CN)₆ and 10 mM K₄Fe (CN)₆ in solution A. After overnight staining, whole tissues were washed briefly in phosphate buffered saline (PBS).

Histologic examination of samples stained with X-gal and counter-staining was processed by cryosection. Slides were fixed briefly in 4% paraformaldehyde, 2 mM MgCl₂, 5 mM EGTA, 0.1 M potassium phosphate buffer pH 7.4, washed with PBS and X-gal stained for overnight at 37 °C. Samples were then washed in PBS and were counterstained with Mayer' hematoxylin to observe tissue structure (Joyner, 2000).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and RT-PCR was performed using Superscript II RT (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. The PCR condition was 94°C for 5min, followed by 35 cycles of at 94°C for 50s, 62°C for 45s, and 72°C for 45s and a final extension step at 72°C for 10 min. Primers for total Runx2 expression were as follows: 5'-TCCTATCTGAGCCAGATGACATCC-3' and 5'-CCGGTCTCCCCCGGTACC-3' (480 bp). For real-time PCR, we used following primers. Primers for type I Runx2 were forward, 5'-CCGGCCACTTCGCTAACTT-3'; and reverse, 5'-TGGTGCTCGGATCTACAGGAA-3' and type II Runx2 (till form) forward, 5'-TCTGGAAAAAAGGAGGGACTATG-3'; reverse, 5'-GGTGCTCGGATCCCAAAGAA-3' Taqman probes were used for: Runx1: 5'-TAGAGGCAAGAGCTTC-3' and Runx3: 5'-ACAATCACCGTGTTACCAA-3'.

Western blot analysis

Western blotting was performed using mouse monoclonal anti-Runx2 antibody followed by HRP-conjugated goat anti-mouse IgG. as previous method (Choi et al, 2001). The desired protein bands on the membrane were detected by using an enhanced chemiluminescence detection system (Amersham Pharmacia Bio-tech, Little Chalfont, UK).

Sex determination and sperm motility assay

For sex determination, genomic DNA from pups was obtained and analyzed the presence of Sry gene by PCR. Primers for Sry PCR were 5'-CACAAGTTGGCCCAGCAGA-3' and 5'-TGTTGGTGGTCATGGAAGT-3' and the PCR reaction was 35 cycles of amplification with initiation at 94°C for 5min, denaturation at 94°C for 30s, annealing at 58°C for 30s and extension at 72°C for 30s.

For sperm motility assay, we performed the sampling of sperm as follow with slight modification (Koyama and Kamimura, 2003). The cauda epididymis was cut with a surgical blade at the side of the corpus epididymis, where the vas deference attached to the epididymis and the latter was placed into a 500 µl drop of 10% FBS D-PBS under mineral oil. Then, sperm was gently squeezed out using forceps and puncturing the cauda with the tips of the syringe. After incubation for 1.5 hours at 37°C, the sperm motility was determined using a hemocytometer.

Statistical analysis

Data was expressed as the mean $\pm$ SD of three or more independent experiments. Differences between two groups were evaluated using the Student's t test. Results were considered significant at $P < 0.05$.

RESULTS

Expression of Runx2 distal promoter (P1) activity in sperms during spermatogenesis

Our previous study showed that activity of the 3 Kb Runx2 promoter derived *LacZ* transgenic mice (3 Kb Tg) is high in testis (Lengner et al, 2002). As shown in Fig 1A, β -gal activity was positive to about the same extent driven by either 3 Kb or 1 Kb Tg mice as well as the Runx2^{+/LacZ} and Runx2/P1^{+/LacZ} mouse models. Histological analysis of 3 Kb Tg mice revealed that the β -gal expression was readily detectable in seminiferous tubules of the testis. β -gal positive cells were found in round spermatids to spermatozoa and in mature sperms, but were not detected in Sertoli or Leydig cells. This β -gal expression pattern was similar to those of 1 Kb Tg and Runx2^{+/LacZ} mice (Figure 1B). Strong positive signals were also observed in epididymus associated with more differentiated spermatids (Figure 2).

These results indicate that 1 Kb distal promoter of Runx2 is sufficient to activate tissue specific Runx2 expression in sperm of testis.

To confirm Runx2 mRNA and protein expression in testis and sperm, total RNA and protein from these tissues of 8 weeks old wild type mice were analyzed by RT-PCR (Figure 3A) and western blot analysis (Figure 3B). Cellular RNA from ATDC5 cells served as a positive control. Verification of Runx2 mRNA present in testis and sperm was confirmed by sequencing of PCR product (data not shown). Indeed, Runx2 protein was also detected by western blot analysis in both testis and sperm. These results indicate that Runx2 is endogenously expressed in isolated mature sperms. The major products of Runx2 65 kDa and 47 kDa were also found in testis, but faintly in sperm. The 45-47 kDa product has been previously reported in glucocorticoid treated osteoblasts (Chang et al, 1998; Prince et al, 2001).

We further addressed that the endogenous type II Runx2 protein was derived from protein encoded by the bone-related P1 promoter in its native locus. In this mouse model, *LacZ* was inserted into exon 1 of the P1 promoter, reflecting only the activity of the P1 promoter and allowing the P2 promoter to encode the type I Runx2 isoform (Liu et al, 2007). The specific Runx2 isoforms were quantified by real-time RT-PCR (Figure 3C). In the heterozygote, we find a 50% reduction of total Runx2 and the two Runx2 isoforms. Interestingly, both Runx1 and Runx3 were equally present in wild type and the Runx2/P1^{+/LacZ} mice. The testis of Runx2/P1^{+/LacZ} was β -gal positive in a similar pattern (data not shown) compared to the 3 Kb Tg and in the Runx2^{+/LacZ} mice which deleted both isoforms. Thus contributing to Runx2 expression in the testis are both Runx2 isoforms required for osteogenesis.

Effects of Runx2 on sex determination and sperm motility

Runt, a *Drosophila* homolog of Runx transcription factors, has been shown to have three different functions including neurogenesis, segmentation and sex determination (Duffy and Gergen, 1994). With regard to the mammalian homolog of Runx, the requirement for sex determination has not been examined. We find Runx2 was expressed in sperm, but not in oocytes (in manuscript preparation). Therefore, it is plausible to hypothesize that Runx2 might be involved in sex determination. If this is the case, the gender of homozygous pups will be affected by the absence of Runx2.

To test this hypothesis, we performed analysis of the male specific gene *Sry* PCR using genomic DNA from Runx2 homozygous pups. Among 9 homozygous mice, four were female and five were male (Figure 4A). This appears to be no gender bias difference in Runx2-null and Runx2/P1^{LacZ/LacZ} (data not shown) and Runx2 $\Delta c/\Delta c$ homozygous mice. This result suggests that Runx2 is not related to the sex determination. In Runx3 null mice, both male and female pups were found (Bae SC, personal communication). Although Runx1 has not been addressed, its presence in testis could contribute to determination of sex. Thus, while Runx2 or Runx3 heterozygote does not exhibit a sex determination, further studies are needed to identify the precise contribution of mammalian Runx transcription factors to sex determination.

We also determined whether Runx2 is related to the sperm motility by examining the isolated sperms from Runx2^{+/LacZ} mice. Mature sperms from Runx2^{+/LacZ} mice are haploid. Therefore, this sperm motility of the isolated sperms would easily observable of the abnormal sperm motility from Runx2^{+/LacZ}. Despite of the presence of Runx2-null sperms, no difference in sperm movement was measured (Figure 4B). This finding is consistent with the fact that heterozygous Runx2 male mice are fertile. These results indicate that the expression of Runx2 in sperm is not related to the function of sex determination or their motility.

Expression of Runx2 in brain

In addition to testis, we checked other non-skeletal tissues to explore the expression of Runx2 promoter activity in detail. In brain, the β -gal activity was strong in cerebellum of 3 Kb Tg, but not in the 1 Kb Tg (Figure 5A). Instead of expression in the cerebellum, Runx2^{+/LacZ} mice showed the positive β -gal staining in the frontal and temporal lobe areas (Figure 5A). The Runx2/P1^{+/LacZ} mouse showed no β -gal activity in the brain of newborn pups (data not shown), suggesting that crosstalk between the P1 and P2 promoters occurs and that the Runx2 in brain mainly derives from the P2 promoter. The β -galactosidase activity of 3 Kb Tg was restricted to granular cells and it was not observed in purkinje cells in cerebellum (Figure 5B). With regard to the expression of Runx2 in Runx2^{+/LacZ} mice, the endogenous Runx2 promoter activity was strong in the hippocampal formation area (Figure 5C). The positive signal was observed in CA1 and CA2 area but not in CA3 area (Figure 5C). Indeed, Runx2 mRNA was detected in both cerebrum and cerebellum by RT-PCR (data not shown). The endogenous expression of Runx2 in hippocampus suggests that Runx2 may play an important role in the hippocampal functions such as learning and memory.

Expression of Runx2 in other non-skeletal tissues

The β -gal activity of three different transgenic mice in other non-skeletal tissues is summarized in Table 1. In addition to sperm and brain, β -gal activity was observed in ovary of Runx2^{+/LacZ} (manuscript in preparation). Another interesting finding was that the β -gal activity in adrenal medulla and hair follicles was observed in the 3 kb Tg (Table 1). These expression patterns, however, were not revealed in Runx2^{+/LacZ} mice indicating that they are not endogenously expressed in these tissues. Runx2 originally has been known as a T cell specific factor (Satake et al, 1995). In this study, however, we couldn't find any positive β -gal staining in the thymus of Runx2^{+/LacZ} mice. It may be due in part to the low resolution of the β -gal staining or that the expression level of Runx2 is too low to detect in thymus. The β -gal activity was not observed in other non-skeletal tissues including kidney, small and large intestine, liver, lung, stomach, spleen, and heart (Table 1). These results indicate that the β -gal positive tissues seem related to the expression of estrogen receptors. Indeed, Runx2 function seems related to the estrogen pathway. For example, Runx2 directly interacts with estrogen receptor α in an estrogen-dependent manner, which enhances Runx2 activity, but inhibits the stimulatory effects of estrogen receptor α on gene expression through consensus ERE (McCarthy et al, 2003). Furthermore, high-dose estrogen-induced osteogenesis is dependent on Runx2 dosage (Juttner and Perry, 2007). In addition, Runx2 is involved in bone loss in estrogen deficiency (Maruyama et al, 2007).

DISCUSSION

Runx2 is known to be an essential factor for skeletogenesis. The expression of Runx2 in non-skeletal tissues as we report herein, including sperm and brain, indicates other important functions of Runx2 in these tissues. These non-skeletal tissues develop normally in the Runx2 null mice. However, our studies are limited to Runx2 null mice at birth and post-natal heterozygous mice. Thus, a remaining question for future investigations is the precise role of Runx2 in testis, brain and ovary. A cDNA profiling study combined with a network association analysis of the hippocampus from schizophrenics uncovered 25 genes, one of which is Runx2 that is significantly downregulated in the disease state (Benes et al, 2007). It is not surprising that Runx2 is found in testis and ovary given several reports that Runx2 is regulated by the sex steroids. For example, Runx2 and androgen receptor mutually repress the transactivation function through change of compartmentalization (Kawate et al, 2007). Estrogen has been most well studied and Runx2 has been shown to increase its expression in estrogen target tissues in bone (McCarthy et al, 2003) and in bone marrow cells (Plant et al, 2002). Furthermore, this increased expression is associated with Runx2 transactivation

through an ERE and Runx response element (Sasaki-Iwaoka et al, 1999) and through AP1 and Runx sites in the very proximal promoter (−93/+7) (Tou et al, 2001). It is now appreciated that Runx2 serves in many cellular functions beyond the activation of tissue-specific target genes, such as osteocalcin and other bone proteins expressed in the mature osteoblasts. For example, Runx regulates cell growth (Pratap et al, 2003) and modulates the expression and activity of growth factors and enzymes involved in early development that are expressed in numerous cells, including the family of matrix metalloproteinases (Pratap et al, 2005), vascular endothelial growth factor (Zelzer et al, 2001), and the TGF- β type I receptor (Kim et al, 2006b). Runx2 has the ability to drive differentiation by promoting exit from the cell cycle (Galindo et al, 2005) and regulating a program of gene expression through its ability to both activate and repress genes as cell progress through stages of maturation (Lian et al, 2004). A recently identified Runx function is its epigenetic role for cell fate determination by its ability to be retained on mitotic chromosomes (Young et al, 2007b). Even more unusual for this family of transcription factors is the ability to contribute to regulation of ribosomal genes and control protein synthesis machinery (Young et al, 2007a). Any or all of these activities may be contributing to specialized tissue-related functions in the non-skeletal organs in which Runx2 promoter activity and endogenous Runx2 protein is presented.

One question arising from transgenic mouse models where only a portion of the Runx2 promoter drives *LacZ* expression is the physiologic relevance of its activity. Certainly the Runx2 3 kb promoter exhibits a nearly similar expression in testis as the *LacZ* knock-in where the endogenous Runx2 gene promoter locus is functional. Notably, the specific deletion of the Runx2/P1 promoter by *LacZ* insertion that allows for a functional P2 promoter and translated Runx2 isoform showed testis expression of β -gal, but no β -gal activity was detected in brain. These findings implicate a potential crosstalk between the two P1 and P2 promoters for regulating isoform expression. Notably, in the absence of Runx2 from the P1 promoter, the Runx2/P2 driven protein was also reduced, but the overall 50% reduction in Runx2/P1 from one allele and Runx2/P2 from both alleles was not sufficient for expressing *LacZ* in brain. The Runx2/P2 promoter has not been studied for functional regulatory elements and may be subject to regulatory factors that differ between skeletal and non-skeletal tissues. During embryonic bone development, the Runx2 isoforms are differentially expressed in cartilage zones during endochondral bone formation (Choi et al, 2002) and in developing incisors (Chen et al, 2002). During osteoblast differentiation, type I Runx2 remains at a constitutive level, while type II Runx2 isoform increases with the onset of bone formation (Drissi et al, 2000).

Another question arising from the non-skeletal expression of Runx2 is the mechanisms for its activation. Runx2 is autoregulated with more than seven Runx2 sites within the proximal promoter and 5' UTR regions (Elango et al, 2006). The presence of Runx2 could arise from the homeodomain protein Dlx5 which is expressed in mouse brain and in testis (Kimura et al, 2004), as Dlx5 is a strong activator of the Runx2 gene (Hassan et al, 2007).

In conclusion, the present studies have identified very specific Runx2 expression in ovary, testis, the process of spermatogenesis and in specific compartments of the brain. These observations provide compelling evidence for future investigations on Runx2 functional activities by cell type specific knock-outs that cannot be identified by current mouse models.

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Fig. 1.

The β-gal activity in testis of 3 Kb and 1 Kb Runx2 distal promoter transgenic and Runx2^{+/LacZ} mice. (A) β-gal activity is positive in 3 Kb, 1 Kb and Runx2^{+/LacZ} mice. Whole tissues of 4-month old mice were stained for overnight with X-gal. (B) Promoter activity of Runx2 of seminiferous tubules in testis. Cryosection tissues (5 μm) of testis were stained with X-gal for overnight. The 3 Kb and 1 Kb Tg mice strongly expressed β-gal activity. Also, the β-gal activity weakly appeared in Runx2^{+/LacZ} mice. The *LacZ* expression was positive only in a certain stage of cells during spermatogenesis in all three kinds of mice. Expression of β-gal was observed from round spermatids in 3 Kb, 1 Kb Tg and Runx2^{+/LacZ} mice. Promoter activity was not observed in Sertoli cells and Leydig cells.

Fig. 2. Expression of β -galactosidase in mature sperms

Promoter activity was strongly detected in epididymis spermatids and sperms in 3 Kb and 1 Kb transgenic mice and *Runx2*^{+/LacZ} mice. Cryosection tissues of epididymis of 4-month old mice were stained with X-gal for overnight. S; mature sperm.

Fig. 3.

Expression of Runx2 in isolated sperms. (A) Total RNA and proteins were isolated from testis and sperm of 8 weeks old wild type mice for RT-PCR using primers that detect total Runx2 levels. Arrow indicates Runx2 signals as 480 bp. Runx2 expressing ATDC5 chondrocyte was used as positive control. (B) Western blot analysis in wild type mice. Runx2 protein was detected as two major bands in both testis and sperm. (C) Expression of Runx2 isoforms in Runx2/P1^{+/LacZ}. Real-time PCR was performed as described in materials and methods for Runx1, 2 and 3 expression. P1, Runx2 type II mRNA; P2, Runx2 type I mRNA. * P<0.05.

Fig. 4.

Sex determination by *Sry* typing and sperm motility assay. (A) Genotyping for sex determination. Genomic DNA was isolated from whole tissue of paraffin block or tail. PCR analysis was conducted as described in materials and methods. M, 1 Kb plus ladder (Invitrogen). (B) The motility assay of sperms from Runx2^{+/LacZ} mice of 4-month old mice was not changed. The values are the means $\pm$ S.D (N=3 in each group). WT; wild type mouse. HE; heterozygous mouse.

Fig. 5. Runx2 promoter activity in brain. (A) β -galactosidase activity is positive in 3 Kb Tg and Runx2^{+/LacZ} mice. (B) LacZ positive cells were observed in cerebellum in 3 Kb Tg mice. The β -gal activity was appeared granular layer (GL) but not detected in purkinje cells (arrow). (C) The longitudinal section of brain in Runx2^{+/LacZ} mice. LacZ positive cells were observed in frontal lobe area (F) and hippocampal area CA1 and CA2 but not in CA3 in Runx2^{+/LacZ} mice. Whole tissues and cryosection tissues of 4-month old mice were stained for overnight with X-gal.

TABLE 1

Tissue	β -galactosidase activity		
	3Kb TG	1Kb TG	Runx2 ^{+/LacZ}
Kidney	–	–	–
Small intestine	–	–	–
Large intestine	–	–	–
Liver	–	–	–
Ovary	–	–	+
Lung	–	–	–
Stomach	–	–	–
Spleen	–	–	–
Thymus	–	–	–
Cerebrum	–	–	+
Cerebellum	+	–	–
Heart	–	–	–
Testis	+	+	+
Epididymis	+	+	+
Adrenal gland	+	–	–
Skin	+	–	–