A

```
                                                                                                38-57 loop
Rabbit(JW)      MGTLQG-LLL WLLLG-TGGA QAPRGPLRPL CRPVNATLAA ENEACPVCIT FTTSICAGYC PSMVRVLPAA LPPVPQPVCT [ 80]
Rabbit(NZW)     .....-...  .....-....  ..........  ..........  ..........  ..........  ..........  .......... [ 80]
Rabbit(Dutch)   .....-...  .....-....  ..........  ..........  ..........  ..........  .........T  .......... [ 80]
Mouse           .ER...-...  ....S-PSVV W.S.......  ..........  ...F......  ..........  ..........  .......... [ 80]
Rat             .ER...-...  ....S-PSVV W.S.......  ..........  ...F......  ..........  ..........  .......... [ 80]
S.Hamster       ..R...-...  .....-P.VV W.S.......  ..........  ..DV......  ..........  ..........  .A........ [ 80]
Mastomys        .ER...-...  ....S-PSVV W.S.......  ..........  ...F......  ..........  ........T.  .......... [ 80]
M.Gerbil        .ER...-...  ....S-P.ML W.C...P.A.  .....T....  .K.F......  ..........  ..........  .......... [ 80]
Guinea pig      .EM...-...  G...S-.S.V W.S......Y  ...I......  .K....I.V.  ..........  ...M...TT. RR....L... [ 80]
Cattle          .EMF..-...  .....-VA.V W.S.......  .Q.I......  .K........  ..........  ...K....VI  ...M..R... [ 80]
Sheep           .EM...-...  .....-VA.V W.S.......  .Q.I......  .K........  ..........  L..KQ...VI  ...M..R... [ 80]
Pig             --M..R-...  ....S-VA.V W.S.......  ...I......  ..........  ........D.  ..........  .......... [ 80]
Cat             .EM...L...  ....LNV..V WTS.E.....  ...I......  ........V.  ...T......  ...M......  .......... [ 80]
C.E.Macaque     .EM...-...  ....S-M...  R.S.E.....  ...I......  .K........  VN........  .T.M...Q..  .L.L..V... [ 80]
Human           .EM...-...  L...S-M...  W.S.E....W  .H.I..I..V  .K.G......  VN.T......  .T.M...Q.V  ...L..V... [ 80]
                                                    93-100 determinant loop
Rabbit(JW)      YRELRFASIR LPGCPPGVDP EVSFPVALSC RCGPCRLSSS DCGGPRAQPL ACDLPHLPGL LFL [143]
Rabbit(NZW)     ..........  ..........  ..........  ..........  ..........  ..........  ...  [143]
Rabbit(Dutch)   ..........  ..........  ..........  ..........  ..........  S.........  ...  [143]
Mouse           ....A...V.  ..........  I.........  ..........  ......T..M  ..........  .L.  [143]
Rat             ........V.  ..........  I.........  ..........  ......T..M  T.........  .LF  [143]
S.Hamster       ....H.T.V.  ..........  M.........  ..........  ..........  .....R....  ...  [143]
Mastomys        ........V.  ..........  I.........  ..........  ......T..M  ..........  .L.  [143]
M.Gerbil        ........V.  ..........  M.........  ..........  ......T...  T.N.......  ...  [143]
Guinea pig      .D........  ..........  V.........  H..T....N.  ....L.G..S  ..E......F  F..  [143]
Cattle          .H......V.  ..........  M.........  H.........T  ......T...  ...H.P..DI  ...  [143]
Sheep           .H......V.  ..........  M.........  H.........T  ......T...  ...H.P..DI  ...  [143]
Pig             ....S.....  ..........  T.........  H.........  ..........  ...R.L....  ...  [143]
Cat             ........V.  ..........  V.........  ..........  ..........  ...R.P....  ...  [143]
C.E.Macaque     .H.V..D..Q  ....L.....  V.........  .....HR.T.  .....KDH..  T..H.Q....  ...  [143]
Human           ..DV..E...  .....R....  V.........  ......R.T.  .....KDH..  T..H.Q.S..  ...  [143]
```

B

←Rabbit→

NZW	100.0	100.0	63.6	63.6	77.3	63.6	68.2	68.2	68.2	72.7	59.1	59.1	77.3	72.7
100.0	**JW**	100.0	63.6	63.6	77.3	63.6	68.2	68.2	68.2	72.7	59.1	59.1	77.3	72.7
98.3	98.3	**Dutch**	63.6	63.6	77.3	63.6	68.2	68.2	68.2	72.7	59.1	59.1	77.3	72.7
93.4	93.4	91.7	**Mouse**	100.0	86.4	100.0	86.4	81.8	72.7	77.3	68.2	63.6	72.7	72.7
92.6	92.6	91.7	97.5	**Rat**	86.4	100.0	86.4	81.8	72.7	77.3	68.2	63.6	72.7	72.7
92.6	92.6	90.9	91.7	90.1	**S.Hamster**	86.4	81.8	68.2	72.7	77.3	63.6	63.6	68.2	68.2
93.4	93.4	91.7	98.3	97.5	90.9	**Mastomys**	86.4	81.8	72.7	77.3	68.2	63.6	72.7	72.7
90.9	90.9	90.1	90.9	91.7	88.4	90.9	**M.Gerbil**	72.7	68.2	72.7	63.6	63.6	77.3	77.3
81.0	81.0	79.3	78.5	77.7	76.9	80.2	76.9	**Guinea pig**	77.3	81.8	72.7	68.2	77.3	81.8
84.3	84.3	83.5	83.5	82.6	82.6	84.3	82.6	77.7	**Cattle**	95.5	77.3	72.7	72.7	72.7
82.6	82.6	81.8	81.8	81.0	81.0	82.6	81.0	76.0	98.3	**Sheep**	81.8	77.3	77.3	77.3
93.4	93.4	91.7	90.1	88.4	90.1	89.3	86.0	80.2	85.1	83.5	**Pig**	63.6	68.2	68.2
91.7	91.7	90.1	89.3	88.4	89.3	89.3	86.0	81.0	85.1	83.5	91.7	**Cat**	68.2	68.2
77.7	77.7	76.9	74.4	75.2	75.2	74.4	74.4	71.9	75.2	73.6	76.9	79.3	**C.E.Macaque**	90.9
73.6	73.6	73.6	71.1	71.9	71.1	71.1	71.1	66.9	71.1	69.4	72.7	76.9	86.8	**Human**

C

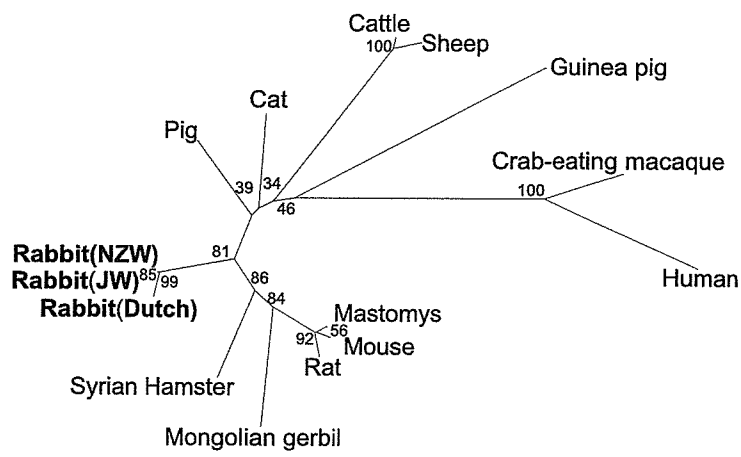

Fig. 2. (A) Alignment of LHB sequences from various mammals. The LHB sequences from JW, NZW, and Dutch rabbits are shown in the top three rows. A dot indicates an amino acid residue that is identical at the corresponding position in the JW sequence (top row). Dashes indicate sequence gaps inserted by the CLUSTAL W software. The cysteine residue positions (shaded) and putative N-glycosylation sites (boxed) are highly conserved in most mammals. (B) Homology matrix for the signal (upper-right triangle) and mature (lower-left triangle) LHB sequences of 15 different animals. (C) Neighbor-joining tree of the LHB amino acid sequences predicted for 15 different animals. Bootstrap values are shown near each node. M. Gerbil, Mongolian gerbil; S. Hamster, Syrian hamster; C.E. Macaque, Crab-eating macaque.

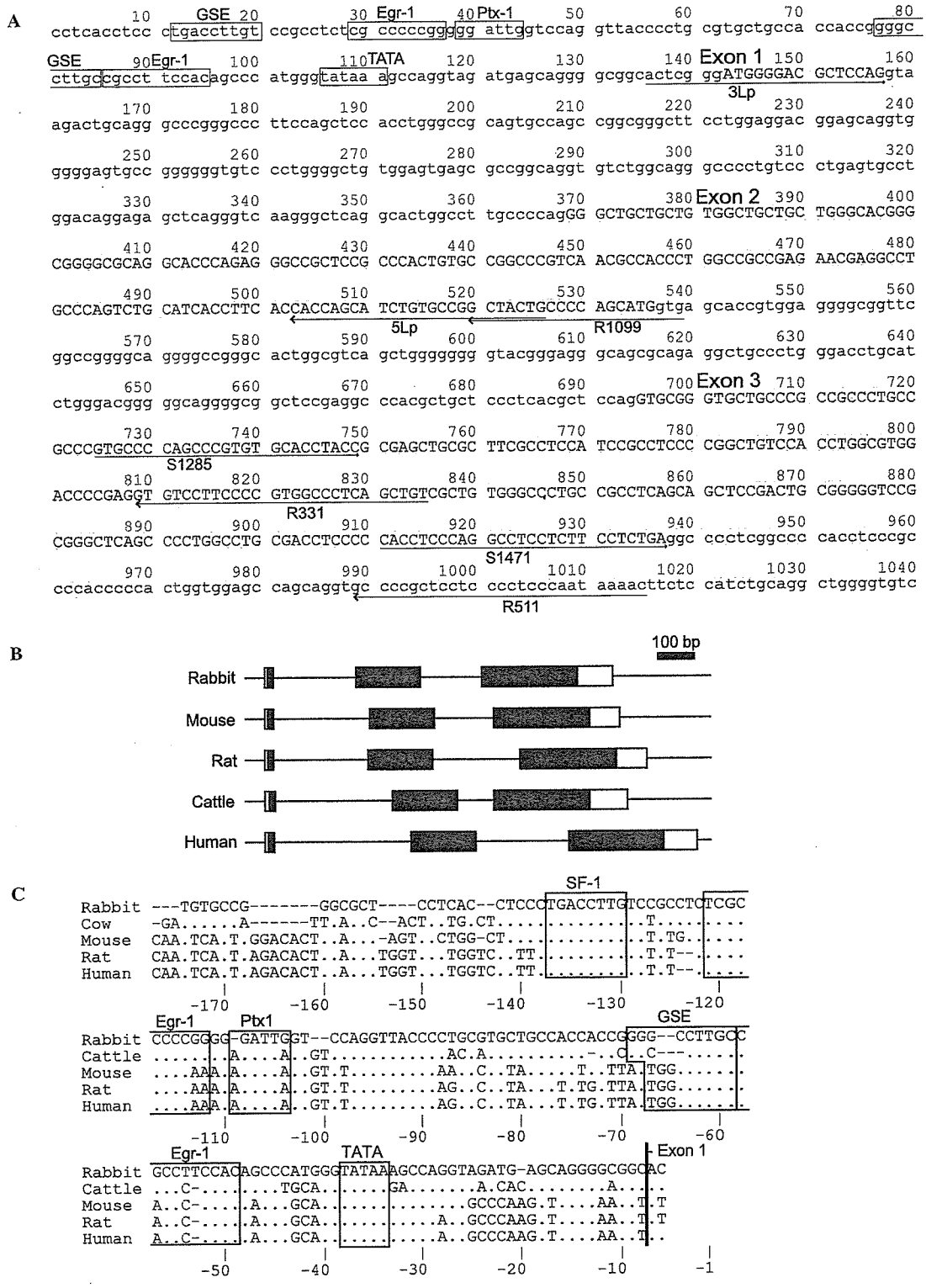

Fig. 3. Genomic sequence of the NZW rabbit LHB gene. (A) Putative TATA-box and binding sequences for GSE, Egr-1, and Ptx-1 are boxed. The rabbit LHB gene contains three exons (shaded) and two introns. Uppercase letters indicate a coding sequence. Arrows indicate the orientations and positions of the primers used for intron determination and genomic walking. (B) Schematic representation of the exon–intron structures of five mammals. Open boxes indicate 5′- or 3′-untranslated regions. Closed boxes indicate coding sequences. (C) Comparison of the 5′-flanking sequences of the LH gene in five mammals. Putative sequences for various factors are boxed as in (A).

The NZW, JW, and Dutch breeds form a distinct group in the molecular phylogenetic tree of LHB proteins (Fig. 2C), as suggested by our previous phylogenetic analysis of common glycoprotein hormone α-subunit (Suzuki et al., 2002) and FSHB (Noguchi et al., 2006). The sequences of the mature rabbit and rodent LHB proteins are highly homologous (~90%, Fig. 2B), whereas the rabbit and rodent signal sequences are considerably less homologous (<65%, Fig. 2B). Thus, all three glycoprotein subunits of rabbit LHB are phylogenetically distant from their rodent counterparts, which supports the proposition that the phylogeny of rabbit is peculiar (Graur et al., 1996).

The high similarity between the LHB genomic sequences of rabbit and other mammals suggests that transcription is regulated similarly in both groups (Fig. 3). The rabbit genomic LHB sequence (Fig. 3A), which has been deposited in the DDBJ/EMBL/GenBank databases under Accession No. AB234232, has an exon configuration similar to that of other LHBs, i.e., three exons and two introns (Fig. 3B). In addition, the 5′-upstream regions of the rabbit and other LHB genes are highly conserved (Fig. 3C). In particular, their promoter regions contain putative binding sites for SF-1 (gonadotroph-specific element; GSE), Egr-1, and Ptx1. Since the binding sequences for these transcription factors are highly conserved in rabbit and other mammals, these factors may function in the rabbit pituitary as they do in other mammals (Reviewed by Gajewska and Kochman, 2001).

Rabbit LH would be the best and most natural hormone for inducing ovulation in rabbits. The LHs of sciurognath rodents, pigs, or cats might be also superior to LH of human origin at inducing ovulation in rabbits, based on the phylogenetic position of rabbits and the homology of mature LHB proteins, although human chorionic gonadotropin (hCG) is easily available and, therefore, commonly used for inducing ovulation in rabbits. Mature rabbit LHB is more similar to mature sciurognath rodent, pig and cat LHB (>90% similarity) than to mature human LHB (74% similarity). The biological potency of LH varies considerably among species (Reichert et al., 1973), and the β-subunit is responsible for this variability (Pierce and Parsons, 1981). Interestingly, rabbit LHB is not highly homologous to either human LHB or hCG-β, in contrast to rabbit and human FSHB, which exhibit high-level homology (Noguchi et al., 2006). In particular, at both the 38–57 loop and the 93–100 determinant loop (Fig. 2A), which are thought to be important for bioactivity, rabbit LHB is more similar to rodent LHB than to human LHB or hCG. Hormone-receptor sequences must also be taken into account, however, since the ligand-receptor interaction is critical for hormone function; sequence analysis of the rabbit LH receptor will aid in clarifying these issues. Our determination of the genomic and cDNA sequences of the LH β-subunit in the rabbit should aid development of improved superovulation technology for the rabbit.

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References

Bousfield, G.R., Perry, W.M., Ward, D.N., 1994. Gonadotropins. In: Knobil, E., Neill, J.D. (Eds.), The Physiology of Reproduction. Raven Press, Ltd., New York, pp. 1749–1792.

Gajewska, A., Kochman, K., 2001. GnRH pulsality and the differential activation of the rat luteinizing hormone subunit genes in the anterior pituitary gland. Neuro. Endocrinol. Lett. 22, 435–440.

Glenn, S.D., Nahm, H.S., Ward, D.N., 1984. The amino acid sequence of the rabbit lutropin beta subunit. J. Protein Chem. 3, 259–273.

Graur, D., Duret, L., Gouy, M., 1996. Phylogenetic position of the order Lagomorpha (rabbits, hares and allies). Nature 379, 333–335.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.

Koura, M., Handa, H., Noguchi, Y., Takano, K., Yamamoto, Y., Matsuda, J., Suzuki, O., 2004. Sequence analysis of cDNA encoding follicle-stimulating hormone and luteinizing hormone β-subunits in the Mongolian gerbil (*Meriones unguiculatus*). Gen. Comp. Endocrinol. 136, 406–410.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA 3: Integrated Software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5, 150–163.

Noguchi, Y., Takano, K., Koura, M., Uchio-Yamada, K., Matsuda, J., Suzuki, O., 2006. Sequence analysis of cDNA encoding rabbit follicle-stimulating hormone β-subunit precursor protein. Gen. Comp. Endocrinol. 147, 231–235.

Pierce, J.G., Parsons, T.F., 1981. Glycoprotein hormones: structure and function. Ann. Rev. Biochem. 50, 465–495.

Reichert, L.E., Leidenberger, F., Trowbridge, C.G., 1973. Studies on luteinizing hormone and its subunits: development and application of a radioligand receptor assay and properties of the hormone-receptor interaction. Recent Prog. Horm. Res. 29, 497–532.

Suzuki, O., Mochida, K., Yamamoto, Y., Noguchi, Y., Takano, K., Matsuda, J., Ogura, A., 2002. Comparison of glycoprotein hormone α-subunits of laboratory animals. Mol. Reprod. Dev. 62, 335–342.

Suzuki, O., Koura, M., Noguchi, Y., Takano, K., Yamamoto, Y., Matsuda, J., 2003. Optimization of superovulation induction by human menopausal gonadotropin in guinea pigs based on follicular waves and FSH-receptor homologies. Mol. Reprod. Dev. 64, 219–225.

Takano, K., Koura, M., Noguchi, Y., Yamamoto, Y., Uchio-Yamada, K., Matsuda, J., Suzuki, O., 2004. Sequence analysis of cDNA encoding follicle-stimulating hormone and luteinizing hormone β-subunits in the Mastomys (*Praomys coucha*). Gen. Comp. Endocrinol. 138, 281–286.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

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臨床分子内分泌学4

—生殖内分泌系—

II. ゴナドトロピン（性腺刺激ホルモン）（LH，FSH）

ゴナドトロピンの生理作用

卵子発育

鈴木　治

ゴナドトロピンの生理作用

卵子発育

Effect of gonadotropins on oocyte growth

鈴木 治

Key words：成熟能獲得，受精能獲得，胚発生能獲得，卵胞発育培養，胚性遺伝子活性化

はじめに

卵子の発育・成熟は卵胞発育とともに進行する．その進行がゴナドトロピン(卵胞刺激ホルモン(FSH)と黄体化ホルモン(LH))による制御を受けていることは明白であるが，卵子自身はゴナドトロピンの受容体を発現しておらず，それゆえゴナドトロピンの生理作用は，卵胞を構成している細胞，すなわち顆粒層細胞(granulosa cell)と莢膜細胞(theca cell)を介して二次的にもたらされる．更に卵胞内ではautocrine/paracrineに働く様々な因子が関与しており，その解析は容易ではない．そこで，卵胞の構成要素を変え，かつ液性因子の解析が可能となるような実験系として，様々な卵胞発育培養系が開発されている．

本稿では，マウスの卵胞発育培養系で得られた知見を中心に，卵子発育へのゴナドトロピンの関与について概説する．

1. 卵子の発育と成熟

卵子形成(oogenesis)は長期にわたる現象である．まず胎生期に卵祖細胞が増殖し，減数分裂を開始して第一減数分裂前期の途中で停止する．核は卵核胞(germinal vesicle)という形態をとり，卵子(卵母細胞(oocyte))は休眠状態となる．性成熟に至って卵胞発育が再開され，原始卵胞が一次卵胞，二次卵胞と発育するに従い，卵子もその径を増大させる．卵胞腔(antrum)が形成され始める頃に卵子は最大径に達する．一方卵胞は卵胞腔の拡大に伴いその径が更に増大して胞状卵胞(antral follicle)となり，卵丘(cumulus oophorus)が形成される．その後，LHサージにより卵子は減数分裂を再開し，卵核胞が消失して(卵核胞崩壊(germinal vesicle breakdown: GVBD))，第一極体を放出し，第二減数分裂中期(MII)に到達し，再度減数分裂が停止する．大部分の哺乳動物ではこの時点で排卵が生じる．減数分裂が再開する前の卵子の径が増大していく過程を卵子発育(oocyte growth)，減数分裂が再開してから受精可能なMII卵子に達する過程を卵子成熟(oocyte maturation)と呼び分けている．

2. ゴナドトロピンの卵胞への作用

ゴナドトロピンの卵胞への作用は多岐にわたる[1]．FSHのβサブユニットやFSH受容体のノックアウトマウス，および性腺機能低下マウス(hpgマウス)の観察から，前胞状卵胞(preantral follicle)までの卵胞形成にはFSHの関与は低く，FSHは専ら初期胞状卵胞のそれ以降への発育を促す．また，顆粒層細胞のアポトーシス

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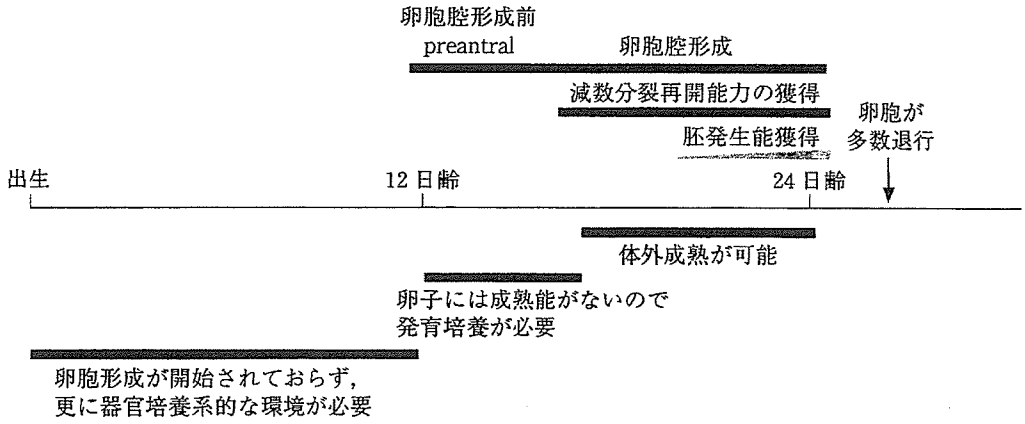

図1 マウスの初回卵胞発育

を低減させ，最終的にLH受容体の発現を促し，LHサージの準備をさせるという働きもある．一方，LHのβサブユニットやLH受容体のノックアウトマウスでも胞状卵胞の形成がなされるが，排卵・黄体形成を欠くことから，LHは卵胞発育に必須ではなく，減数分裂再開刺激，排卵制御，黄体形成などが主たる役割である．

3. マウスの初回卵胞発育(first wave of folliculogenesis)

雌マウスでは性成熟後は4-5日周期で排卵を繰り返すが，性成熟前にも幼若期にほぼ同調した一過性の卵胞形成がみられる(図1)．雄でも幼若期に一過性の精子形成がみられ，これらをfirst wave(本稿では初回発育と訳したが，確たる邦訳はないようである)と呼んでいる．この初回発育の時期には成熟段階が揃った卵胞卵子を比較的大量に採取することが可能で，卵胞発育の段階に対応した卵子の性質を調べることができる．

初回発育においても卵子は，成熟個体と同様，卵胞の発育とともに様々な能力を有するようになる(～能を獲得する，と表現する)．若干の系統差はあるが，12日齢頃は前胞状卵胞の状態で，16日齢頃に徐々に卵胞腔が形成され始め24日齢頃には胞状卵胞となる．しかし，これらの卵胞は排卵せず，閉鎖退行する．胞状卵胞への発育過程で卵子は徐々に減数分裂の再開が可能となり(成熟能獲得)，受精能，受精後の胚発生能(developmental competence)を獲得する．なお，この初回発育由来卵子の個体への発生能は確認されているものの[2]，成熟個体の卵胞形成とは異なる可能性もある点は留意すべきである[3]．

4. 卵胞発育培養

現在，それぞれに工夫を凝らした培養系が数多く報告されているが，前胞状卵胞の卵胞培養には大きく分けて3つのパターンがある(図2-A)[4]．ポイントは'構成要素がどこまで単純化されているか'である．第一の方法(例えばNayuduの方法[5])では卵巣から針などを用いて機械的に卵胞を摘出して培養する．この場合，卵胞構造のほとんどの構成要素を保持している．第二の方法(例えばRoyの方法[6])では，酵素処理によって卵胞を単離し，寒天ゲル内に包埋して培養する．この場合は基底膜および莢膜細胞層が消失した'卵子顆粒層細胞複合体'の状態である．第三の方法(Eppigの方法[2])でも酵素処理によって卵子顆粒層細胞複合体(図2-B)を得，培養は平板上で行う(詳細は後述参照)．第二の方法での培養では最終的に卵胞腔形成を伴うのに対し，第三の方法では壁側顆粒層細胞群が構成されず，ドーム状の卵丘構造のみが形成される(図2-C)．著者はEppigの方式による卵胞培養を学んだのでこの方法について記載する．

Eppig方式の卵胞培養は，初期にはWaymouth MB752培地にピルビン酸を加えたもの

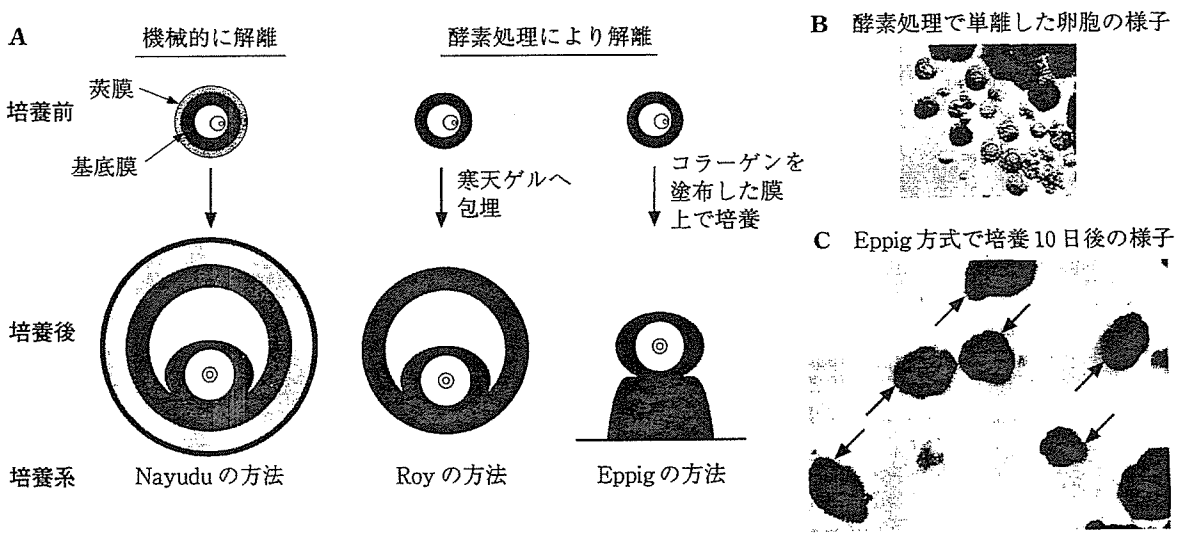

図2 卵胞培養系（文献[4]より改変）
A: 卵胞培養の3パターン，B，C: 実際の培養の様子．

を基本培地としていたが[7]，近年はMEM-alphaを基本としている[8]．血清添加培地では5％ウシ胎児血清を加え，無血清培地ではウシ血清アルブミン，fetuin，insulin，transferrin，seleniteを添加する．

実際には12日齢のマウス卵巣からコラゲナーゼとピペッティングにより前胞状卵胞を単離し（**図2-B**），コラーゲンコートメンブレンインサート上にて，5％CO_2，5％O_2，90％N_2の気相下で10日間培養する．培養後，卵丘構造が形成されるので（**図2-C**），卵丘細胞卵子複合体を回収し，体外成熟，体外受精に供する．

なお，この方法は基本的にマウスにのみ有効である．他の動物では，顆粒層細胞があたかも卵子の存在を忘れたかのようにメンブレンや培養皿の底に遊離・伸展して，裸の卵子が培養液中に浮いている，という状態になるため，卵子は発育できない．ただし，ウシでは高分子物質の高濃度添加による優れた方法が最近報告されており[9]，他の動物種への応用が期待される．

5. 発生能獲得とFSH

Eppig方式によるマウスの卵胞発育培養において無血清条件下ではFSHは不要で，むしろinsulinとFSHの同時添加は卵子の質（受精後の胚発生能）を低下させる[7]．insulin存在下でFSHは濃度依存的に卵胞培養由来胚の2細胞を超える率，桑実胚形成率，胚盤胞形成率などを低下させる（**図3**）．その際，第一卵割でのDNA合成やRNA合成量に影響はなかった．そこで2細胞を超える率の低下，いわゆる2-cell blockが生じることに注目し，胚性遺伝子活性化zygotic gene activation（ZGA）のマーカー蛋白質であるtranscription requiring complex（TRC）[10]の合成状況を調べた．

^{35}Sメチオニンで2細胞期胚を2時間ラベルした後，胚のTriton-X不溶分画をSDS-PAGEで分析すると**図4-A**のようなオートラジオグラムが得られる．TRCは近接する3本のバンド（68，70，73 kDa）として現れる．FSHの存在，非存在下で卵胞発育培養して得られた卵子に由来する胚を同一時刻にラベルした場合は，FSH添加濃度依存的にTRCの合成が低下し（**図4-B**），卵胞培養時のFSHの添加によりZGAが阻害されることが確認できた．更に**図4-C**のようにラベルする時間をずらして経時的変化を調べると，全体的にFSH添加群でTRC合成量が低い傾向があるが（一部に有意差あり），卵割後12時間では差がなかった．これは合成量の低下だけでなく，合成時期遅延の可能性も示唆された．

このことは胚発生能が卵子発育時の出来事，

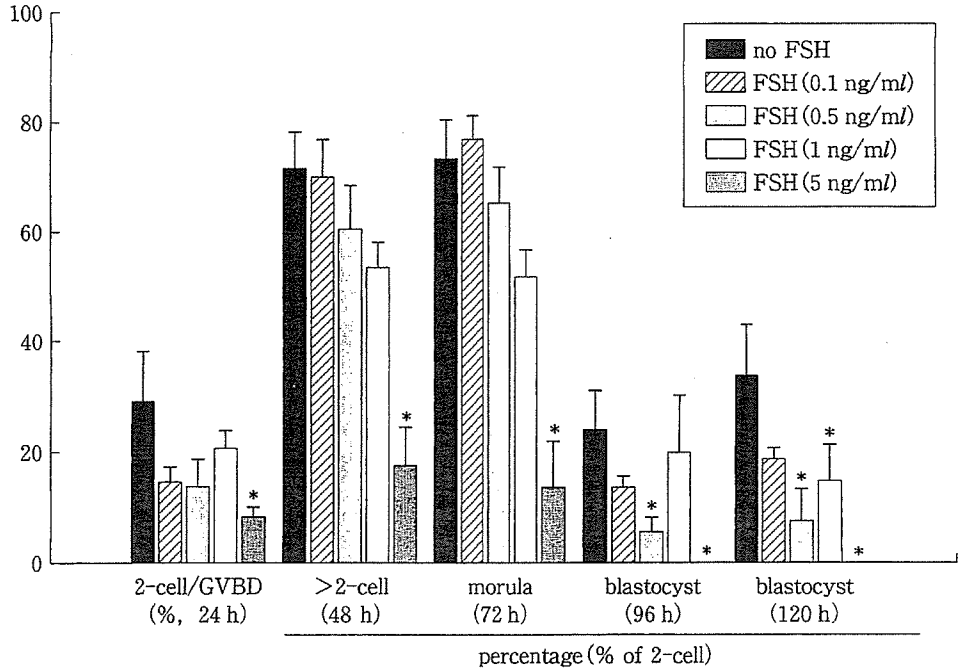

図3 FSH添加濃度と体外培養成績

ヒツジFSHを各濃度で含む培地で培養した卵子を体外受精ののち，体外培養したもの．*はFSH不含培地に比べ有意差があることを示す．

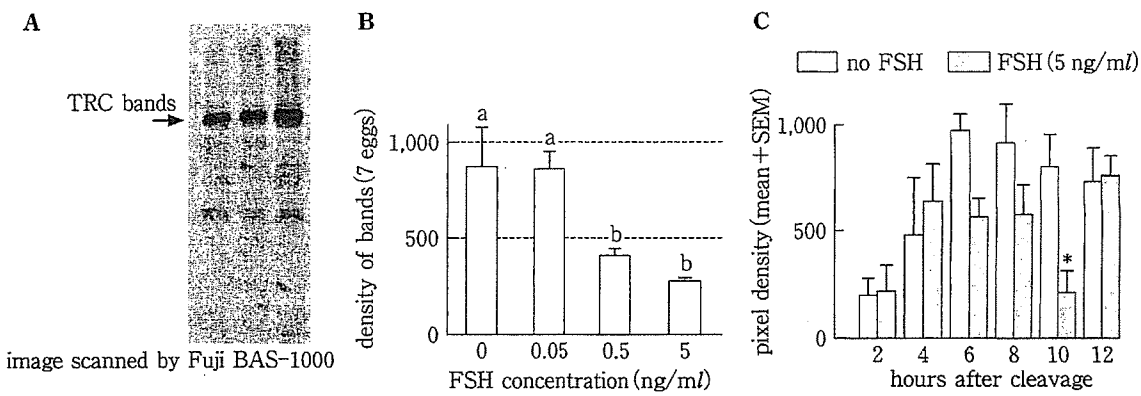

図4 TRC合成量の観察

A: オートラジオグラムでのTRCの見え方の例．B: FSH濃度とTRC合成量との関係．異なるラベル(a, b)間で有意な差がある．C: TRC合成の経時的変化に対するFSH添加の影響．*はFSH不含培地に比べ有意差があることを示す．

恐らくは卵子の遺伝子発現に大きく依存しており，卵胞培養時のFSH+insulinがこうした遺伝子発現に何らかの悪影響を与えるのであろう．卵子発育時の卵子での遺伝子発現がその後の胚発生能に影響する例は幾つか知られている[11]．著者も発生能獲得に関与する候補遺伝子を幾つか見いだしており[12]，更なる検索が必要と思われる．

卵胞全体ではなく，卵子顆粒層細胞複合体のみというシンプルな構成の卵胞培養系においてはinsulin+FSHが卵子の発生能を低下させるという結果はどう解釈すべきなのだろうか．卵子形成は*in vivo*ではFSHおよびinsulinに曝露されて進行しており，培養系の結果は*in vivo*と

は異なっているように思える．しかし，FSHの影響が卵丘細胞と壁側顆粒層細胞で異なる点を考慮する必要がある．しかも，卵丘を囲んでいる卵胞液中にはFSH binding inhibitorの存在が報告されている[13]．こうした物質が卵丘細胞のFSH受容体へのFSHの結合を防いでいるというメカニズムが*in vivo*でも働いているのかもしれない．しかし，卵胞液にはFSHのアゴニストや調整因子の存在も報告されており，混沌としているのが現状のようである．

おわりに

最近，成熟個体の卵巣に生殖幹細胞(germline stem cell)の存在が示された[14]．更に生殖幹細胞は骨髄から血行性に供給されることが報告され[15]，卵子形成メカニズムは更に複雑化の様相を呈してきた．ゴナドトロピン受容体をもち，生殖幹細胞と密接に関係する細胞が新たに骨髄組織で発見されるのだろうか？

なお，本稿で著者が示したデータはJackson LaboratoryのJ.J. Eppig博士の研究室に著者が留学した際に得たものである．Eppig博士と研究室の皆さんに深く感謝を申し上げたい．

文献

1) Demeestere I, et al: Impact of various endocrine and paracrine factors on in vitro culture of preantral follicles in rodents. Reproduction 130: 147-156, 2005.
2) Eppig JJ, Schroeder AC: Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization in vitro. Biol Reprod 41: 268-276, 1989.
3) Liu X, et al: Effects of growth hormone, activin, and follistatin on the development of preantral follicle from immature female mice. Endocrinology 139: 2342-2347, 1998.
4) Eppig JJ: Further reflections on culture systems for the growth of oocytes in vitro. Hum Reprod 9: 974-976, 1994.
5) Nayudu PL, Osborn SM: Factors influencing the rate of preantral and antral growth of mouse ovarian follicles in vitro. J Reprod Fertil 95: 349-362, 1992.
6) Roy SK, Greenwald GS: An enzymatic method for dissociation of intact follicles from the hamster ovary: histological and quantitative aspects. Biol Reprod 32: 203-215, 1985.
7) Eppig JJ, et al: Factors affecting the developmental competence of mouse oocytes grown in vitro: Follicle-stimulating hormone and insulin. Biol Reprod 59: 1445-1453, 1998.
8) O'Brien MJ, et al: A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. Biol Reprod 68: 1682-1686, 2003.
9) Hirao Y, et al: In vitro growth and development of bovine oocyte-granulosa cell complexes on the flat substratum: Effects of high polyvinylpyrrolidone concentration in culture medium. Biol Reprod 70: 83-91, 2004.
10) Conover JC, et al: Stage-specific expression of a family of proteins that are major products of zygotic gene activation in the mouse embryo. Dev Biol 144: 392-404, 1991.
11) De Sousa PA, et al: Temporal patterns of embryonic gene expression and their dependence on oogenetic factors. Theriogenology 49: 115-128, 1998.
12) Suzuki O, et al: Search for genes involved in developmental competence in mouse oocytes using suppression subtractive hybridization. Reprod Fertil Dev 16: 245-246, 2004.
13) Simoni M, et al: The follicle-stimulating hormone receptor: Biochemistry, molecular biology, physiology, and pathophysiology. Endocr Rev 18: 739-773, 1997.
14) Johnson J, et al: Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 428: 145-150, 2004.
15) Johnson J, et al: Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. Cell 122: 303-315, 2005.

各種実験動物の性腺刺激ホルモン配列比較と過排卵技術の改良

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各種実験動物の性腺刺激ホルモン配列比較と過排卵技術の改良

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現在、多種の実験動物が各々の特性を生かして様々な研究分野で用いられている。発生工学技術の発展による実験動物の効率的増産技術、凍結保存技術、遺伝子改変技術をこうした実験動物全体に効率よく応用できれば、科学の発展に大いに寄与するものと考えられる。

発生工学技術を支える技術は多々あるが、発生工学では卵子・胚を対象とするため、卵子・胚の品質向上・入手の効率化は発生工学技術全般の向上をもたらす。その意味で過排卵誘起技術の改良は発生工学技術の多方面への応用に対する波及効果が高く、慎重に考慮すべき課題であろう。

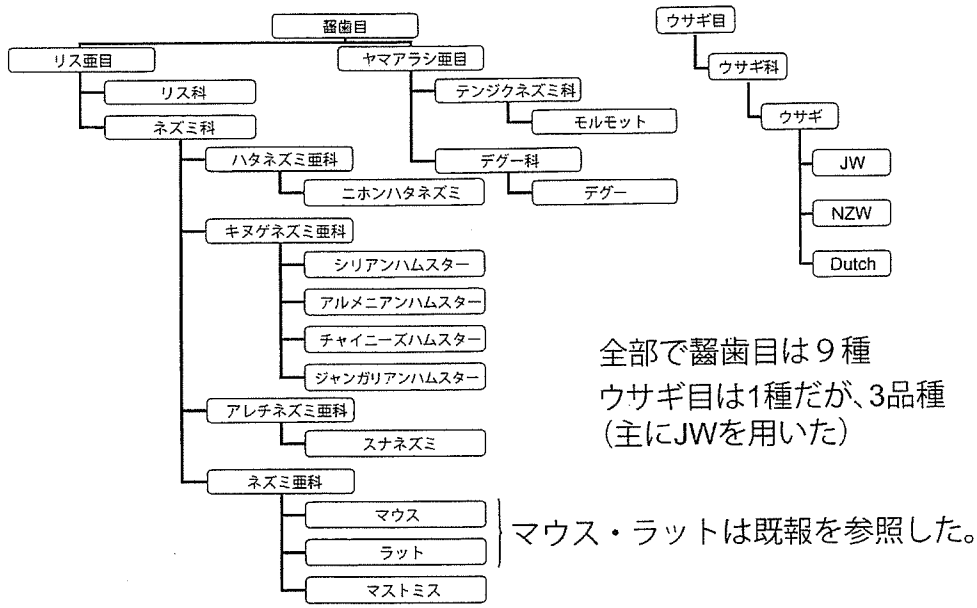

Fig 1. 本研究で用いた実験動物の系統分類。

実験動物の過排卵誘起はホルモン投与によってなされるのが一般的である。最も汎用されるホルモンは妊馬血清性性腺刺激ホルモンとヒト絨毛性性腺刺激ホルモンの組み合わせである。実際、非常に有効なホルモンではあるものの、動物種や系統によって卵胞は発達するが排卵率が低いとか、交尾率に影響するとか、ほとんど効かない例（モルモットなど）などが知られており、馬やヒト由来のホルモン製剤が齧歯類をメインとする実験動物の過排卵誘起に本当に最適なのだろうかという疑問がある。さらに、これらホルモンの選択は「対象動物に適しているから」ではなく、「製剤として入手が容易だから」という理由が大きいのではないだろうか。となれば、もっと対象動物に合ったホルモン製剤を用いるべきではないだろうか。特に近年、リコンビナント製剤等ホルモン製剤としても入手の選択肢が広がっていることから、改めてホルモン製剤の選択基準を考えるべきではないかと考えている。実験動物のほとんどが齧歯目に属するとはいえ、齧歯目内の動物ですら繁殖学的特性は多様であり（表１）、ホルモン処理を行うに当たっては各種動物への最適化が必要であろう。

そこで動物種本来の性腺刺激ホルモンのアミノ酸配列との類似性から過排卵誘起処理に使用するホルモン製剤の選択基準が考えられないかと考え、これまでに 10 種（スナネズミ、ハムスター４種、マストミス、ニホンハタネズミ、モルモット、デグー、ウサギ；図１）の実験動物の性腺刺激ホルモンやその受容体の配列決定を行い、既報のマウス・ラット、家畜、ヒトの配列との比較を行った。本稿では主にリガンド側である性腺刺激ホルモンの比較について述べる。

Table 1. 各種実験動物の繁殖学的特徴

	マウス・ラット	ハムスター類	ニホンハタネズミ	モルモット	ウサギ
性周期	不完全（4～5日）	不完全（～4日）	不明瞭	完全性周期（～20日）	連続発情
排卵	自然	自然	交尾	自然	交尾
黄体の活性化	交尾刺激	交尾刺激	（交尾排卵）	自然に活性化	（交尾排卵）
リッターサイズ	大きい	大きい	中等度（4匹程度）	小さい	大きい
妊娠期間	短い（～20日）	短い（15～18日）	短い（22～25日）	長い（～65日）	中間（約1ヶ月）
新生仔	無毛、開眼	無毛、開眼	無毛、開眼	被毛、開眼	無毛、開眼
その他				膣閉鎖膜	

性腺刺激ホルモンは、下垂体で産生・分泌される卵胞刺激ホルモン（FSH）と黄体化ホルモン（LH）、

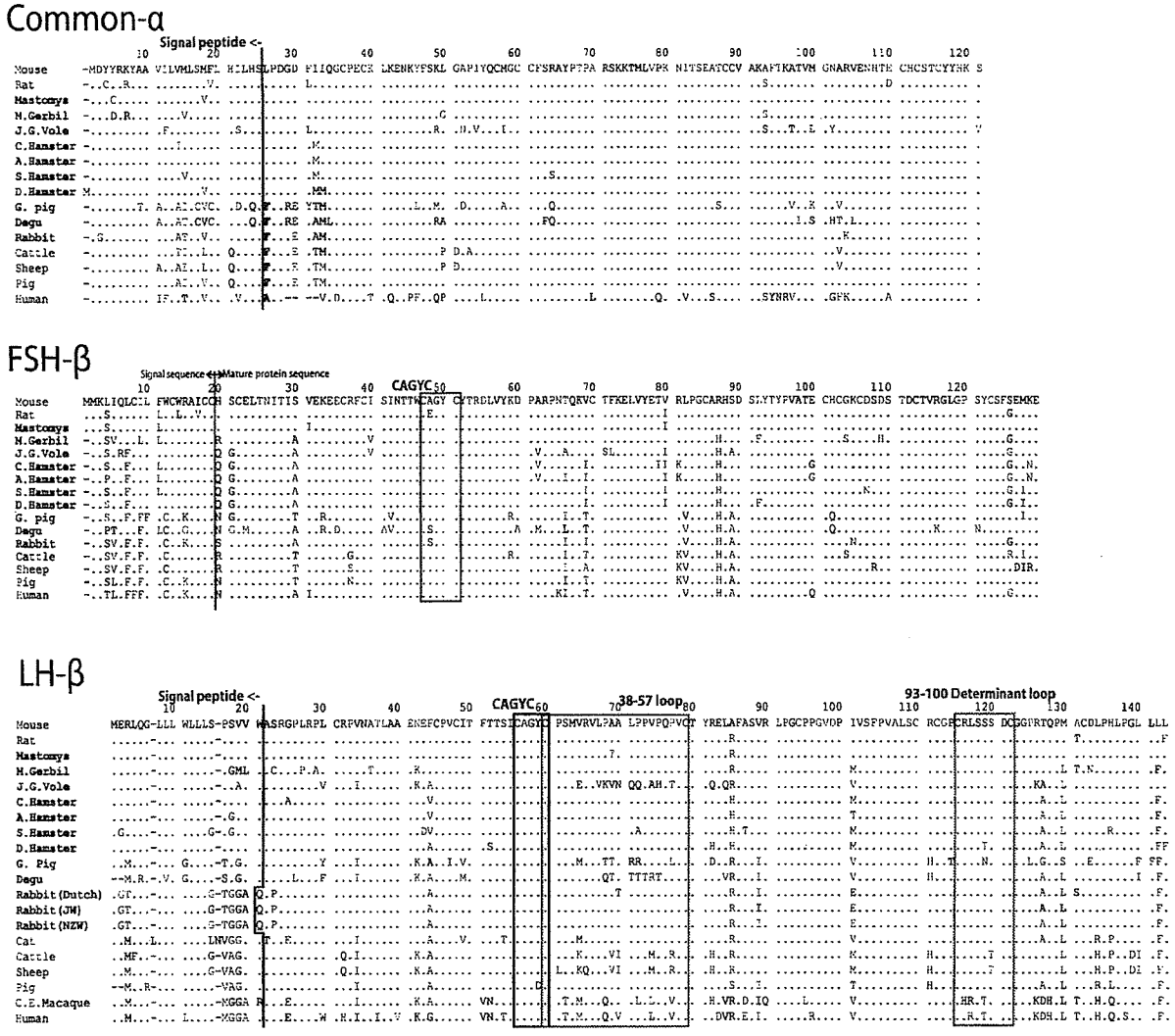

Fig 2. 性腺刺激ホルモンを構成する3サブユニットのアミノ酸配列。CLUSTAL W ソフトウエアにより整列した。各略称は、M.Gerbil (Mongolian Gerbil、スナネズミ)、J.G.Vole (Japanese Grass Vole、ニホンハタネズミ)、C.Hamster (Chinese Hamster, チャイニーズハムスター)、A.Hamster (Armenian Hamster, アルメニアンハムスター)、S.Hamster (Syrian Hamster, シリアンハムスター)、D.Hamster (*Djungarian* Hamster, ジャンガリアンハムスター)、G.pig (Guinea pig、モルモット) である。

霊長類および馬の胎盤で産生分泌される絨毛性(胎盤性)性腺刺激ホルモン(CG)がある。これらホルモンは、甲状腺刺激ホルモン (TSH) とも共通するαサブユニット (Common-α) とホルモン特異的なβサブユニット (FSHβ、LHβ、CGβ) からなるヘテロダイマーである。今回用いた10種の動物ではCGは産生されないので(論文的には産生される種もあると言われているが、コンセンサスを得られていないと思う)、下垂体のみを用いた。各種実験動物の下垂体より Total RNA を抽出し、SMART-RACE 法 (Clontech) によって各種ホルモンの cDNA 配列を決定した。遺伝子特異的プライマーは、ラットの配列を基本とし、既報のマウス、牛、羊、豚との相同性からプライマー結合部位を選択した。Common-α および LHβ の cDNA は約 600 塩基 (polyA を除く)、FSHβ cDNA は 3'- 非翻訳領域が長い点が特徴で種によってバラツキがあるが 1,500 ～ 2,000 塩基対程度の長さである。シグナル配列を含むアミノ酸配列は、若干の例外があるが、Common-α が 120 残基、FSHβ が 130 残基、LHβ は 143 残基である (これらは、Boucefield *et al.*, 1994 の総説に詳しい)。

cDNA 配列情報から得たアミノ酸配列を図2に示す。おおむね種間で高い相同性を示すが、細かい点で亜目レベル、亜科レベルを反映したような違いが見られる。例えば、Common-α では成熟蛋白の N 末端がリス亜目ではロイシンだが、ヤマアラシ亜目やウサギ、家畜ではフェニルアラニンである。ヒトはアラニンで異なる上、さらに4残基の欠損が見られる。FSHβ では、ネズミ亜科のみシグナル配列の N 末のメチオニン

が２つ重なっており、一方、成熟蛋白のＮ末端は亜科レベルで共通性が見られる。LHβ では、シグナル配列が豚とデグーで２残基短く、一方、ウサギ LHβ の成熟蛋白のＮ末はグルタミンで、おそらくピログルタミル化されているという (Glenn *et al.*, 1984)。確認はしていないが、ハムスターの４種すべてで FSHβ の成熟蛋白Ｎ末端がウサギ LHβ と同様グルタミンであり、それゆえピログルタミル化されている可能性がある。

アミノ酸配列の全体的な相同性を見てみると、Common-α は機能的制約がきついせいか、成熟蛋白の相同性が非常に高く、わずかにＮ末側にバラツキが見られる程度である。FSHβ も、種間で非常に相同性が高い。一方、LHβ は他の２つのサブユニットに比べ種間のバラツキが大きく、特に受容体結合部位として重要な 38-57 loop や 93-100 determinant loop において、霊長類とそれ以外では比較的ホモロジーが低いようである。

MEGA3.1 ソフトウエアにて、各サブユニットについて分子系統樹を書いてみると（図３）、Common-α はヒトを除き比較的まとまっているのに対し、FSHβ ではリス亜目齧歯類と家畜を両極としてウサギ、ヒト、ヤマアラシ亜目が中間に来る（ただし家畜寄り）、LHβ ではリス亜目齧歯類と霊長類を両極として間にウサギ、家畜、ヤマアラシ亜目が位置することがわかる。

これらのことから、ヤマアラシ亜目以外の齧歯類では、ヒトや家畜のホルモンとのホモロジーは低く、何らかの方法でいわゆる「齧歯類型」製剤を作るべきではないか、と感じた。一方、ヤマアラシ亜目の実験動物（モルモットやデグーなど）は、他の齧歯類よりも家畜やヒトに類似し、ヒト製剤の有効性が示唆された。ちなみにモルモットの FSH 受容体（AY082514）はヒトの受容体との類似性が高く、ヒト FSH 製剤を用いるとモルモットで過排卵を誘起できることを確認している（Suzuki, *et al.*, 2003）。

なお、本研究で決定した配列、およびデータベースから参照した配列の Accession number について表 2、3 に挙げておいた。

こうした各種実験動物の性腺刺激ホルモンや受容体の配列データが今後の過排卵誘起法改良の分子的基礎となれば幸いである。個人的には齧歯目全体を俯瞰するためにリスのデータを追加したいと考えているが、入手が困難なため、なかなか実現しそうにないのが残念である。

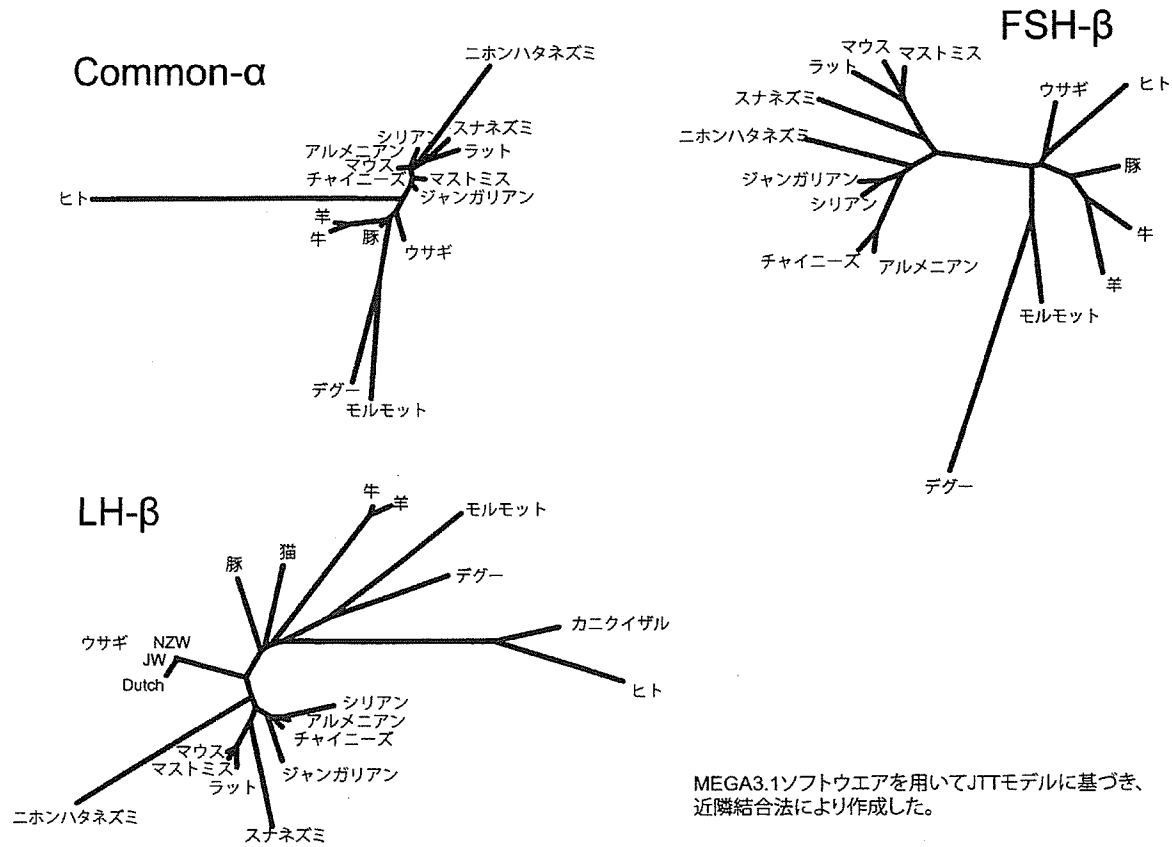

Fig 3. 性腺刺激ホルモンの３サブユニットの近隣接合法による分子系統樹。

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参考文献

一般的な総説として：

Bousfield, G. R., Perry, W. M., Ward, D. N., 1994. Gonadotropins. In Knobil, E., Neill, J. D. (eds.), The physiology of reproduction. 2nd Edition, Raven Press, Ltd., New York, 1749-1792. (2006年にThe physiology of reproductionの第3版が出たが、配列比較の参考には第2版のほうが詳しい)

ウサギのLHβのアミノ酸配列とピログルタミル化について：

Glenn, S. D., Nahm, H. S., Ward, D. N. (1984) The amino acid sequence of the rabbit lutropin beta subunit. J. Protein Chem. 3:259-273.

本研究に関する論文：

Koura, M., Handa, H., Noguchi, Y., Takano, K., Yamamoto, Y., Matsuda, J., Suzuki, O. (2004) Sequence analysis of cDNA encoding follicle-stimulating hormone and luteinizing hormone β-subunits in the Mongolian gerbil (*Meriones unguiculatus*). Gen. Comp. Endocrinol. 136:406-410.

Noguchi, Y., Takano, K., Koura, M., Uchio-Yamada, K., Matsuda, J., Suzuki, O. (2006) Sequence analysis of cDNA encoding rabbit follicle-stimulating hormone β-subunit precursor protein. Gen. Comp. Endocrinol. 147:231-235.

Suzuki, O., Mochida, K., Yamamoto, Y., Noguchi, Y., Takano, K., Matsuda, J., Ogura, A. (2002) Comparison of glycoprotein hormone α-subunits of laboratory animals. Mol. Reprod. Dev. 62:335-342.

Suzuki, O., Koura, M., Noguchi, Y., Takano, K., Yamamoto, Y., Matsuda, J. (2003) Optimization of superovulation induction by human menopausal gonadotropin in guinea pigs based on follicular waves and FSH-receptor homologies. Mol. Reprod. Dev. 64:219-225.

Suzuki, O., Koura, M., Noguchi, Y., Takano, K., Uchio-Yamada, K., Matsuda, J. (2006) Analyses of the cDNA and genomic DNA sequences encoding the luteinizing hormone β-subunit precursor protein in the rabbit. Gen. Comp. Endocrinol. (in press)

Takano, K., Koura, M., Noguchi, Y., Yamamoto, Y., Uchio-Yamada, K., Matsuda, J., Suzuki, O. (2004) Sequence analysis of cDNA encoding follicle-stimulating hormone and luteinizing hormone β-subunits in the Mastomys (*Praomys coucha*). Gen. Comp. Endocrinol. 138:281-286.

Table 2. 本研究で新規登録した遺伝子配列の Accession Number

Species	Common-α	FSHβ	LHβ
Mongolian gerbil	AF303351	AY376547	AY369077
Mastomys	AF307149	AY458603	AY353073
Japanese grass vole	AF307150	AB262180	AB262179
Syrian hamster	AF307148	AB241062	AY353074
Armenian hamster	AB235912	AB235911	AB233028
Chinese hamster	AB248597	AB248599	AB248598
Djungarian hamster	AB250761	AB252645	AB250762
Guinea pig	AF257213	AF257212	AY373317
Degu	AB262181	AB262182	AB262183
Rabbit	AF318299 (JW)	AY614704 (JW)	AY614703 (JW)
			AB235913 (NZW)
			AB235914 (Dutch)

Table 3. 既報遺伝子配列の Accession Number

Species	Common-α	FSHβ	LHβ
Mouse	J00643	NP_032071	NM_008497
Rat	V01252	P18427	J00749
Pig	D00767 & D00768	P01228	NM_214080
Sheep	X16977	P01227	NM_001009380
Cattle	X00050	NP_776485	NM_173930
Human	NM000735	P01225	NM_000894
Cat	-	-	NM_001009277
Crab-eating macaque	-	-	AJ781396

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Sequence analysis of cDNA encoding rabbit follicle-stimulating hormone β-subunit precursor protein

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Abstract

To understand the molecular basis of the rabbit's efficient superovulation, we determined the cDNA sequence of the follicle-stimulating hormone (FSH) β-subunit precursor protein using a combination of 5′- and 3′-rapid amplification of cDNA ends (RACE) with pituitary cDNA libraries of the Japanese White rabbit and compared it with those of other mammals. RACE experiments detected at least three transcripts for the FSHβ precursor protein in the libraries. The transcripts had lengths of 457, 1621, and 1767 bp, from the 5′-end to the poly(A) site. The shortest and mid-length transcripts had the putative polyadenylation signal sequence AATAAA at nucleotides 436 and 1601, respectively, whereas the longest form had an ATTAAA sequence at nucleotide 1745 of the cDNA sequence. These transcripts are likely to be polyadenylation variants of one large transcript because they share the same coding sequence for the precursor protein (130 amino acid residues in length). However, only a few shortest variants seem to be formed because the shortest variants were not detected by Northern blot analysis. Phylogenetic analysis of the deduced amino acid sequence indicates that the rabbit is phylogenetically closer to humans than to the other mammals, suggesting that an FSH preparation from human sources would be superior as a follicle stimulant for the induction of superovulation.

Keywords: cDNA; FSHβ; Phylogeny; Rabbit

1. Introduction

The induction of superovulation using gonadotropin treatments is a basic technique for manipulating embryos in laboratory animals. To understand the molecular basis of this technique, we have examined and compared the cDNA sequences of the pituitary gonadotropin subunits in several animals: the α-subunits in seven laboratory animals (Suzuki et al., 2002), and the β-subunits in Mongolian gerbil (Koura et al., 2004) and mastomys (Takano et al., 2004). In this study, we determined and characterized the cDNA and deduced amino acid sequences of the rabbit follicle-stimulating hormone (FSH) β-subunit precursor protein in rabbit. The goal was to find better ways of inducing ovarian follicular growth in female rabbits. We also performed a phylogenetic analysis of the deduced protein sequence. This paper discusses the possibility of using sequence information as a criterion for selecting gonadotropins for rabbit superovulation.

2. Materials and methods

2.1. RNA extraction, RT-PCR, and sequencing

We determined the full sequence of FSHβ cDNA in Japanese White rabbit using a combination of 5′- and 3′-rapid amplification of cDNA ends (RACE), followed by direct sequencing of the RACE products. We used a SMART RACE cDNA amplification kit (BD Biosciences Clontech, Palo Alto, CA) for 5′-RACE. We used a different system for the 3′-RACE (described below) because our preliminary experiments revealed that, in this particular situation, the results were insufficient. Total RNA

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was extracted from rabbit pituitary tissues (Funakoshi, Tokyo, Japan) using an RNeasy Mini kit (Qiagen, Hilden, Germany). A cDNA library for the 5′-RACE was prepared from the total RNA by reverse transcription with Superscript II (Invitrogen, Carlsbad, CA) according to the kit protocol. A cDNA library for the 3′-RACE was prepared from the total RNA by reverse transcription with Superscript III (Invitrogen) and an oligo(dT) primer (AAC TGG AAG AAT TCG CGG CCG CAG GAA TTT TTT TTT TTT TTT TTT) at 50 °C for 90 min. The RACE reactions were performed using these libraries, a DNA polymerase mixture (HotStarTaq Master Mix, Qiagen), an adaptor primer (the universal primer mix included in the kit or the primer AAC TGG AAG AAT TCG CGG CCG CAG GAA for 5′- or 3′-RACE, respectively), and gene-specific primers. PCR amplifications were performed using a Hybaid PCR Express thermal cycler (Thermo Electron K.K., Tokyo, Japan) as follows: 94 °C for 15 min (denaturation and enzyme activation) and 40 cycles at 94 °C for 2 s and 68 °C for 5 min. To determine the 3′-end, nested PCR amplifications for primer walking were performed using the primary 3′-RACE products (diluted 1:50), a DNA polymerase mixture, nested adaptor primer (CTG GAA GAA TTC GCG GCC GCA GGA ATT), and gene-specific primers under the following conditions: 94 °C for 15 min; 40 cycles at 94 °C for 10 s, 60 °C for 10 s, and 72 °C for 1 min; and 72 °C for 10 min. Fig. 1B shows the positions of the gene-specific primers used in the RACE reactions. First, 5′-RACE was conducted with primer 5Fp (TTC CTT CAT TTC ACT GAA GGA GCA GTA). Following this, primer walking was performed in 3′-RACE reactions with a total of five primers: 3Fp1 (TAC CTT CAA GGA GCT GGT GTA CGA), 3Fp2 (TGG CAG AGG GGG AGT TCC AGG AAT TGA), 3Fp3 (GGG CTT CAA GAG CAG GGC CAG AAA CCT), 3Fp4 (CCT GAG CTC GGA TTT TCC AAG CCC TTC A), and 3Fp5 (GGA GGA AGG AAA TTG CTG CTT TCT AAG). The primer 5Fp was designed from the mouse FSHβ sequence (NP_032071 from GenBank) using Primer3 software (Rozen and Skaletsky, 1998), after which the newly determined partial sequences were used to design the other primers using Primer3. Additional primers were used to confirm the accuracy of the sequence. The RACE products were gel-purified and sequenced directly using a DYEnamic ET Terminator Cycle sequencing kit (Amersham Biosciences, Piscataway, NJ) with a DNA sequencer (RISA384, Shimadzu Biotech, Kyoto, Japan). Full sequences were obtained by combining overlapping sequences of 5′- and 3′-RACE products. The appearance of SMART II oligo- and putative poly(A) sequences, respectively, determined the 5′- and 3′-ends of the cDNA sequences in the RACE products.

2.2. Northern blot analysis

Northern blot analysis was performed using NorthernMax-Gly Kit (Ambion, Austin, TX) with 10 μg of pituitary total RNA and biotinylated RNA size markers (BrightStar Biotinylated RNA Millennium Markers, Ambion). Biotinylated DNA probes, 404-base-long, were generated by RT-PCR with primers S1 (GGC TTC AGT CTA CAG TTT TCC CCA GAC) and R404 (CCC CAG GCC TCG TAC AGT GCA GTC AGT; see

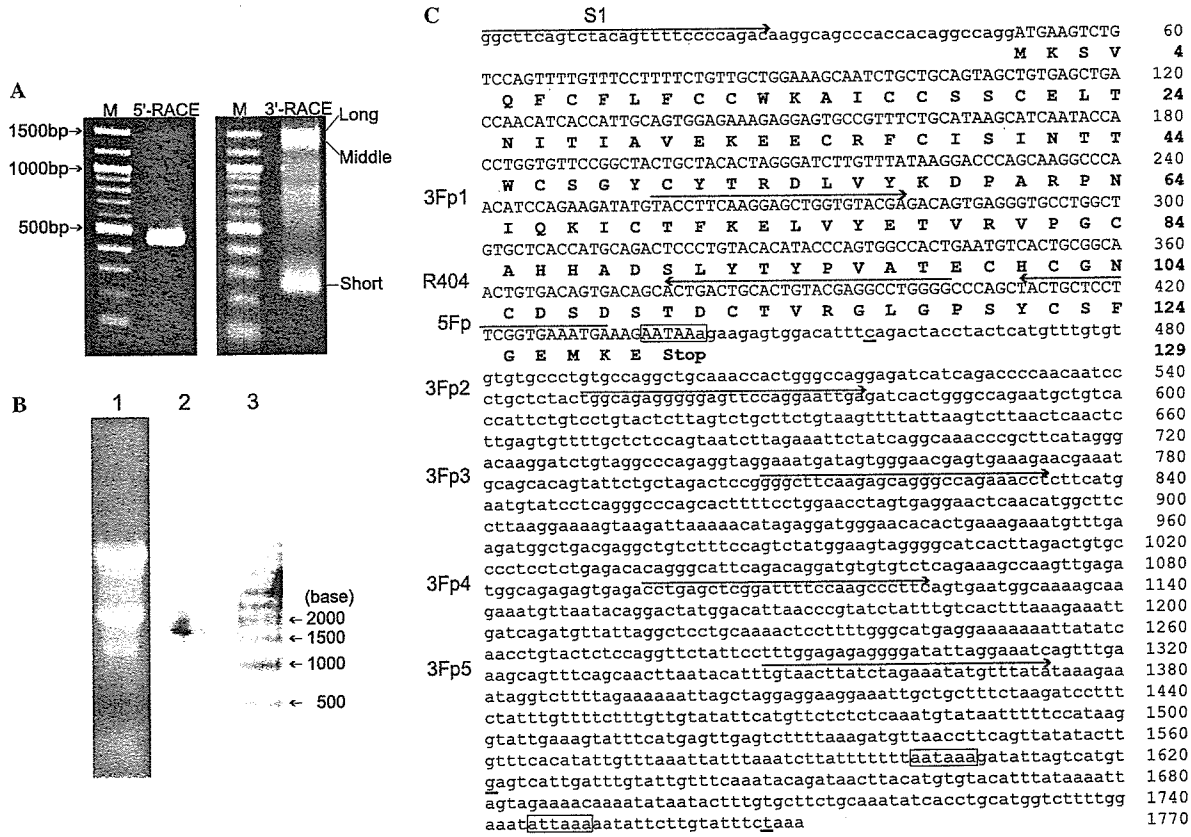

Fig. 1. (A) The products of rapid amplification of cDNA ends (RACE) were resolved on 2% agarose gels, stained with ethidium bromide, and photographed under UV illumination. Note three distinct bands in the 3′-RACE product lane. Lane M, size markers. (B) Northern blot analysis (lanes 2 and 3) of follicle-stimulation hormone (FSHβ) transcripts. One distinct band of ~1.8 kb is visible, whereas an expected shortest band (~0.6 kb) is not detectable. Lane 1, Electrophoretic gel stained with ethidium bromide; lane 2, pituitary total RNA; lane 3, RNA markers. (C) The FSHβ cDNA and deduced amino acid sequences (in bold face). Lowercase letters indicate both 5′- and 3′-untranslated regions, and uppercase letters indicate a coding sequence. Putative polyadenylation signal sequences are boxed, and putative polyadenylation sites are underlined. Arrows indicate primer positions (S1, R404, 5Fp, 3Fp1, 3Fp2, 3Fp3, 3Fp4, and 3Fp5). The cDNA sequence has been listed in GenBank under Accession No. AY614704.

Fig. 1C for primer locations) and labeled using LabelIT biotinylation kit (Mirus Bio, Madison, WI). After overnight hybridization at 42 °C, the hybridization signals and size markers were detected by chemiluminescence using BrightStar BioDetect Kit (Ambion) and photographed using a CCD camera (LAS-3000, Fuji Photo Film, Tokyo, Japan).

2.3. Molecular phylogenetic analysis

Additional mammalian FSHβ protein sequences were retrieved from GenBank as follows (accession numbers are shown in parentheses): mouse (NP_032071), rat (P18427), pig (P01228), cattle (NP_776485), sheep (P01227), horse (P01226), human (P01225), Mongolian gerbils (AAQ83633), mastomys (AAR21602), guinea pig (Q9JK69), and Djungarian hamster (*Phodopus sungorus*; Q9QYB0). The amino acid sequences of these species and the rabbit sequence were aligned using Clustal W (Thompson et al., 1994). A neighbor-joining tree containing the FSHβ protein sequences of 10 species (the Djungarian hamster was omitted because AF106914 was a partial sequence) was constructed using MEGA3 software (Kumar et al., 2004), based on the Jones–Taylor–Thornton (JTT) matrix model (Jones et al., 1992).

3. Results and discussion

Fig. 1C shows the full-length FSHβ cDNA sequence based on RACE products (Fig. 1A). The RACE experiments detected at least three transcripts for the FSHβ precursor protein in the rabbit pituitary cDNA library. These transcripts had lengths of 457, 1621, and 1767 bp, from the 5′-end to the poly(A) site. The shortest and mid-length transcripts had the putative polyadenylation signal sequence AATAAA at nucleotides 436 and 1601, respectively, whereas the longest form had an ATTAAA sequence

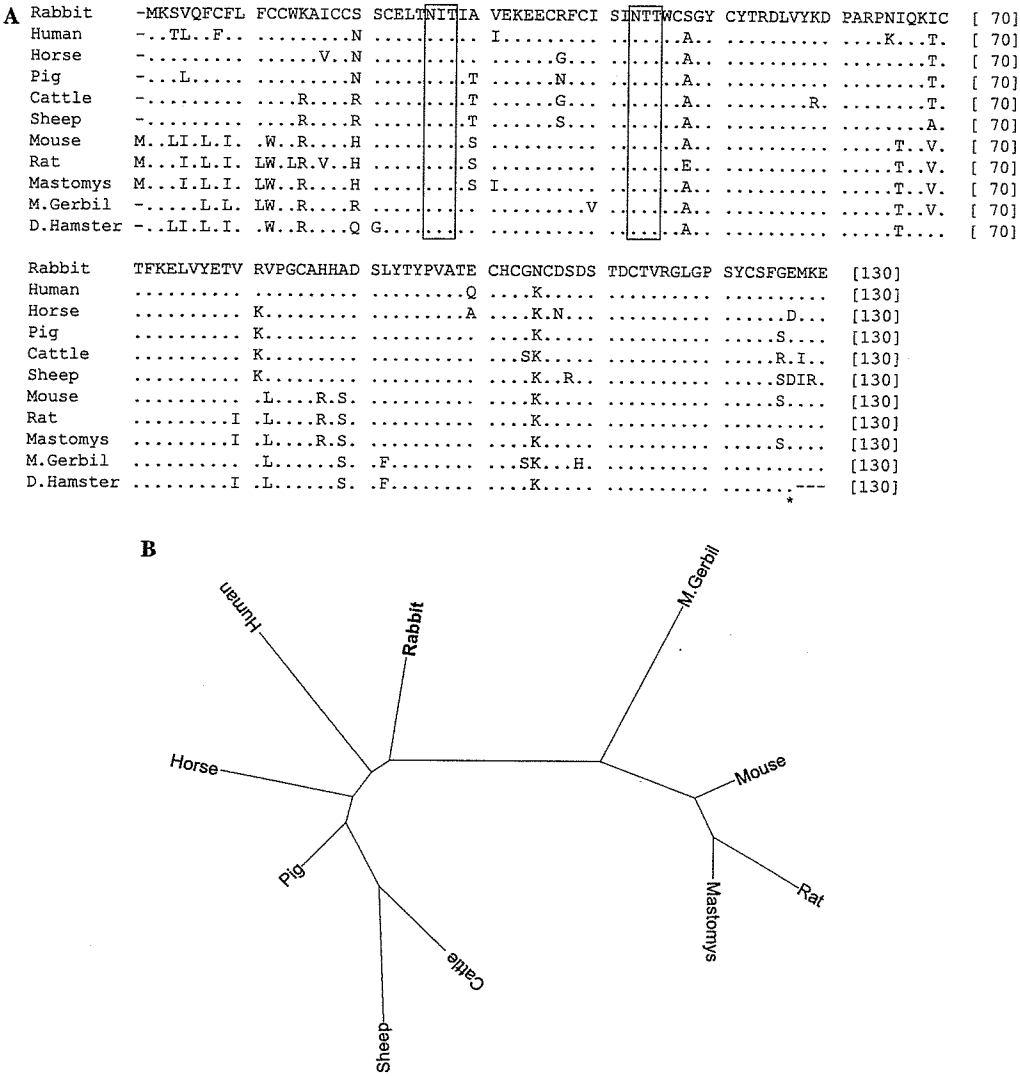

Fig. 2. (A) Alignment of various mammals' follicle-stimulating hormone (FSHβ) precursor protein sequences. The mouse, rat, and mastomys belong to the subfamily Murinae, the Mongolian gerbil (M. Gerbil) to Gerbillinae, and the Djungarian hamster (D. Hamster) to Cricetinae. A dot indicates an amino acid residue identical to the corresponding site in the rabbit sequence (top row). Dashes in the sequences indicate gaps inserted by Clustal W software. An asterisk (*) indicates the end of the currently available partial sequence for the Djungarian hamster. The half-cysteine residue positions (shaded) and putative N-glycosylation sites (boxed) are well conserved in all mammals. (B) An unrooted neighbor-joining tree with aligned FSHβ precursor protein sequences constructed using MEGA3 based on the JTT matrix model.

at nucleotide 1745 of the cDNA sequence. It is likely that these transcripts are polyadenylation variants from one large transcript because they share the same coding sequence for the precursor protein (130 amino acid residues in length). However, only a few smallest variants seem to be formed because the smallest variants were not detected by Northern blot analysis (Fig. 1B). Multiple FSHβ mRNA species have also been found in humans; four mRNA species are transcribed using one alternate splicing site and two polyadenylation sites (Jameson et al., 1988). In bovines (Maurer and Beck, 1986) and rats (Gharib et al., 1989), only a single mRNA species is transcribed. Interestingly, the stop codon of rabbit FSHβ was located inside one of the polyadenylation sites as that of the human FSHβ, indicating a further similarity between the two sequences. The mid-length and longest forms of rabbit FSHβ cDNA each have a long 3′-untranslated region (UTR) common to other mammals, whereas all three rabbit FSHβ cDNAs have a 5′-UTR (50 bp in length) that is shorter than that in most rodents (>60 bp) and domestic animals (>70 bp) and longer than that in chickens (44 bp, Shen and Yu, 2002).

The alignment of the deduced amino acid sequences indicates an overall similarity between FSH β-subunit precursor proteins in rabbits and other mammals (>85%, Fig. 2A). The positions of the half-cysteine residue and the putative N-glycosylation site were well conserved in rabbit compared with those in other mammals. Interestingly, the rabbit FSHβ amino acid sequence contains a CSGYC sequence at the same position in which most mammals (except rats) have the unique β-subunit sequence CAGYC, which researchers consider to be a key structure for binding to common α-subunits (Gharib et al., 1990). The rabbit CSGYC sequence is similar to sequences in birds (chicken: Shen and Yu, 2002; quail: Kikuchi et al., 1998; ostrich: Koide et al., 1996) and amphibians (bullfrog: Hayashi et al., 1992; Japanese toad: Komoike and Ishii, 2003), suggesting that rabbits have an evolutional proximity to birds and amphibians. However, the second residue does not seem to be functionally important, as rat FSHβ contains a different residue (CEGYC) in the corresponding position (Gharib et al., 1989). The phylogenetic analysis of the protein (Fig. 2B) indicated that rabbits occupy a position phylogenetically intermediate between rodents and ruminants. In particular, rabbits appear to be more similar to humans than to the other mammals used in this analysis. These results support the theory that rabbits are phylogenetically more similar to primates than to rodents (Graur et al., 1996).

Researchers induce superovulation in rabbits using injections of FSH or eCG in combination with hCG. There is still no agreement as to whether FSH or eCG should be used. Multiple injections of FSH may result in more embryos than would a single injection of eCG (Hirabayashi et al., 2000), and eCG may also induce chromosomal abnormality (Fujimoto et al., 1974) and decrease the number of blastomeres in the blastocyst (Carney and Foote, 1990). Tsiligianni et al. (2004) have countered that eCG is superior because repeated administration of FSH and hCG may cause various problems, such as increased hemorrhagic and cystic follicles. When a choice of follicle growth stimulant is based on the similarity to endogenous hormones, the overall similarity of rabbit FSH to the FSH of other mammals indicates that most commercially available FSH preparations would be suitable; a FSH preparation from human origins, such as human menopausal gonadotropins (hMG), might be a better choice because of its greater homology (Fig. 2B). Research has shown that administration of hMG in rabbits induces a superovulatory response superior to the response from FSH injections (Kanayama et al., 1995). Human recombinant FSH is now commercially available; it could become a commonly used superovulation agent because this type of preparation prevents the common problem of LH activity, which often occurs when researchers use a naturally extracted preparation.

In summary, we determined the cDNA sequences of the FSH β-subunit in rabbit. This information should provide a molecular basis for improving superovulation technology in the rabbit.

References

Carney, E.W., Foote, R.H., 1990. Effects of superovulation, embryo recovery, culture system and embryo transfer on development of rabbit embryos in vivo and in vitro. J. Reprod. Fertil. 89, 543–551.

Fujimoto, S., Pahlavan, N., Dukelow, W.R., 1974. Chromosome abnormalities in rabbit preimplantation blastocysts induced by superovulation. J. Reprod Fertil. 40, 177–181.

Gharib, S.D., Roy, A., Wierman, M.E., Chin, W.W., 1989. Isolation and characterization of the gene encoding the beta-subunit of rat follicle-stimulating hormone. DNA 8, 339–349.

Gharib, S.D., Wierman, M.E., Shupnik, M.A., Chin, W.W., 1990. Molecular biology of the pituitary gonadotropins. Endocr. Rev. 11, 177–199.

Graur, D., Duret, L., Gouy, M., 1996. Phylogenetic position of the order Lagomorpha (rabbits, hares and allies). Nature 379, 333–335.

Hayashi, T., Hanaoka, Y., Hayashi, H., 1992. The complete amino acid sequence of the follitropin beta-subunit of the bullfrog, *Rana catesbeiana*. Gen. Comp. Endocrinol. 88, 144–150.

Hirabayashi, M., Hirao, M., Hochi, S., Kimura, K., Hirasawa, K., Ueda, M., 2000. Effects of superovulatory protocols on the recovery and subsequent developmental potential of rabbit zygotes (in Japanese). J. Exp. Anim. Tech. 35, 23–28.

Jameson, J.L., Becker, C.B., Lindell, C.M., Habener, J.F., 1988. Human follicle-stimulating hormone beta-subunit gene encodes multiple messenger ribonucleic acids. Mol. Endocrinol. 2, 806–815.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.

Kanayama, K., Nariai, K., Sankai, T., Endo, T., 1995. Superovulation induction by human menopausal gonadotrophin in rabbits. J. Vet. Med. Sci. 57, 543–544.

Kikuchi, M., Kobayashi, M., Ito, T., Kato, Y., Ishii, S., 1998. Cloning of complementary deoxyribonucleic acid for the follicle-stimulating hormone-beta subunit in the Japanese quail. Gen. Comp. Endocrinol. 111, 376–385.

Koide, Y., Papkoff, H., Kawauchi, H., 1996. Complete amino acid sequences of follitropin and lutropin in the ostrich, *Struthio camelus*. Eur. J. Biochem. 240, 262–267.

Komoike, Y., Ishii, S., 2003. Cloning of cDNAs encoding the three pituitary glycoprotein hormone beta subunit precursor molecules in the Japanese toad, *Bufo japonicus*. Gen. Comp. Endocrinol. 132, 333–347.

Koura, M., Handa, H., Noguchi, Y., Takano, K., Yamamoto, Y., Matsuda, J., Suzuki, O., 2004. Sequence analysis of cDNA encoding follicle-stimulating hormone and luteinizing hormone β-subunits in the Mongolian gerbil (*Meriones unguiculatus*). Gen. Comp. Endocrinol. 136, 406–410.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA 3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. Brief. Bioinform. 5, 150–163.

Maurer, R.A., Beck, A., 1986. Isolation and nucleotide sequence analysis of a cloned cDNA encoding the beta-subunit of bovine follicle-stimulating hormone. DNA 5, 363–369.

Rozen, S., Skaletsky, H.J., 1998. Primer3. Code available at <http://www-genome.wi.mit.edu/genome_software/other/primer3.html/>.

Shen, S.T., Yu, J.Y., 2002. Cloning and gene expression of a cDNA for the chicken follicle-stimulating hormone (FSH)-beta-subunit. Gen. Comp. Endocrinol. 125, 375–386.

Suzuki, O., Mochida, K., Yamamoto, Y., Noguchi, Y., Takano, K., Matsuda, J., Ogura, A., 2002. Comparison of glycoprotein hormone α-subunits of laboratory animals. Mol. Reprod. Dev. 62, 335–342.

Takano, K., Koura, M., Noguchi, Y., Yamamoto, Y., Uchio-Yamada, K., Matsuda, J., Suzuki, O., 2004. Sequence analysis of cDNA encoding follicle-stimulating hormone and luteinizing hormone beta-subunits in the Mastomys (*Praomys coucha*). Gen. Comp. Endocrinol. 138, 281–286.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tsiligianni, T., Saratsi, A., Besenfelder, U., Anastasiadis, A., Vainas, E., Saratsis, P., Brem, G., 2004. The use of cytological examination of vaginal smears (CEVS) in the selection of rabbits for superovulation. Theriogenology 61, 989–995.

Efficient Production of Intersubspecific Hybrid Mice and Embryonic Stem Cells by Intracytoplasmic Sperm Injection

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ABSTRACT Recently, mice and embryonic stem (ES) cells with allelic polymorphisms have been used extensively in the field of genetics and developmental biology. In this study, we examined whether intersubspecific hybrid mice and ES cells with these genotypes can be efficiently produced by intracytoplasmic sperm injection (ICSI). Frozen-thawed spermatozoa from wild-derived strains, JF1 (*Mus musculus molossinus*), MSM (*M. m. molossinus*), HMI (*M. m. castaneus*), and SWN (*M. m.* spp.), were directly injected into mature oocytes from laboratory mice ([C57BL/6 × DBA2]F1; *M. m. domesticus*). The in vitro and in vivo developmental capacity of F1 embryos was not significantly different among the groups ($P > 0.05$), and term offspring were efficiently obtained in all groups (27%–34% of transferred embryos). However, the mean body and placental weights of the offspring differed significantly with genotype ($P < 5 \times 10^{-10}$), with the HMI hybrid greatest in both body and placental weights. In an application study using these F1 offspring, we analyzed their mitochondrial DNA using intersubspecific polymorphisms and found the consistent disappearance of sperm mitochondrial DNA in the F1 progeny. In a second series of experiments, we generated F1 blastocysts by injecting MSM spermatozoa into C57BL/6 oocytes and used them to generate hybrid ES cell lines. The ES cell lines were established at a high efficiency (9 lines from 20 blastocysts) and their allelic polymorphisms were confirmed. Thus, ICSI using cryopreserved spermatozoa allows the efficient and immediate production of a number of F1 hybrid mice and ES cell lines, which can be used for polymorphic analysis of mouse genetics. *Mol. Reprod. Dev.* © 2007 Wiley-Liss, Inc.

Key Words: ICSI; mitochondrial DNA; placenta; polymorphism; ES cells

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) can bypass the process of sperm penetration of the cumulus cells and zona pellucida, and the process of fusion with the oolemma. ICSI was first introduced as an alternative assisted-reproduction technique in experiments with hamsters, and has subsequently advanced our understanding of the early events of fertilization (Yanagimachi, 2005; Ogura et al., 2005). This approach is now one of the most successful methods for achieving fertilization in severe cases of human male infertility. In laboratory species, including mice, ICSI has been used frequently for research purposes and the conservation of genetic resources (Ogura et al., 2001; Hirabayashi et al., 2002; Yamauchi et al., 2002; Ogonuki et al., 2003a,b; Ogonuki et al., 2005). When spermatozoa are unable to fertilize oocytes because of spontaneous mutations or gene modifications, experiments with ICSI (but not in vitro fertilization [IVF]) ensure the ability of the sperm genome to support embryonic development (Yanagimachi et al., 2004). The technique can also be advantageous in conventional IVF when mouse spermatozoa have been cryopreserved for genetic resource banking, because frozen-thawed mouse spermatozoa are occasionally unable to fertilize oocytes because of damage to the sperm plasma membrane (Szczygiel et al., 2002; Nishizono et al., 2004).

Interspecific or intersubspecific F1 hybrid mice are invaluable research material in mouse genetics, especially for genetic and gene expression analysis using polymorphisms between the parents. However, the production of F1 offspring by natural mating or conventional IVF is not always efficient or practicable, so it is

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