A variant of Runx2 that differs from the bone isoform in its splicing is expressed in spermatogenic cells

Satoru Kanto, Marcin Grynberg, Yoshiyuki Kaneko, Jun Fujita, Masanobu Satake

Background. Members of the *Runx* gene family encode transcription factors that bind to DNA in a sequence-specific manner. Among the three Runx proteins, Runx2 comprises 607 amino acid (aa) residues, is expressed in bone, and plays crucial roles in osteoblast differentiation and bone development. We examined whether the *Runx2* gene is also expressed in testes.

Methods. Murine testes from 1-, 2-, 3-, 4-, and 10-week-old male mice of the C57BL/6J strain and W/W^v strain were used throughout the study. Northern Blot Analyses were performed using extracts form the murine testes. Sequencing of cDNA clones and 5'-Rapid Amplification of cDNA Ends were performed to determine the full length of the transcripts, which revealed that the testicular Runx2 comprises 106 aa residues coding novel protein. Generating an antiserum using the amino-terminal 15 aa of Runx2 (Met^1 to Gly^{15}) as an antigen, immunoblot analyses were performed to detect the predicted polypeptide of 106 aa residues with the initiating Met^1. With the affinity-purified anti-Runx2 antibody, immunohistochemical analyses were performed to elucidate the localization of the protein. Furthermore, bioinformatic analyses were performed to predict the function of the protein.

Results. A *Runx2* transcript was detected in testes and was specifically expressed in germ cells. Determination of the transcript structure indicated that the testicular *Runx2* is a splice isoform. The predicted testicular Runx2 polypeptide is composed of only 106 aa residues, lacks a Runt domain, and appears to be a basic protein with a predominantly alpha-helical conformation. Immunoblot analyses with an anti-Runx2 antibody revealed that Met^1 in the deduced open reading frame of *Runx2* is used as the initiation codon to express an 11 kDa protein. Furthermore, immunohistochemical analyses revealed that the Runx2 polypeptide was located in the nuclei, and was detected in spermatocytes at the stages of late pachytene, diplotene and second meiotic cells as well as in round spermatids. Bioinformatic analyses suggested that testicular Runx2 is a histone-like protein.

Discussion. A variant of *Runx2* that differs from the bone isoform in its splicing is expressed in pachytene spermatocytes and round spermatids in testes, and encodes a histone-like, nuclear protein of 106 aa residues. Considering its nuclear localization and

differentiation stage-dependent expression, Runx2 may function as a chromatin-remodeling factor during spermatogenesis. We thus conclude that a single *Runx2* gene can encode two different types of nuclear proteins, a previously defined transcription factor in bone and cartilage and a short testicular variant that lacks a Runt domain.

Title page

A variant of Runx2 that differs from the bone isoform in its splicing is expressed in spermatogenic cells

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Abbreviations: aa, amino acid; GST, glutathione-S-transferase; ORF, open reading frame; PBS, phosphate-buffered saline.

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Running title: Novel variant of Runx2 is expressed in testis

Abstract

Background. Members of the *Runx* gene family encode transcription factors that bind to DNA in a sequence-specific manner. Among the three Runx proteins, Runx2 comprises 607 amino acid (aa) residues, is expressed in bone, and plays crucial roles in osteoblast differentiation and bone development. We examined whether the *Runx2* gene is also expressed in testes.

Methods. Murine testes from 1-, 2-, 3-, 4-, and 10-week-old male mice of the C57BL/6J strain and W/W^v strain were used throughout the study. Northern Blot Analyses were performed using extracts form the murine testes. Sequencing of cDNA clones and 5'-Rapid Amplification of cDNA Ends were performed to determine the full length of the transcripts, which revealed that the testicular Runx2 comprises 106 aa residues coding novel protein. Generating an antiserum using the amino-terminal 15 aa of Runx2 (Met^1 to Gly^{15}) as an antigen, immunoblot analyses were performed to detect the predicted polypeptide of 106 aa residues with the initiating Met^1. With the affinity-purified anti-Runx2 antibody, immunohistochemical analyses were performed to elucidate the localization of the protein. Furthermore, bioinformatic analyses were performed to predict the function of the protein.

Results. A *Runx2* transcript was detected in testes and was specifically expressed in germ cells. Determination of the transcript structure indicated that the testicular *Runx2* is a splice isoform. The predicted testicular Runx2 polypeptide is composed of only 106 aa residues, lacks a Runt domain, and appears to be a basic protein with a predominantly alpha-helical conformation. Immunoblot

41 analyses with an anti-Runx2 antibody revealed that Met[1] in the deduced open reading frame of
42 *Runx2* is used as the initiation codon to express an 11 kDa protein. Furthermore,
43 immunohistochemical analyses revealed that the Runx2 polypeptide was located in the nuclei, and
44 was detected in spermatocytes at the stages of late pachytene, diplotene and second meiotic cells as
45 well as in round spermatids. Bioinformatic analyses suggested that testicular Runx2 is a histone-
46 like protein.
47 **Discussion.** A variant of *Runx2* that differs from the bone isoform in its splicing is expressed in
48 pachytene spermatocytes and round spermatids in testes, and encodes a histone-like, nuclear
49 protein of 106 aa residues. Considering its nuclear localization and differentiation stage-dependent
50 expression, Runx2 may function as a chromatin-remodeling factor during spermatogenesis. We
51 thus conclude that a single *Runx2* gene can encode two different types of nuclear proteins, a
52 previously defined transcription factor in bone and cartilage and a short testicular variant that lacks
53 a Runt domain.
54
55 Key words: alternative splicing, cell differentiation, Runx transcription factor, spermatogenesis.
56

57 Introduction

58 The mechanism of spermatogenesis is still largely unknown although genomes and genes are
59 vigorously explored. It's probably because some specific products transcribed and translated using
60 the common genes in somatic cells are responsible for unique events in spermatogenesis (Kleene,

2001; Kleene, 2003).

Runx transcription factors are characterized by the presence of a Runt domain (Kagoshima et al., 1993). This domain contains 130 amino acids (aa) and is responsible for sequence-specific DNA-binding activity and for dimerization with the protein PEBP2β/CBFβ. There are three known *Runx* genes in mammals, *Runx1*, *Runx2*, and *Runx3*, each of which plays important roles in cell proliferation and differentiation as well as in the occurrence of specific human diseases (Wong et al., 2011; Chuang, Ito & Ito, 2013).

Runx2, the topic of this study, is expressed in bone, thymus, testis, and brain (Satake et al., 1995; Komori et al., 1997; Otto et al., 1997; Jenog et al., 2008). In bone and thymus, the *Runx2* transcript contains a Runt domain sequence and the translated product functions as a transcription factor. In bone, gene-targeting studies have demonstrated that *Runx2* is essential for the differentiation of immature osteoblasts into mature osteocytes. In mice lacking the Runt domain of *Runx2*, ossification of the skeletal system is severely impaired and the animals die soon after birth due to a respiratory defect (Komori et al., 1997). Of clinical importance, heterozygous loss of *Runx2* causes cleidocranial dysplasia in humans, which is characterized by hypoplasia/aplasia of the clavicles and fontanelles (Otto et al., 1997; Mundlos et al., 1997).

In the thymus, *Runx2* appears to function as an oncogene because the insertion of a retroviral genome near to the *Runx2* locus in mice results in its overexpression and subsequently the occurrence of T-cell leukemia (Stewart et al., 1997). In addition, overexpression of a *Runx2* transgene in the T-cell lineage perturbs the differentiation of thymocytes, mainly at the β selection

81 stage, and produces a population that predominantly consists of immature CD8+ thymocytes
82 (Vaillant et al., 2002).

83 *Runx2* is also expressed in the testis. This was originally reported by Satake *et al*. (Satake et
84 al., 1995) and subsequently by Ogawa *et al*. (Ogawa et al., 2000). According to Ogawa *et al*.
85 (Ogawa et al., 2000), the testicular *Runx2* transcript displays several unique features. First, it is
86 remarkably shorter (~1.8 kb) than the transcripts found in bone (6.3 and 7.4 kb), mainly due to the
87 premature termination of the testicular transcript within exon 8. Second, as a result of alternative
88 splicing and fusion between exons 1 and 3, a new stop codon is generated in exon 3. The deduced
89 open reading frame (ORF) encodes a polypeptide of only 106 aa residues. In addition, there are
90 two methionine codons within exon 1 of this ORF, Met^{1} and Met^{69}. Ogawa *et al*. (Ogawa et al.,
91 2000) predicted that Met^{69} is the translation initiation codon because the nucleotide sequence
92 adjacent to Met^{69} is in better agreement with Kozak's rule than the sequence adjacent to Met^{1}
93 (Kozak, 2002). However, if Met^{69} was the start codon, then the encoded polypeptide would only
94 be 38 aa residues long. Furthermore, because the alternative splicing removes exon 2, which
95 encodes the amino-terminal portion of the Runt domain, the testicular *Runx2* transcript cannot
96 encode a Runt domain-containing transcription factor.

97 In this study, we investigated the possibility that Met^{1} rather than Met^{69} is used as the
98 initiation codon for the translation of the testicular *Runx2* transcript because the environment for
99 translation in testicular cells is distinct from that in somatic cells. Furthermore, we examined the
100 expression pattern of the putative 106-aa polypeptide in relation to the differentiation stages of

101 testicular germ cells. We propose that the single *Runx2* gene can encode two distinct types of
102 protein: a small protein expressed in the testis that lacks a Runt domain, and a previously defined
103 Runt-containing transcription factor that is expressed in bone and thymus.
104
105 **Materials and methods**
106 Mice were maintained in the Animal Facility of the Institute of Development, Aging, and Cancer,
107 Tohoku University, an environmentally controlled and specific pathogen–free facility. Animal
108 protocols were reviewed and approved by the Animal Studies Committee of the Tohoku
109 University (relevant approval number: 2013-IDAC-Animal-013).
110 **Northern Blot Analysis**
111 Testes were isolated from 1-, 2-, 3-, 4-, and 10-week-old male mice of the C57BL/6J strain and
112 from 10-week-old male mice of the W/W^{v} strain. Spermatocyte and spermatid fractions were
113 prepared from the cell suspension of C57BL/6J testes (Mays-Hoopes et al., 1995). Total
114 cytoplasmic RNA was prepared from testes using Isogen (Nippon Gene, Toyama, Japan).
115 Poly(A)$^{+}$ RNAs were selected using Oligo(dT)-Latex (Takara, Otsu, Japan) and 2 μg of sample
116 was electrophoresed through a 1% (w/v) agarose gel containing 2.2 M formaldehyde. RNA was
117 transferred from the gel to a membrane, and the membrane was hybridized with a ^{32}P-labeled
118 probe as described previously (Chiba et al., 1997). The probes were prepared either from a
119 HindIII-NotI fragment of murine *Runx2* cDNA (corresponding to nt 282 through to nt 473 in
120 NM_001146038.2) or from a cDNA fragment of murine *PEBP2β/CBFβ*.

121

122 **cDNA Cloning and Sequencing**

123 A cDNA library prepared from murine testicular poly(A)$^+$ RNA was provided by Y. Nishina
124 (Osaka University, Osaka, Japan). A ^{32}P-labeled HindIII-NotI fragment of murine *Runx2* cDNA
125 was used as the probe. The library was screened under a stringent condition according to the
126 standard method. The cDNA inserts from each of the five isolated clones were excised from the
127 pAP3neo vector and subcloned into the pBluescript II vector. The entire length of the insert was
128 sequenced using the dideoxy-dye terminator method.

129

130 **5'-Rapid Amplification of cDNA Ends (5'-RACE)**

131 To determine the full length of the transcripts, 5'-RACE was performed following the manual
132 supplied by the manufacturer (Life Technologies). A gene-specific primer (5'-
133 TGTAAATACTGCTTGCAGCC-3') was annealed to poly(A)$^+$ RNAs and cDNA was synthesized.
134 After degrading RNA with RNase H, purified cDNA was tailed with dCTP and TdT. The dC-
135 tailed cDNA was amplified with the anchor primer and a nested gene-specific primer (5'-
136 GTGACCTGCAGAGATTAACC-3'). The double-stranded cDNA was subcloned into the
137 pBluescript II vector and sequenced.

138

139 **Bioinformatic Analysis**

140 The subcellular localizations of proteins were predicted using the PSORT program (Nakai &

141 Horton, 1999). The secondary structures of proteins were predicted using the PSIPRED
142 (McGuffin, Bryson & Jones, 2000), SAM-T99-2d (Karplus et al., 1999), and Profsec (Rost &
143 Eyrich, 2001) programs. These programs were downloaded from the BioInfo MetaServer
144 (http://bioinfo.pl/). The PSI-BLAST program (Altschul et al., 1997) was used for homology
145 searches. Three iterations were used before full saturation was reached. The domain architecture of
146 proteins was analyzed using the SMART tool (Letunic et al., 2002).
147
148 **Immunoblot Analysis**
149 Proteins were extracted from testes using RIPA buffer and 10 μg of the sample was subjected to
150 8% or 10% (w/v) SDS-polyacrylamide gel electrophoresis (PAGE). The ORF of testicular *Runx2*,
151 which encodes a polypeptide of 106 aa, was fused in frame to glutathione-S-transferase (GST)
152 using the pGEX vector. The plasmid was transfected into *E. coli* and transformed bacteria were
153 lysed in sample buffer after induction with isopropyl-β-D-thiogalactoside. Two micrograms of
154 protein was separated by SDS-PAGE. Proteins were transferred from the gel to a membrane and
155 the blotted membranes were blocked with TBS-T buffer, which contained 20 mM Tris-HCl pH 7.4,
156 150 mM NaCl, and 0.1% (v/v) Tween 20. The primary antibody was anti-Runx2 serum, which was
157 raised in rabbit using the amino-terminal 15 aa residues (MLHSPHKQPQNHKCG) of murine
158 testicular Runx2 as an antigen. In some cases, the antiserum was preabsorbed with an excess
159 amount of antigen peptide. The secondary antibody was alkaline phosphatase-conjugated goat
160 anti-rabbit IgG (Promega, Madison, WI). The antibodies were diluted appropriately in TBS-T.

Immunologically reacted products were detected using the BCIP/NBT Color Development Substrate Kit (Promega, Madison, WI).

Immunohistochemistry of Testicular Preparations

To prepare frozen sections, testes from adult male C57BL/6J mice were cut into three pieces and fixed in Zamboni solution for 6 hr at 4°C with agitation. The tissues were immersed sequentially in 10% (w/v), 15%, and 20% sucrose solutions prepared in phosphate-buffered saline (PBS) for 2 hr each at 4°C; embedded in OCT compound (Miles Laboratories, Berkeley, CA); and kept frozen at −80°C until use. The tissues were cryostat-sectioned (7-μm thick sections), air-dried, and post-fixed for 20 min in 4% (w/v) paraformaldehyde prepared in PBS.

The sections were then treated with methanol containing 0.3% (v/v) hydrogen peroxide for 15 min followed by PBS containing 3% (w/v) skimmed milk and 10% (v/v) goat serum for 30 min. In immunohistochemistry, the anti-serum was affinity-purified using a peptide (MLHSPHKQPQNHKCG)-linked Sepharose 4B column. The sections were incubated with appropriately diluted, affinity-purified anti-Runx2 antibody at 4°C overnight, followed by biotinylated goat anti-rabbit IgG for 30 min. The primary antibody was detected using an ABC Kit (Vector Laboratories, Burlingame, CA). The sections were post-stained with methyl green, and coverslips were mounted on glass slides.

Results

Testicular Runx2 Is Transcribed Specifically in Germ Cells

To gain insight into the significance of *Runx2* transcript in testes, we first used W/W^v mice as well as wild-type C57BL/6 mice. W/W^v mice lack germ cells except spermatogonia because of mutations in the *c-Kit* gene (Kubota et al., 2009). RNA was extracted from testes and processed for Northern blot analysis (Fig. 1). The *Runx2* transcript was detected as a broad band of ~1.8 kb length in wild-type testis, but not in W/W^v testis (Fig. 1, *A,* lanes 1 and 2). By contrast, the *PEBP2β/CBFβ*transcript, which was used as a control, was detected in both RNA samples tested (Fig. 1, *B,* lanes 1 and 2). This indicates that *Runx2* is expressed specifically in germ cells, not in somatic cells.

Next, spermatocytes at the pachytene stage and spermatids were purified from a cell suspension prepared from wild-type testis, and then RNA analyzed. The *Runx2* transcript was detected in both the spermatocyte and spermatid fractions (Fig. 1, *A,* lanes 3 and 4). The differentiation of germ cells proceeds in a synchronous fashion immediately after birth; therefore, RNA was prepared from the testes of newborn mice and analyzed (Fig. 1, *A,* lanes 5–8). A band of 1.8 kb was detected in 4 week-old testis, which largely contains germ cells at the spermatid stage. Thus, testicular *Runx2* was transcribed in germ cells at the spermatocyte and spermatid stages.

Determination of The Structure of The Testicular Runx2 Transcript

Although the study by Ogawa *et al*. predicted the ORF of *Runx2* to encode a 106-aa protein (Ogawa et al., 2000), the cDNA and aa sequences have not been published or registered in a public

201 database. Only the junction sequence between exons 1 and 3 is available in the literature.
202 Therefore, we decided to independently clone *Runx2* cDNAs from a library prepared from murine
203 testis. The nucleotide sequence that was determined from the obtained cDNA clones and 5'RACE
204 is presented in Figure 2, *A* and has been deposited at NCBI (accession number, DQ458792). The
205 AUG codons of Met^{1} and Met^{69} as well as the termination codon are indicated in bold.
206 We next compared this cDNA sequence with the murine genomic sequence of *Runx2*
207 (AB013129) (Xiao et al., 1998). The so-called exon 1 could actually be split into two small exons
208 that are separated by an intronic sequence of 197 nucleotides. We tentatively designated these
209 smaller exons as 1U (U for upstream) and 1D (D for downstream). The predicted coding region of
210 each exon is shown in Figure 2, *B*. Exon 1U harbors the Met^{1} codon, whereas exon 1D includes the
211 Met^{69} codon. Exons 1U and 1D are also transcribed in bone *Runx2* (Xiao et al., 1998) and the ORF
212 of bone *Runx2* has been predicted (NM_009820) (Ducy et al., 1997). The amino-terminal 87 aa
213 residues are common to both the testicular and bone ORFs, whereas the carboxy-terminal 19 aa
214 residues derived from exon 3 are unique to the testicular ORF.
215
216 **Bioinformatic Analysis Suggests that The Testicular Runx2 Variant Is Authentic**
217 The predicted aa sequence of the testicular Runx2 variant is shown in Figure 3, *A*. We performed
218 bioinformatic analysis as a first step to examine the theoretical likelihood that this is an authentic
219 protein. According to the PSORTII program, the Runx2 variant had a 43% probability of being a
220 nuclear protein. Moreover, the variant appears to be a basic protein; out of 106 aa residues, 22 are

221 basic and only 6 are acidic. Secondary structure assignment programs suggested that Runx2 has
222 both α-helical and β-sheet structures (indicated by H and E, respectively; Fig. 3, *A*). In particular, a
223 stretch of 55 aa in the amino-terminus are folded into two distinct α-helices. A basic, nuclear
224 protein with a high α-helical content is reminiscent of a histone. On the other hand, three carboxy-
225 terminal Cys residues may adopt a globular structure with a sulfur bridge (indicated by green, Fig.
226 3, *A*).

227 The amino-terminal part of the Runx2 variant (from Met^{1} to Ser^{46}) showed weak but
228 significant similarity to a 48-aa sequence of a murine protein of unknown function that is
229 expressed in the eye, namely, Q7TPL8/33942100 (SWISS and NCBI IDs, respectively; Fig. 3, *B*).
230 Q7TPL8 possesses features that suggest it is a transcription factor (Fig. 3, *C*). At the amino-
231 terminus, it contains a KRAB (Kruppel-associated box) domain that may function as a
232 transcription-repression domain, and at the carboxy-terminus, it harbors eight zinc fingers that
233 may function as nucleic acid-binding structures. The region of Q7TPL8 that shows similarity to
234 Runx2 is located immediately before the stretch of zinc fingers. This feature suggests that the
235 amino-terminal motif of 46 aa residues in Runx2 represents a functional domain. In addition,
236 testicular Runx2 also retains a stretch of 19 aa residues (from Met^{69} to Trp^{87}) that corresponds to an
237 important transcription activation domain in bone Runx2 (Thirunavukkarasu et al., 1998).

238 Taken together, these observations increase the probability that testicular Runx2 is an
239 authentic protein.

240

241 **Met[1] Is Used as An Initiation Codon in The Testicular Runx2 Variant**

242 We next examined whether Met[1] is indeed used as an initiation codon in testis. We generated an
243 antiserum using the amino-terminal 15 aa of Runx2 (Met[1] to Gly[15]) as an antigen. A protein extract
244 was prepared from mouse testis and processed for immunoblot analysis. A clear band of
245 approximately 11 kDa was detected (Fig. 4, *A*, lane 1), whereas inclusion of an excess amount of
246 antigen peptide in the immunoreaction abolished this band (Fig. 4, *A*, lane 2). The upper bands
247 were non-specific. Lysates from bacteria expressing a GST-Runx2 ORF fusion protein were run as
248 controls in lanes 3 and 4, and GST-Runx2 was detected with the same antiserum. It is therefore
249 highly likely that this ORF is expressed in testis as a polypeptide of 106 aa residues.

250

251 **Testicular Runx2 Is A Differentiation Stage-dependent Nuclear Protein**

252 To verify that the truncated variant of Runx2 is expressed, we performed immunohistochemistry
253 on frozen sections of testes (Fig. 4, *B*). Panel a shows the staining pattern in mouse testis that was
254 probed with the affinity-purified anti-Runx2 antibody raised against the Met[1]-to-Gly[15] peptide.
255 Positively stained cells were detected within seminiferous tubules. This immunostaining was
256 specific for Runx2 because it completely disappeared when the antibody was preabsorbed with an
257 excess amount of antigen peptide (panel b).

258 Interestingly, the distribution pattern of positive cells appeared to differ from tubule to
259 tubule. Therefore, each seminiferous tubule containing positive cells were classified according to
260 the differentiation stage. This was judged by the morphology of cells and nuclei (see panel c–i).

Runx2 staining was detected in the following differentiation stages: late pachytene spermatocytes at stages VIII and X (panels c and d), diplotene spermatocytes at stage XI (panel e), cells of second meiotic phase at stage XII (panel f), and round spermatids of spermiogenesis phase 1, 2/3, and 5 at stage I, II/III, and V, respectively (panels g, h, and i). In short, the Runx2 variant was detected in various stages of differentiation, from late pachytene spermatocytes to round spermatids. It must be noted that detection of Runx2 protein by immunohistochemistry (Fig. 4, *B*) and detection of *Runx2* transcript by Northern blot (Fig. 1) coincides in terms of germ cell specific expression and expression in pachytene spermatocytes to spermatids.

Finally, as seen in the enlarged view in Figure 4, *C,* positive staining was always restricted to the nuclei, indicating that testicular Runx2 is a nuclear protein. The protein was detected as multiple foci in the nuclei of germ cells. The original picture of immunohistochemistry observed under differential interference contrast microscope can be seen as supplement figures (Fig. S1, Fig. S2).

Discussion

This study showed that testicular Runx2 is located in the nucleus, appears to be a basic protein, and has a predominantly α-helical conformation. These characteristics are somewhat reminiscent of histone proteins. The protein was detected in spermatocytes at the late pachytene and diplotene stages as well as in round spermatids. At the pachytene and diplotene stages, genetic information is exchanged between a pair of homologous chromosomes through homologous recombination.

281 Thereafter, in post-meiotic and round spermatids, chromatins containing a haploid genome start
282 preparing to remodel their structures. The testicular Runx2 protein detected in this study might be
283 involved in the aforementioned processes. In this context, it is worth noting the histone variants
284 that are expressed specifically in male germ cells. For example, a testicular variant of linker
285 histone 1 is detected in pachytene spermatocytes and persists until the round spermatid stage
286 (Brock, Trostle & Meistrich, 1980; Drabent et al., 1996). Likewise, TH2A and TH2B (testicular
287 variants of the core histones H2A and H2B) and H3t (a testicular variant of the core histone H3)
288 are expressed in round spermatids. It would be interesting to determine whether testicular Runx2 is
289 incorporated into nucleosomes and play roles in loosening their structures, as histone variants are
290 suggested to do (Rathke et al., 2014).

291 Jeong *et al.* reported the expression of the Runx2 transcription factor in mice sperm (Jeong
292 et al., 2008). Their immunoblot analyses using a monoclonal antibody against Runx2 detected
293 proteins of 47 and 65 kD in lysates from testes and sperm. Therefore, the observations of Jeong *et*
294 *al.* (Jeong et al., 2008) appear to be substantially different from our results. However, it must be
295 noted that our Northern blot analyses could detect, albeit faintly, bands larger than the major 1.8-
296 kb band (see fig. 1). If such larger transcripts harbor the Runt domain sequence, they probably
297 encode the Runx transcription factor that is found in bone and T cells. Thus, the findings of Jeong
298 *et al.* (Jeong et al., 2008) and the current study may not be contradictory.

299 Although this study used murine testes, the *Runx2* gene is conserved among mammals,
300 thus suggesting a possible extrapolation of our findings to other species. In this sense, we note that

301 the two homologous transcripts are found in the NCBI database. They are AB573882.1 and
302 AB573881.1, and are reported to be expressed in a periosteum tissue of rat. In these rat transcripts,
303 splicing appears to skip exon 2 and fuse exons 1 and 3 as in the same way as a murine testicular
304 *Runx2* transcript. Whether this rat *Runx2* transcript is expressed as a protein or not is not known at
305 present.
306
307 ## Conclusion
308 In conclusion, a variant of *Runx2* that differs from the bone isoform in its splicing is
309 expressed in pachytene spermatocytes and round spermatids in murine testes, and encodes a
310 histone-like, nuclear protein of 106 amino acid residues. Considering its nuclear localization and
311 differentiation stage-dependent expression, Runx2 may function as a chromatin-remodeling factor
312 during spermatogenesis.
313
314
315 **Author's contribution:** SK carried out genetic studies and immunoassays. MK carried out
316 bioinformatics analysis. YK carried out northern blot analysis. JF carried out northern blot analysis.
317 MS conceived of the study, participated in its design and drafted the manuscript.
318
319 **Competing Interest:** None
320

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Figure 1(on next page)

Northern blot analysis of Runx2 expression in testis

Fig. 1- RNA was prepared from 10-week-old W/W^{v} and C57BL/6J mouse testes (lanes 1 and 2, respectively), from pachytene spermatocytes and round spermatids (lanes 3 and 4, respectively), and from 1-, 2- 3- and 4-week-old C57BL/6J mouse testes (lanes 5, 6, 7 and 8, respectively). The probes were cDNAs of the murine Runx2 (A) (a HindIII-NotI fragment corresponding to nt 282 through to nt 473 in NM_001146038.2) and *PEBP2β/CBFβ* (B). The numbers alongside the gels show the sizes of the transcripts in kb.

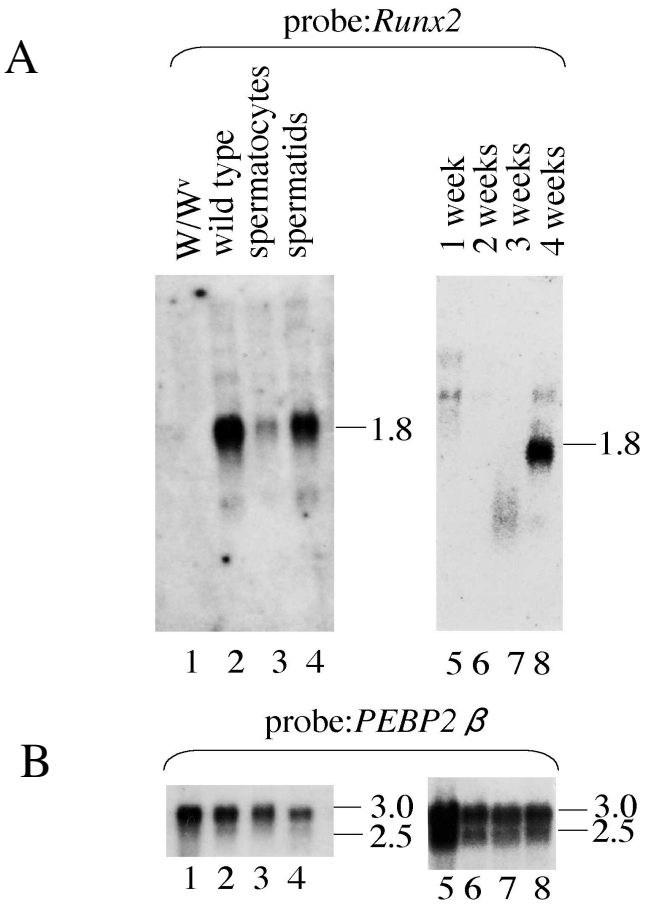
A
probe:Runx2
W/Wv
wild type
spermatocytes
spermatids
1 week
2 weeks
3 weeks
4 weeks
1.8
1.8
1 2 3 4
5 6 7 8
B
probe:PEBP2 β
3.0
2.5
3.0
2.5
1 2 3 4
5 6 7 8

Figure 2(on next page)

The nucleotide sequence and the structure of testicular Runx2

Fig. 2 - (A) The nucleotide sequence of the testicular Runx2 transcript (1,480 nucleotides) is shown. The two ATG codons represent the Met1 and Met69. The TGA represents the termination codon. The two forward slashes indicate the boundaries between exons 1U and 1D and between exons 1D and 3. The underlined sequence is a poly(A)-addition signal. (B) Comparison of open reading frames that are assigned to the testis- and bone-derived Runx2 transcripts is shown. The numbers represent the exon numbers and the boxes represent the coding regions.

A

aaaagattgagaaagagggagggaagagagcaaggggaagccacagtggtaggcagtcccactttactttgagt
actgtgaggtcacaaaccacatcgattctgtctctccagtaatagtgcttgcaaaaaataggagttttaaagctt
ttgctttttggattgtgtga**atg**cttcattcgcctcacaaacaaccacagaaccacaagtgcggtgcaaacttt
ctccaggaagactgcaagaaggctctggcgtttaaatggttaatctctgcaggtcactaccagccaccgagacca
accgagtca/
tttaaggctgcaagcagtatttacaacagagggcacaagttctatctggaaaaaaaaggagggact**atg**gcgtca
aacagcctcttcagcgcagtgacaccgtgtcagcaaagcttcttttgg/
ggttgtagccctcggagaggtaccagatgggactgtggttaccgtcatggccgggaa**tga**tgagaactactccgc
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tcactaccacacgtacctgccaccaccctaccccggctcttcccaaagccagagtggacccttccagaccagcag
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aaaaaaaaaaaaaaaaa

B

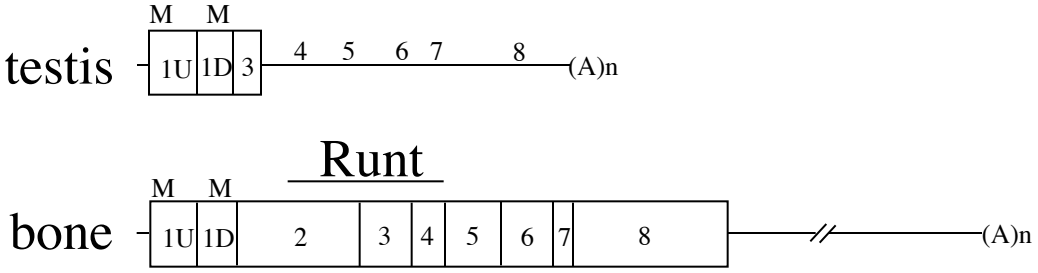


Figure 3(on next page)

Bioinformatic analysis of the testicular Runx2 polypeptide

Fig. 3 - (A) Secondary structures such as α-helices and β-sheets are indicated by H and E, respectively. The two Met and five Cys residues are indicated. The two forward slashes indicate the boundaries of the exons. (B) A homologous motif found in testicular Runx2 and Q7TPL8 proteins is shown. Identical or similar aa are indicated. (C) The domain architecture of the Q7TPL8 protein is shown. The circle and boxes represent the KRAB and C2H2-type zinc finger domains, respectively. The region showing similarity to testicular Runx2 is indicated by a red line.

A testicular Runx2:

```
            HHHHHHHHHHHHHHHHHHHHH          HH HHHHHHHH
MLHSPHKQPQNHKCGANFLQEDCKKALAFKWLISAGHYQPPRPTES/FKAASSIYN

   EEEEE    EEE  HHHHH       EEE         EEE
RGHKFYLEKKGGTMASNSLFSAVTPCQQSFFW/GCSPRRGTRWDCGYRHGRE
```

B

```
testicular Runx2 :  MLHSPHKQPQNHKCGANFLQEDCKKALAFKWLISAG--HYQPPRPTES
Q7TPL8:             AFHHKSLLPQYQSARADEQQSDCKELMKIYFYVSSPTQHHGPPPPEKP
```

C Q7TPL8:

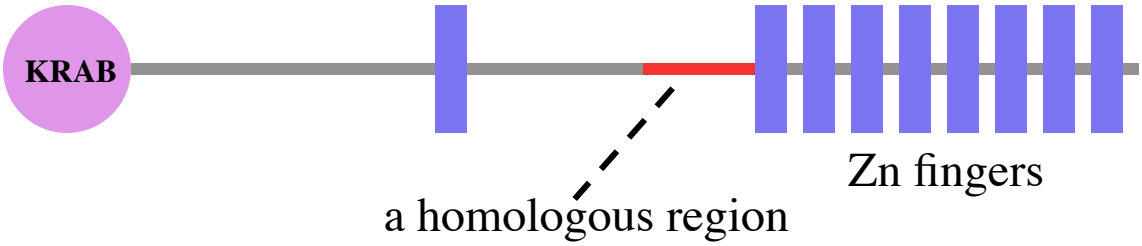
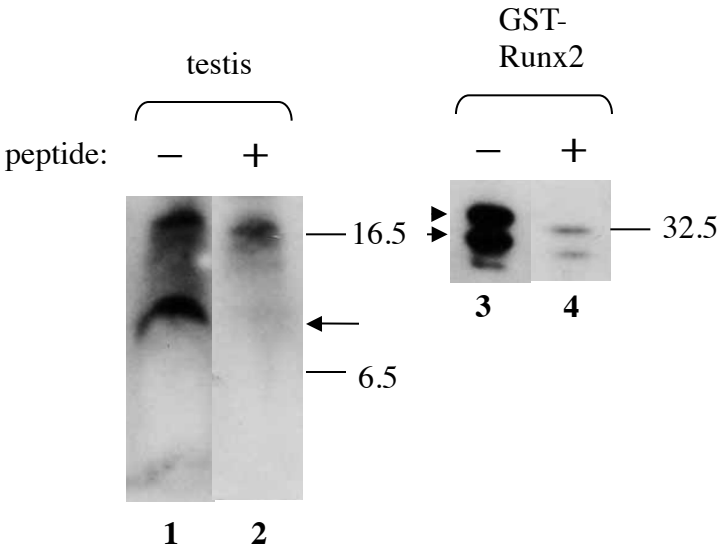


Figure 4(on next page)

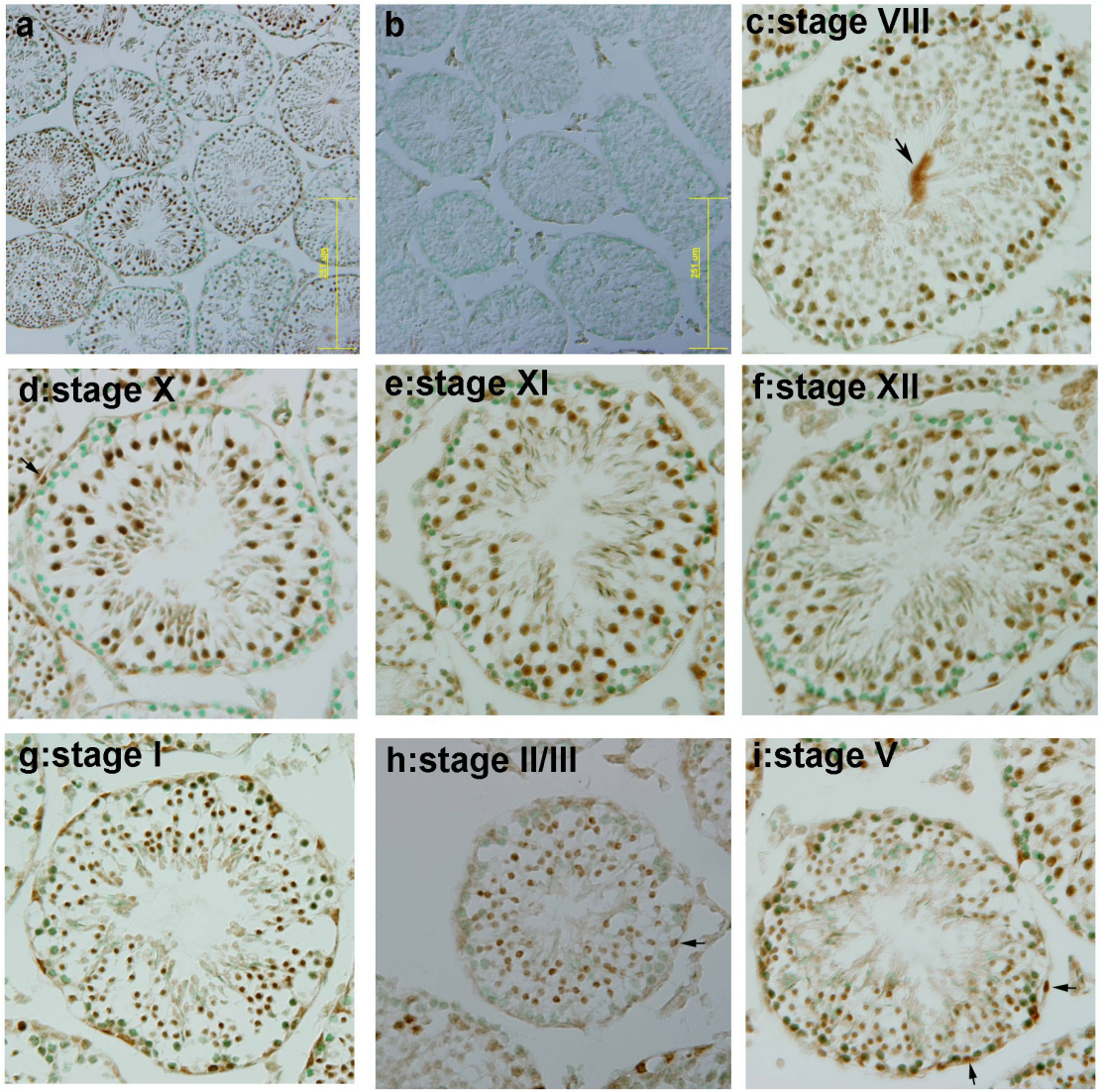
Immunoblot detection of Runx2 protein in testis and its localization in nuclei of spermatogenic cells dependent on seminiferous-stage

Fig. 4 - (A) Immunoblot detection of Runx2 is shown. Lanes 1 and 2 contain protein from testes, whereas lanes 3 and 4 contain protein from GST-Runx2-transfected bacteria. The membrane was probed with anti-Runx2 serum, which was preabsorbed with the antigen peptide (lanes 2 and 4) or was not (lanes 1 and 3). The arrow and arrowhead indicate testicular Runx2 and the GST-Runx2 fusion protein, respectively. (B) Immunohistochemical staining of Runx2 protein in testis is shown. Testes from adult C57BL/6J mice were stained with the affinity-purified anti-Runx2 antibody and counterstained with methyl green. In b, the antibody was preabsorbed with the antigen peptide. The yellow scale bars correspond to 251 μm. Arrow in panel c: this staining was not disappeared when the antibody was preabsorbed with the antigen peptide. Arrow in panel d, h and i: nuclei of spermatogonia. (C) Enlarged view of positive staining in nuclei is shown. a: pachytene spermatocytes, b: round spermatids.

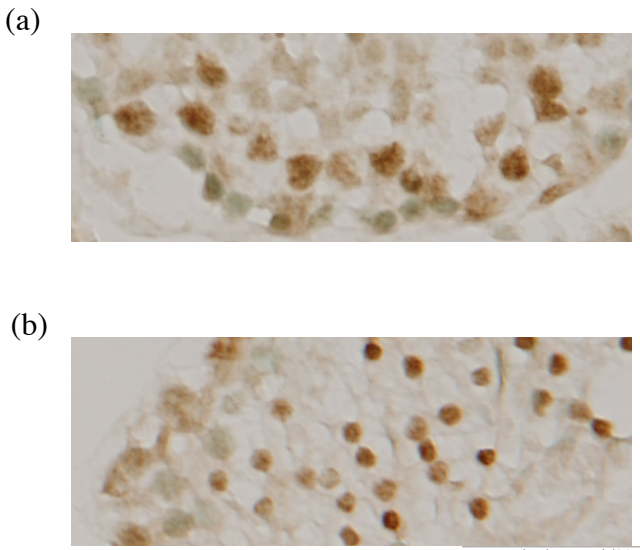
A


testis
GST-
Runx2
peptide:
–
+
–
+
16.5
32.5
6.5
1
2
3
4

B


a
b
c:stage VIII
d:stage X
e:stage XI
f:stage XII
g:stage I
h:stage II/III
i:stage V

C


(a)
(b)