ABSTRACT

HAN, SANG-OH. Identification of DNA Sequences Necessary and Sufficient for Activin Induction of Ovine Follicle Stimulating Hormone Beta-Subunit. (Under the direction of Dr. William L. Miller).

Follicle stimulating hormone (FSH) is an alpha/beta heterodimer central to vertebrate reproduction, and its synthesis depends on expression of its beta-subunit (FSHB). Activin is the primary inducer of FSHB, and previous work from this laboratory showed that induction depends on sequences between -169/-158 bp of the ovine FSHB promoter. Deletion and promoter substitution studies suggested, however, that other 5' and 3' sequences might also alter activin induction. The work described here was designed to identify all sequences required for activin induction of ovine FSHB.

These studies used mutant or wild type ovine FSHB promoter/reporter constructs (wild type = oFHSBLuc; -4741 bp of 5' promoter plus 3' exon/intron 1 linked to luciferase) which were analyzed using transient expression in transformed gonadotropes (LBT2 cells) plus expression in transgenic mice in some cases. First, eleven successive 5' deletions were made to -195 bp which progressively decreased induction by activin from 9.5-fold to 1-fold (no induction). Also five more deletions internal to this region were made to -175 bp, but these deletions were replaced with exogenous DNA to maintain the original spacing. Also 3' exon/intron deletions with replacement sequences were made. When correct spacing was maintained, none of the changes in the 5' or 3' exon/intron regions altered induction of FSHB by activin. Deletions between -90 bp and the TATA box revealed one important site (-68/-58 bp) that was necessary for normal basal expression and activin induction in LBT2

cells. This region contained a Pitx1 binding site and partially overlapping putative Runx binding site. Follow-up transgenic studies showed that this Pitx1 site was responsible for 99 % of oFSHBLuc expression *in vivo*, but had no effect on activin action. The putative Runx binding site had no effect on oFSHBLuc expression *in vivo*, but significantly degraded gonadotrope-specific expression of oFSHBLuc. Finally, to determine if the oFSHBLuc TATA box was important for activin induction, it was replaced with a minimal rat prolactin promoter (contains a TATA box), and full activin induction was maintained. Substitution with a thymidine kinase minimal promoter (no TATA box) prevented activin induction.

IN SUMMARY, these studies plus previous results from our laboratory show that sequences between -169/-58 bp plus a minimal TATA box promoter are necessary and sufficient for robust activin-induced expression of oFSHBLuc in LBT2 cells. They also show the Pitx1 site between -68/-63 bp is necessary for 99 % of ovine FSHB expression *in vivo* but has no effect on activin induction. Finally, the putative Runx site that overlaps Pitx1 was found important for gonadotrope-specific expression of oFSHBLuc.

Identification of DNA Sequences that are Necessary and Sufficient for Activin Induction of ovine Follicle Stimulating Hormone beta-subunit.

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Biochemistry

Raleigh, North Carolina

2009

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DEDICATION

To my wife, *Eun-yi Han*, and my children, *Jooney Han, Jeehe Han, and Chahney Han*, for their continuous encouragement, belief in my ability to obtain Ph.D in Biochemistry, and support during my time of study.

To my parents for their endless love and teaching me not to give up under any condition when pursuing a worthy goal.

BIOGRAPHY

Sang-oh Han was born on February 13th, 1968 in Samcheok, Korea. He received his Bachelor degree from the Department of Chemistry at Kyungpook National University at Taegu, Korea in 1992. After working at COSMO Industry Co. Ltd. in Chung-ju, Korea from 1993 to 1995, he joined the graduate school of the Department of Chemistry at Seoul National Univeristy in Seoul, Korea. He received his Master degree from the Department in 1999 with a thesis entitled "Synthesis and characterization of poly(α-Aminobutyl-L -glycolic acid) as a biodegradable polymer for complexation with plasmid DNA." He worked as a research associate in the Department of Pharmaceutics and Pharmaceutical Chemistry at the University of Utah in Salt Lake City, Utah, USA from 1999 to 2002. In this job, he developed and synthesized many polymer-based gene carriers that led to the publication of 17 articles in well respective journals on Gene Therapy plus 3 patents under the direction by Dr. Sung Wan Kim who was an Academy of Science Member. Sang-oh Han received an Outstanding Research Award at the 4th Annual meeting of the American Society of Gene Therapy in Seattle, WA, USA in 2001. Due to personal financial problems, he worked again at Expression Genetics, Inc., Huntsville, AL, USA as a scientist II from 2002 to 2004. He continued to work developing and synthesizing gene carriers, and published one paper during this time. He joined the Ph. D program of the Department of Molecular and Structural Biochemistry at North Carolina State University in 2004, where he has studied activin-mediated induction of follicle stimulating hormone beta-subunit under the direction of Dr. William L. Miller.

He married Eun-yi Han in 1998, and has two sons, Jooney Han and Chahney Han, and a daughter, Jeehe Han.

ACKNOWLEDGMENTS

I would like to express my heartfelt thanks to my major advisor, Dr. William L. Miller. I am forever indebted to him, not only for his continuous support, but for our fruitful discussions, his creative suggestions and instruction in the English language. My gratitude is also extended to my committee members, Dr. Paul F. Agris, Dr. Robert B. Rose, and Dr. Jun Nonomiya-Tsuji for their insightful comments and generous attitudes throughout the course of my graduate research.

I would like to thank past and present members of Dr. Miller's lab, Joyce Wu, Pei Su, Farideh Shafiee-Kermani, Jesse Gore, Jingjing Jia, and Kiti Ho. I would also like to thank the faculties, post-doctoral, and graduate students in the Deparment of Molecular and Structural Biochemistry who helped my work and shared instruments and reagents during my graduate research.

I would like to express my thanks to Dr. Sung Wan Kim (University of Utah), Dr. Ram I Mahato (University of Tennessee) , and Dr. Minhyung Lee (Hanyang University). They were, are, and will be my endless teachers and friends during my research life.

I would also like to thank my parents, brothers, sister, sisters-in-law, and brothers-in-law for their emotional and financial support during my graduate studies. Without their devotion and support, this project would not have been possible. Finally, I would like to thank my wife**,** Eun-yi, and my children**,** Jooney, Jeehe, and Chahney for their endless love, encouragement and devotion. I will never forget their support as long as I live.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Regulation of Follicle Stimulating Hormone Beta subunit

Literature Review

I. Follicle stimulating hormone (FSH) and luteinizing hormone (LH)

Regulation of reproduction is complex and involves a network of communication systems with endocrine, paracrine and/or autocrine hormones within and from the pituitary, gonads and other reproductive tissues. Gonadotropes in the anterior pituitary are important cells for vertebrate reproduction. They comprise 3-12% of the pituitary and produce follicle stimulating hormone (FSH) and luteinizing hormone (LH) (1, 2). These hormones are the key effecters for development and gonadal function such as folliculogenesis in females and spermatogenesis in males. The gonadotropins, FSH and LH, are involved with regulation of reproduction in all vertebrates including fish (3), birds, amphibians (4), and mammals (5). The hormones are highly conserved in amino acid sequence within the same subclass of animals such as mammals, birds, amphibians, and fish (4, 5). The similarity is reduced when compared across classes, however. Both FSH and LH are produced specifically in the pituitary for all known species.

Receptors for FSH and LH reside in different end organ cells. For example, FSH in males binds to a single gene copy FSH receptor on Sertoli cells leading to mature sperm, while LH binds the LH receptor on Leydig cells causing production of androgens (3). In females, FSH binds the FSH receptor on granulosa cells leading to stimulation of follicular growth and egg maturation, while LH binds the LH receptor on theca cells to aid in folliculogenesis (6). Both hormones require each other for their proper function in reproduction. Both FSH and LH are α/β heterodimeric glycoproteins. The α-glycoprotein subunit (αGSU) is common for both hormones and its production in gonadotropes is higher

than either of the β-subunits. The β-subunits are hormone-specific and rate-determining for overall hormone synthesis and secretion.

IA. Reproductive hormones and regulation of the estrous cycle

Gonadotropin-releasing hormone (GnRH) from the hypothalamus is a critical factor controlling the estrous cycle in mammals. Secretion of GnRH is pulsatile in both genders, but pulsatility changes dramatically during the estrous cycle in females (7). As shown in Fig. 1, the level of GnRH secretion from the hypothalamus is low during metestrus and diestrus, but the level is increased dramatically during proestrus, and then rapidly declines again during estrus. Production of the LH β-subunit (LHB) is mainly stimulated by gonadotropin-releasing hormone (GnRH) at high pulse frequency. Regulation of follicle stimulating hormone β-subunit (FSHB) is quite different from LH. Serum FSHB is low during low GnRH frequency, and increases as GnRH frequency increases, but FSHB decreases during the highest GnRH frequency of the estrous cycle in female rats (7). The authors suggested that the difference might be due to other effectors, such as activin, inhibin, and follistain, that are well known effectors for FSHB regulation.

IB. The relationship between αGSU and reproduction

The functions of both hormones, FSH and LH, have been studied in mice with gain- or loss-of-function modifications. Disruption of the αGSU gene causes disruption of LH and

FSH plus thyroid stimulating hormone (TSH; also contains αGSU), but does not disrupt growth during the first 2-3 weeks in mice. After 4.5 weeks, however, the body sizes of

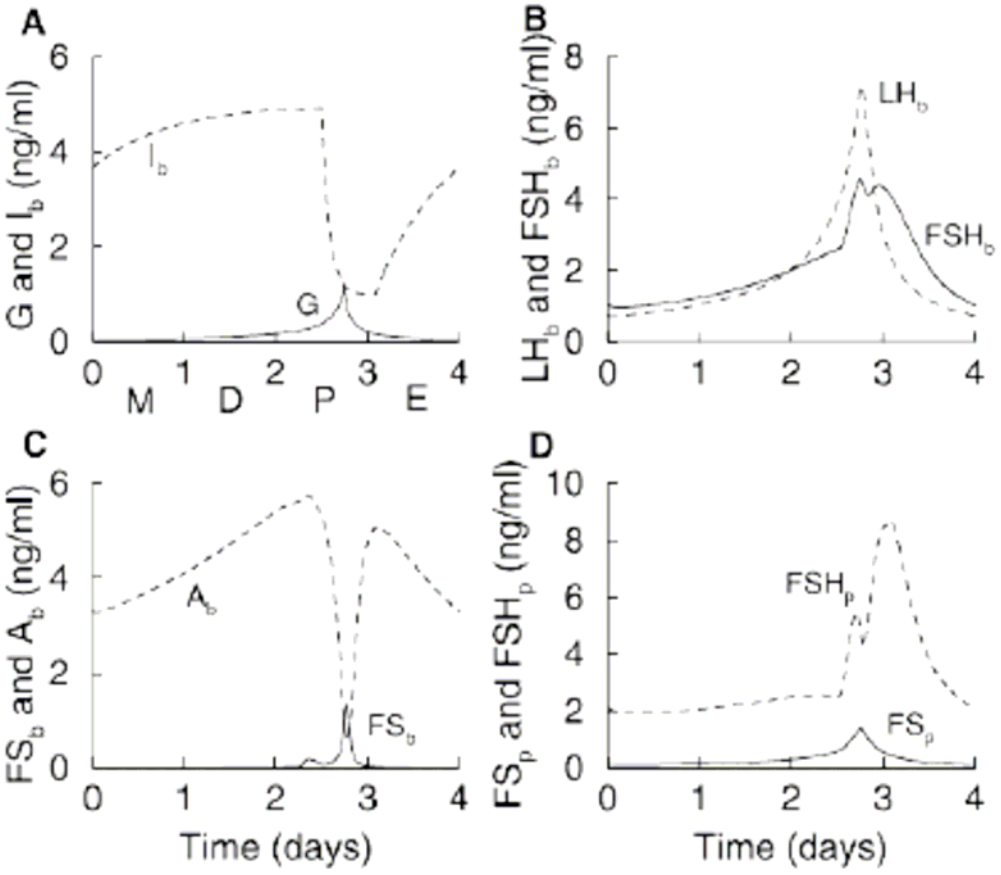

Fig. 1. Time-courses with variable hormones during estrous cycle (Adapted from [7])
(A)The time course of inhibin (I) and GnRH (G) during the rat estrous cycle. (B) FSH in the ratis supressed by inhibin during the first two days of the cycle but the level increases during proestrous of estrus by increasing GnRH pulses, but inhibin is decreased at this time. (C) The Follistatin level is increased, but the level is not affected by inhibin. (D) FSH secretion is two clear peak on proestrous, but the second peak may not be directly stimulated by GnRH. Subscript b: level in plasma, subscript p: level in intracellular
A: activin, I: inhibin, G:GnRH, FS: Follistatin

αGSU disrupted mice were half that of normal mice (8). This result means that αGSU is not important at the developmental stage, but the protein is important for growth during puberty since FSH and LH control estrogen and testosterone produced by gonads. The testosterone level in the αGSU-knockout mice was undetectable compared to 4.17±0.29 ng/ml in normal males. In addition, the size of the testis was much smaller and spermatogenesis was blocked at the first meiotic division. In female αGSU-knockouts, the size of the ovary and uterus is very small indicating that these female mutants lack estrogen. Both male and female mutants are infertile.

IC. The relationship between FSH and reproduction

Serum FSH increases as a woman approaches reproductive age (9), but becomes even more elevated as women approach menopause (late 30s to mid 40s). This increase of FSH with age is related to a declining level of gonadal inhibin which normally inhibits the positive action of activin produced in the pituitary by competitively binding to the activin receptor. Rising FSH is not only related to aging but also to diseases such as premature ovarian failure and fragile X syndrome that displays an increased incidence of dizygotic twining and early onset of ovarian failure (9). A mouse model was generated with gain-of–function over-expressed human FSH (hFSH) in multiple tissues (10-12). Gain-of–function male mice showed enlarged seminal vesicles, increased progesterone levels, and are infertile (11). The mutant females showed highly hemorrhagic and cystic ovaries, increased estradiol and progesterone, and were also infertile.

By contrast, low levels of FSH cause primary amenorrhea in women and azospermia in men (15). Mice were also produced without a functional FSHB gene (FSHB knock-out) (14). Surprisingly, FSHB-deficient male mice were fertile despite having small testes and a reduced number of epididymal sperm with reduce mobility. The mutants were not altered in terms of accessory glands and Leydig cell numbers in the interstitium, and the testosterone level was not altered in serum as well. In contrast, the female FSHB knock-outs were infertile with a block in folliculogenesis prior to antral follicle formation. Female mutants had small ovaries and thin uteri. Serum LH was not altered in FSHB deficient male mutants, but LH was increased in female mutants. Kumar *et al* (14) demonstrated that the discrepancy might be related to negative feed back regulation through steroids which affect the GnRH surge during the estrous cycle.

ID. The relationship between LH and reproduction

A deficiency in LH caused amenorrhea in women and hypogonadism, plus a failure of gonadal function for men. Male mice lacking LH [LHβ knock-out (KO) mice) were infertile with reduced testis size and accessory glands (15). They also had reduced testosterone levels both in their serum and testis. Leydig cells were also affected by the mutation with a reduction in number and size in males. Spermatogenesis was stopped at the stage of spermatocyte formation, and sperm were not processed properly into elongated spermatids. However, FSH levels were not affected in LHβ KO mice. LHβ KO females were similar to

males in being hypogonadal and infertile. Primary and secondary follicles were normally formed, but antral follicles were abnormal. The theca layer was not impaired by the mutation.

IE. The treatment of human infertility with FSH

The first treatment of humans with FSH was reported in 1993 for infertility in a woman with a tubal rupture (16). The Chinease hamster ovary cell (CHO)-derived recombinant human FSH treatment successfully solved this infertility with a healthy girl who did not show any abnormality by the pidiatric exam. FSH treatment has been used successfully for infertile women for ovulation induction and for ovarian stimulation with in-vitro fertilization (IVF) since that first success (17). Daily injection of FSH, however, was required because of the short half-life of FSH (32±12 h). The problem was solved by creating a long-acting FSH agonist, which could successfully solve the infertility problem of a 32-year-old woman with a 7-year history of primary infertility (17).

According to the results, phenotypes of this human disease are well matched to animal models, and the results clearly showed that FSH and LH are the critical factors for vertebrate reproduction. In addition, FSH treatment can solve infertility problems in women. The clinical trials supporting this fact were performed only with in-vitro-fertilization (IVF), however, because of the variation of results obtained from many research groups using natural fertilization.

II. Activin and activin ihibitors regulating FSHB expression

As described in the above section, LHB regulation was clearly related to GnRH pulsatility and a surge of this activity from the hypothalamus. Regulation of FSHB, however, involved much more than GnRH. Much evidence indicates that activin and its antagonists, inhibin and follistatin, are the main regulators of FSHB and FSH production overall.

IIA. Activin

Activin is a member of the transforming growth factor-β (TGFβ) superfamily which includes inhibin, bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs), TGFβs, and mullerian inhibitory factor (MIF) (20). Activin is produced as a homodimer or heterodimer with two β-subunits, β_A and β_B, resulting in three possible molecular species such as activin A (β_A-β_A), activin B (β_B-β_B), and activin AB (β_A-β_B). All three isoforms were originally purified from porcine follicular fluid (21, 22). Inhibin, an activin antagonist, consists of the activin β-subunit combined with a different, but similar α-subunit, resulting in two forms: inhibin A (α-β_A) and inhibin B (α-β_B) (23). Activin receptors are composed of two single membrane spanning serine-threonine kinases designated ActRIB, also known as ALK4, and ActRIIB (24). Activin binds to ActRIIB which then recruits and activates ActRIB to phosphorylate down stream signaling elements such as Smads. ActRIIB is constitutively phosphorylated and does not need further activation for downstream signaling. In addition, activin can bind to ActRIIB without

assistance from ActRIB, but the binding cannot initiate the signal transduction without ActRIB (25). By contrast, activin cannot bind directly to ActRIB without ActRIIB (26). Kinase activity of both receptors is important for activin signaling to activate target gene expression. Both kinase-deficient ActRIIB or ActRIB were unable to induce transcriptional activity. Kinase-deficient ActRIIB failed to phosphorylate ActRIB. Both mutant receptors, however, could bind each other in the presence of activin. This result showed that activin is required to assemble the ActRIIB and ActRIB complex. ActRIIB phosphorylates ActIB, and the phosphorylated ActIB activates down stream signaling.

ActRII-deficient mice behave like FSHB-deficient mice (27) suggesting that ActRII is integral to FSHB expression. ActRII deficient male mice were fertile although fertility was delayed and the mice had small testes. ActRII deficient female mice were infertile, and had thin uteri and small ovaries. The result was exactly the same as observed for FSHB-deficient mice (14). FSH expression was suppressed in both female and male mutants, but serum LH was not altered in this mutant (27). The result clearly showed that activin receptors, ActRIB and ActIIB, are major signaling elements for FSHB expression.

Production of activin is widespread, although the testis and ovary are significant distributors, and frequently overlap with follistatin production sites. Activin stimulates follistatin expression. In addition, often activin production overlapps with sites of activin action (18, 19). Inhibin was also detected in the pituitary, but the major expression of inhibin was related to granulosa cells. The signaling mechanism of activin and its counter effectors is shown in Fig. 2. Follistatin and inhibin are the extracellular antagonists of activin signaling.

Follistatin blocks activin signaling by directly binding to it and sterically keeping it from interacting with its receptor, whereas inhibin binds to the activin receptor and exclusively blocks activin binding to its receptor.

IIB. Inhibin

As described in the above section, inhibin is a member of the TGFβ superfamily which includes molecules like activin. Inhibin is similar to activin in structure with both sharing β-subunits. Inhibin blocks the ability of activin to induce FSHB expression by blocking the activin-receptor interaction (28-29, Fig. 2). The affinity of inhibin for actRIIB is 10 times less than that of activin without betaglycan, but the affinity is comparable to activin in the presence of betaglycan. Betaglycan is the protein necessary for inhibin binding to ActIIB, but not for activin (30).

Inhibin acts as a circulating feedback regulator of activin activity for FSHB expression. Although it is produced at a number of sites, the granulosa cells of the ovary and Sertoli cells of the testis are the major source of inhibin (31-33). Interestingly, inhibin expression is increased by FSH in granulosa cell culture (32). Inhibin is also increased throughout the human menstrual cycle (34). The concentration of inhibin in plasma is increased rapidly during the early follicular phase on the day after the intercycle rise in FSH, and then falls progressively during the remainder of the follicular phase. Therefore, it can be concluded that FSH stimulates inhibin expression, and its expression blocks the ability of activin to induce

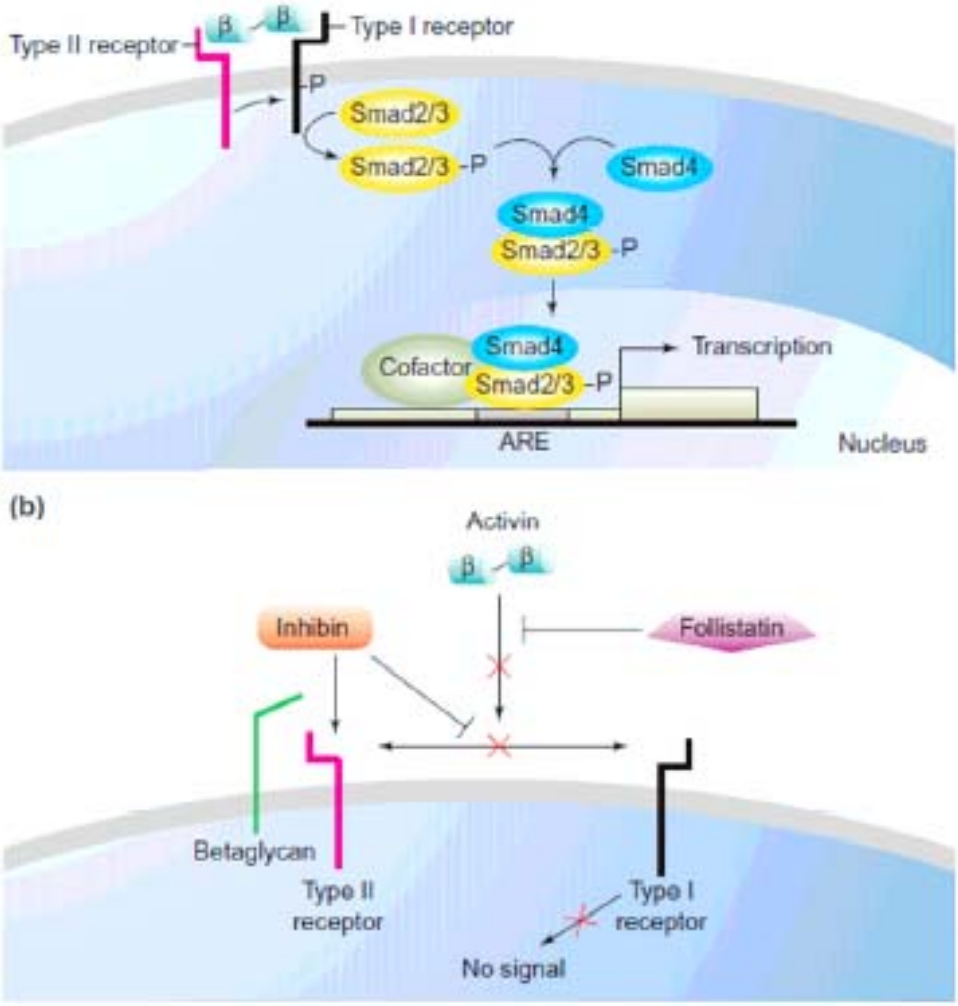

Figure 2 Model for activin signaling (Adapted from [18])
(a)Activin binds to the actRIIB, which recruits actRIB. The actRIB tansduces the signal by phosphorylating Smad2/Smad3. Once phosphorylated, Smad2/Smad 3 dimerizes with Smad4. Finally the dimer is tranlocated into nucleus, where the Dimer can directly bind to the activin response element (ARE)
(b) Extra cellular antagonists of activin are inhibin and follistatin. Follistatin binds activin and prevents binding of activin to its receptor. Inhibin binds to actRIIB with assistant of betaglycan, and prevents access of activn to the receptor.

FSH expression in a negative regulatory feedback mechanism. Inhibin is also induced by FSH in Sertoli cell culture (35, 36). Mice were produced that overexpressed inhinbin by a GeneSwitch system (37). Inhibin-overexpressing mice had small testes in males and were blocked in folliculogenesis at an early stage in females. The result was similar to actRII KO mice.

IIB. Follistatin

Like inhibin, follistatin also blocks the ability of activin to induce FSHB. The mechanism is different from inhibin, however. Inhibin blocks activin action by inhibiting interaction with ActRIIB, but follistatin blocks activin action by directly binding and inactivating activin (Fig. 2). Follistain has no homology to either α- or β-subunits of inhibin/activin (19). Follistatin is a single-chain glycoprotein with three different molecular weights caused by altered mRNA splicing. Each type of follistatins can bind activin and block its function *in vitro* as well as *in vivo* (19).

The level of follistatin does not seem to change during the estrous cycle (19), but the level was shown to increase after treatment with high doses of GnRH in ovariectomized (OVX) female rats (38). GnRH seems to stimulate follistatin expression, but follistatin expression might be regulated by other factors during the estrous cycle. Follistatin is also considered an autocrine/paracrine regulator because its expression is up-regulated by activin (19, 39).

Expression of follistatin is also related to the developmental stage of certain organs such as the hindbrain, somites, vibrissae, teeth, epidermis, and muscle. Follistatin-deficient mice are retarded in growth, and show a decrease in muscle mass, and defects in their skeletons, ribs, and teeth (40). These mice die within hours of birth. Follistatin-deficient mice show more serious defects than activin-deficient mice during development so it is thought that follistatin has more functions than simply counter balancing the reproductive effect of activin.

III. DNA binding elements for FSHB expression

Analysis of the FSHB promoter has been performed for several species of mammals including sheep (41-45), pig (46-48), rat (49-52), mouse (53-57), cow (58, 59), and human (6, 55, 60). As described above, activin, inhibin and folistatin are the major regulators of FSHB expression. The signaling pathway for activin is thought to involve Smads as second messengers leading to gene regulation. Although FSHB expression is related to Samds in mice and rats, Smads themselves are not sufficient to explain activin induction of ovine FSHB for at least two reasons (24): 1) dominant negative Smad3 cannot suppress FSHB expression by activin, and 2) FSHB expression by activin requires 20-24 h for full activation of FSHB transcription instead of 2-6 h which is the normal time-course for Smad2/3 activation and inactivation. These results imply that FSHB expression might require partner protein(s) and that the function of partner proteins might be as important or more so for FSHB expression. Partner proteins are not easy to identify, however, because at least 70 genes are up- or down-regulated by activin in LβT2 cells according to DNA microarray

analysis (61). A number of publications have implicated Smads and Smad binding elements (SBEs) as being critical and direct effecters for FSHB expression (6, 8, 42).

The next section will deal with the location of important sequences in the FSHB promoter, and some important factors involved in FSHB induction by activin will be described.

IIIA. 5'-deletion mutations

A general method for finding important promoter sequences is deleting sequences until function is lost. An effecter binding sequence is revealed by this method because the critical sequences are removed or destroyed by the mutation. After finding a region of critical elements by deletion mutation, putative elements are revealed based on DNA binding sequences. Finally, the putative elements are mutated and tested in transgenic animal. Serial deletions are one method that is useful for finding important promoter sequences needed for promoter function (62). Many research groups have followed this method to identify critical sequences in FSHB promoters.

The size of FSHB promoter studied varies according to species: –1028/+7 for human FSHB (55), -1195/+1 for mouse FSHB (55), -4700/+759 for ovine FSHB (63), -2320/430 for porcine FSHB (48), -2300/+44 for bovine FSHB (59) and -2000/+1709 for rat FSHB (64). All 5'-sequences of FSHB promoters have high homology (6) implying they contain useful and conserved sequences for their promoter function.

Sequenctial deletion mutations from the 5'-region were made for rat FSHB (rFSHB) (50), and the minimal sequences required for activin induction were found within –338 bp of the

transcription start site. This minimal promoter was shown to be sufficient for activin to induction of rFSHBLuc expression in LβT2 cells. The rFSHB promoter was deleted between –472bp and –140bp in a stepwise manner (49). The mutations did not affect either basal or fold-induction by Pitx1 up to the Pitx1 binding site at –54/-48 bp, but further deletion to –50bp showed significant reductions of basal as well as Pitx1 induced expression of FSHB expression in transformed rat growth hormone producing cells, GH_3-1' cells. GnRH was also unable to stimulate FSHB expression when the –54/-48 region was mutated. Unfortunately, the study did not use activin as a stimulator for rFSHB expression. However, the result was unexpected because a Samd-binding site is located at –266/-259 bp of the rFSHB that was deleted during this mutation study without any effect on its expression. The authors only emphasized the importance of the Pitx1 binding site at –54/49 bp. The result implied that Smads might be important for activin induced FSHB expression but not basal expression.

Mouse FSHBLuc (mFSHLuc), consisting of –1990/+1 bp linked to the luciferase gene in a pGL3 plasmid, was also truncated sequentially between –1990 bp and –257 bp. Fold-induction by activin was gradually decreased by the stepwise deletion until –398bp, and then completely gone after further deletion to –257 bp of mFSHB in LβT2 cells (55). The result from this last deletion was expected because the mutation removed a putative Smad binding site at –267/-260 bp. However, results from other mutations were not expected because there are no putative Smad binding sites in the distal promoter region. The authors demonstrated that there might be important sequences between –1990 and –398 bp of mFSHB, but did not

identify any specifically. These results were also found by another group with a similar pattern of decreasing fold-induction caused by serial deletions (47). The authors also postulated that multiple activin-responsive elements might be within the deleted regions. Reduction of FSHB expression by deletion was also observed *in vivo* (43). In those studies, –4700/+759 oFSHBLuc was expressed in a pituitary specific manner in transgenic mice, but –750/+759 oFSHBLuc or –215/+759 oFSHB were not expressed.

IIIB. Smads

Smads are second messengers for signals generated by TGFB family members like activin. When activin binds to its type II receptor (ActIIR), that receptor activates the activin type I receptor (ActIR) which, in turn, phosphorylates and activates Smad2 and Smad3. Other family members can phosporylate Smads 1, 5 and 8 and also Smads 6 and 7 (61).

Betaglycan is a known accessory protein for the interaction between TGF-β family members and their receptors. The affinity of inhibin for the activin receptor is one-tenth that of activin without betaglycan, but the affinity of inhibin for ActIIBR increases by 10-fold in the presence of betaglycan. Betaglycan helps inhibin bind ActIIB, but not activin (30).

The access of Smad2/Smad3 to ActRIB requires SARA (Smad anchor for receptor activation) and SARA can be blocked by the inhibitory Smads (I-smads) such as Smad6 and Smad 7 (Fig. 3). Inhibition can occur in two ways: 1) competitive binding for the activated ActRIB or 2) recruiting E-3 ubiquitin ligase, also as known Smad ubiquitination regulatory factor 1 (Smurf 1) and Smurf 2, resulting in ubiquitination and subsequent degradation of activated ActIB (29, Fig. 3).

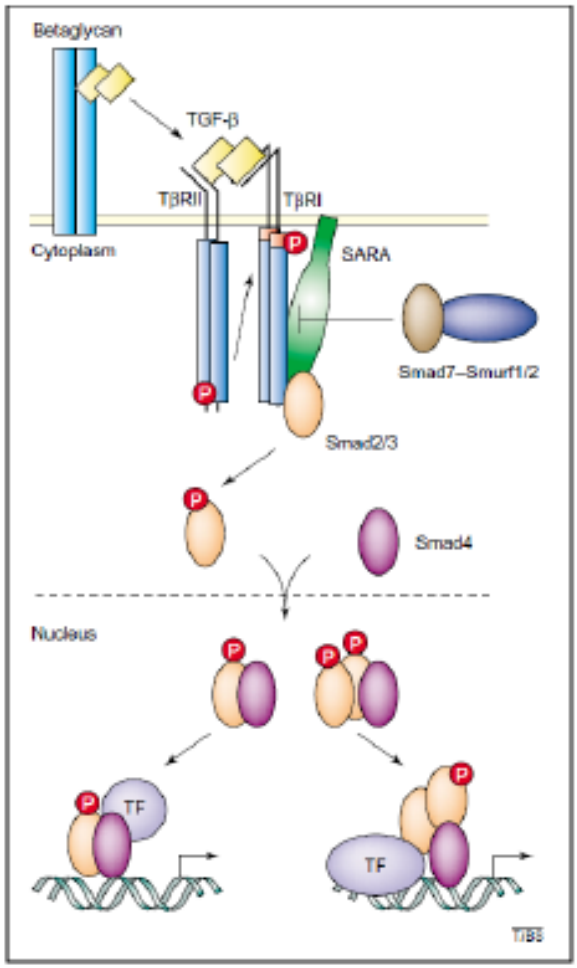

Fig. 3 Mechanisms and regulators for Smad signaling (adapted from [29]

After activin binds to its receptors, ActRIB phosphorylates R-Smads by assistant of SARA. The activity of ActIB is regulated by Smad7 and Smufs. Smad7 inhibits R-samd binding to ActIB, and Smurfs induce ubiquitination and degradation

Activated R-smads form heterodimeric complexes with Smad4. These complexes go into the nucleus and bind promoter sequences in target genes followed by activation of gene expression (Fig. 3). Activin signaling can phosphorylate Smad2 and Smad3 in LβT2 cells, but only Smad3 over-expression could increase mouse FSHB (mFSHB) expression among R-smads including Smad1, Smad2, Smad3, Smad4, and Smad5 (54). Smad3 also increased expression of rat

FSHB (8). Over-expression of both Smad3 and Smad 4 further increased expression of rFSHBLuc in LβT2 cells. Although mFSHB expression by activin was significantly attenuated by inhibiting Smad2 in cells (8), rFSHB was not affected by this inhibition (52). The result implied their might be species differences between rFSHB and mFSHB regulation, however, both promoters were significantly affected by inhibition of Smad3, implying that Smad3 might be the critical factor for both rFSHB and mFSHB expression. Ovine FSHB (oFSHB) expression was also increased in a dose-dependent manner with increasing Smad3 over-expression (24). Gold fish Smad2 and Smad3 could also stimulate gold fish FSHB expression in LβT2 cells (66), implying that Smad3 over-expression could stimulate FSHB expression in all vertebrates. Interestingly, dominant negative Smad3 did not inhibit oFSHB expression in LβT2 cells, implying that Smad3 is not important for oFSHB expression in LβT2 cells (24). This result indicated that Smad3 could stimulate oFSHB expression, but was not a critical factor.

Smad3 strongly binds to a palindromic Smad-binding element (SBE) with a sequence of GTCTAGAC (half site = GTCT or AGAC) (53). Smad2 does not bind directly to DNA, but can bind in conjunction with Smad 4 which does have a DNA binding domain (67).

As shown at Fig. 4, SBEs are located within the mFSHB and rFSHB promoters at –266GTCTAGAC-259 (6, 8). The palindromic consensus SBE of mFSHB promoter was confirmed to be a critical sequence for FSHB expression (8). However, other species, including human, cow, sheep, buffalo, and pig, do not have this site that is conserved only in

rodents (mice and rats) (Fig. 4). These other species have a half SBE site at –167GTTTAGAC-160 as in the ovine FSHB (oFSHB) promoter. Although other SBE half-

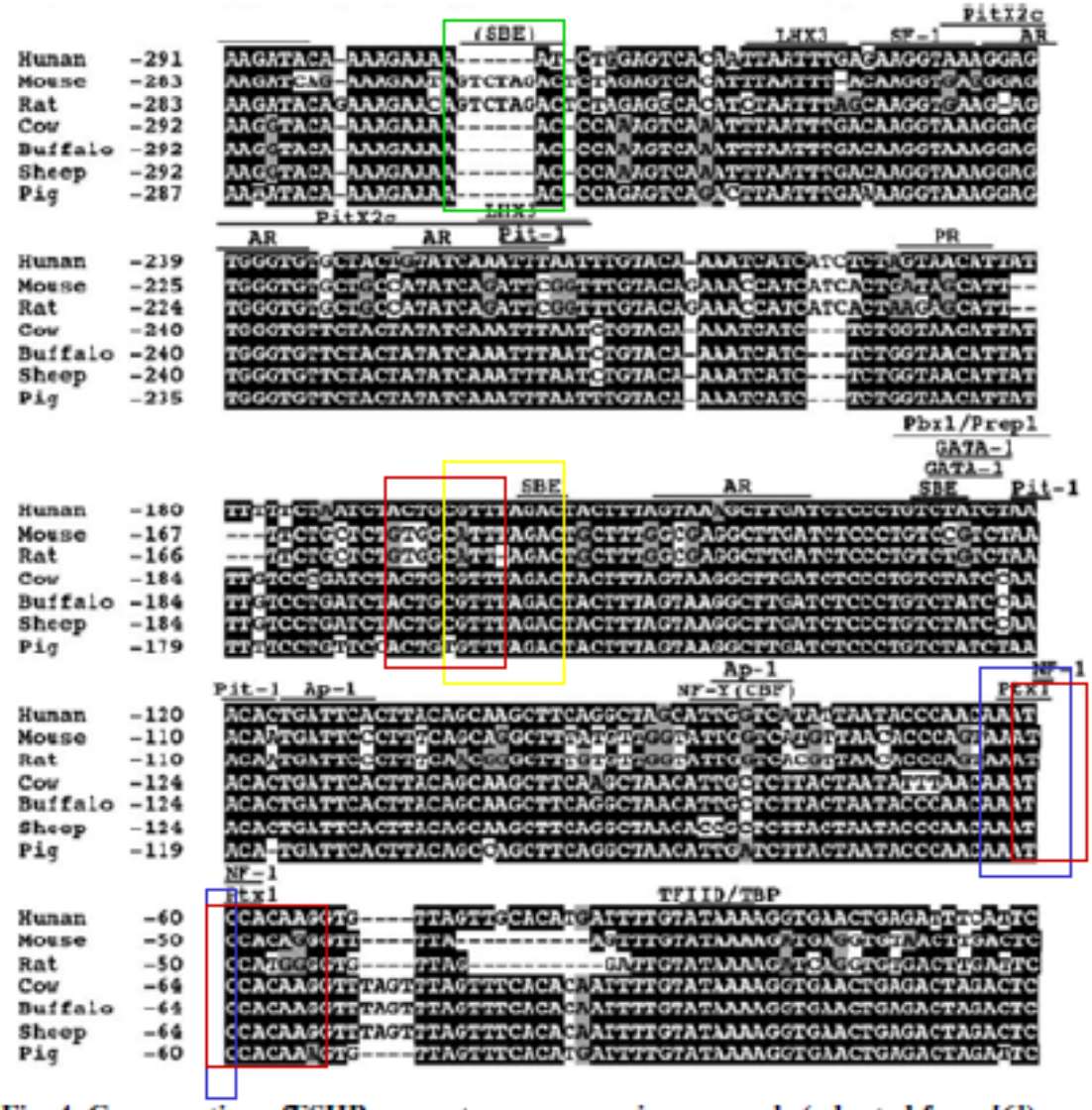

Fig. 4. Conservation of FSHB promoter sequences in mammals (adapted from [6])
Alignment of sequences across species reveals the existence of a palindromic SBE that is unique to only mice and rats (green rectangle). All other species have half-SEB in-167bp region (yellow rectangle). A RUNX1 is located adjacent half SEB at –170 region, and the other located with ovelapping Pitx1 site –60 region which is conseved through mammals (Pitx1 binding site:blue rectangle, RUNX1: red rectangle)

sites exist at several places in the ovine FSHB promoter (–973/-962 bp, -453/-442 bp, and -421 bp) in oFSHB promoter, the only function site appears to be at –167/-160 bp (42). However, a dominant negative Smad3 did not block activin induced expression of oFSHB (24). In addition, activin induced FSHB expression occurs over 20-24 h for both oFSHB and mFSHB (24, 55), although mFSHB has palindromic SBE in the promoter. This result suggests that Smads might not be critical at the -167 bp site for FSHB expression by activin. Perhaps a co-activator(s) or Smad-partner protein are involved. Many research groups are working to understand this puzzle.

IIIC. Pitxs

Pituitary homeobox (Pitx) proteins belong to the bicoid-related subclass of homeodomain genes. These are required for the development of anterior pituitary structures from drosophila to human (68). The nomenclature for this gene family is complicated because different laboratories cloned the same genes and each laboratory provided unique names (69). For example, Pitx1 is also called as Ptx1, P-OTX, BFT, and Brx2. The nomenclature for Pitxs in this thesis follows that devised for the mouse genome database (MGD). The Pitx gene family consists of three bicoid-related homeobox genes: Pitx1, Pitx2 and Pitx3; all have been cloned from the human and mouse genomes.

All three proteins have highly conserved amino acid sequences, especially in the homeodomain region that binds DNA. This fact implies that all Pitx family members can competitively bind to the same DNA sequences if they are expressed in the same cell. Pitx1

and Pitx2 showed 70% homology in the C-terminal region, but Pitx3 showed just 55%. By contrast, they differ in the N-terminal region, a known protein-protein interaction region, which gives them distinct functions (68).

Pitx2 is a critical factor for development of gonadotropes. Pitx1 has been reported as a transcription factor for hormone genes. The functions for Pitx1 and Pitx2 overlap during early pituitary development (70). Pitx1 mutants showed a mild pituitary phenotype, but Pitx2 compensated for a lack of Pitx1. However, Pitx2 mutants exhibited developmental failure when trying to make Rathke's pouch that holds the anterior pituitary in adulthood (71). The result implied that Pitx1 might not compensate for Pitx2, but Pitx2 seemed to substitute for Pitx 1, at least, at the early developmental stage. In addition, over-expression of Pitx2 could increase gonadotrope cells in the pituitary. Both Pitx transcription factors are expressed with similar patterns throughout development and adulthood in gonadotropes (71), implying overlapped function in the pituitary. The pattern of Pitx3 expression has not been investigated yet, although it is clear that Pitx3 is not expressed in the same tissues that express Pitx1 and Pitx2 (72). No Pitx1 mutation has been reported in human diseases, but Pitx2 mutations are responsible for Axenfeld Rieger Syndrom (ARS), an autosomal disease with very low frequency (1/200,000) (68).

Pitx 1 induces pituitary hormones, such as prolactin (PRL) and growth hormone (GH) (72). Interestingly, Pitx1 and Pitx2 can also stimulate many pituitary hormones including FSHB, LHB, αGSU, and thyroid stimulating hormone (TSH) in African green monkey

kidney CV-1 cells (72, 73). These results suggest that Pitx1 might be a factor absolutely needed for FSHB expression.

Pitx proteins recognize the TAA(T/G)CC motif on promoters with its homeodomain. Pitx2 stimulated rFSHB expression in LβT2 cells (50, 51), but mutant Pitx2 could not stimulate expression. The result implied that Pitx2 might be a factor needed for FSHB expression. However, mice with a conditional deletion for Pitx2 in pituitary gonadotropes were normal in puberty and fertility (74), although the authors demonstrated the possibility of compensation by Pitx1.

Pitx1 and Pitx2 are expressed in LβT2 cells but not in Chinese hamster ovary (CHO) cells (56). There are several putative Pitx-binding sites in the promoter for mFSHB such as at –1592/-1587, -963/-958, -941/-936, and –53/-48, which was confirmed by the silico method. Pitx1 bound at this latter site and was identified by the electrophoretic mobility shift assay (EMSA) (56). However, only the sequences at –53AAATCC-48 bp of mFSHB and -54/-48 of rFSHB, normally associated with Pitx binding were confirmed as being critical for mFSHB and rFSHB expression in LβT2 cells (49, 56). These 6 nucleotides are conserved in all mammals studied to date (Fig. 4). In addition, Pitx1/Pitx2 can physically interact with Smad3 (56), suggesting that these proteins might be the partner protein of Smads for FSHB expression.

The critical sequences for Pitx1 action have never been confirmed *in vivo*, however. There are many factors that can be different between *in vivo* and *in vitro* situations, however.

The importance of the Pitx1 DNA binding element has been confirmed both in vitro and in vivo for ovine FSHB expression in Chapter II of this thesis.

IIID. RUNXs

Runt related (RUNX) genes play diverse roles in different biological systems, including cell differntiation. RUNX1 plays a critical role in the formation of hematopoietic stem cells and are frequently associated with human leukaemia. RUNX2 is a key regulator for bone morphogenesis in osteoblast maturation and osteogenesis. Runx3 is a tumor suppressor for

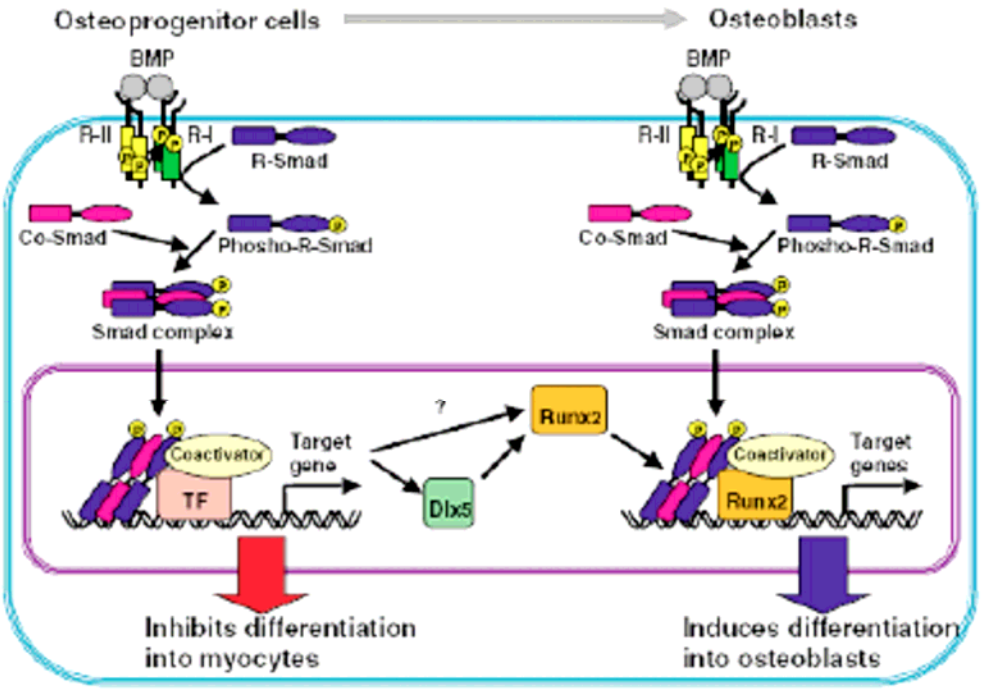

Fig. 5 The relationship between Smad and RUNX for stimulating target genes (adapted from [82])
Smads stimulates the target gene expression including JunB, and JunB stimulates RUNXs, and then the RUNX-Smads complexes stimulate or respress the target genes. The function whether Stimulator or repressor is determined by coactivator(s).

gasteric cancer (75-78). Polymavirus enhancer-binding protein 2/core-binding factor (PEBP2/CBF) is a α/β heterodimer. There are three genes in mammals that encode the α-subunit, which are runt-related genes, such as RUNX1 (also called AML1, PEBP2αB, and CBFA2), RUNX2 (also called AML3, PEBP2αA, and CBFA1), and RUNX3 (AML2, PEBP2αC, and CBFA3), all with structural similarities. These genes share a β-subunit, PEBP2β (also called CBFβ), which does not bind DNA itself but increases binding affinity of RUNX proteins to DNA when they become heterodimers with α-subunits (75-78).

All RUNX proteins bind to the same DNA motif, termed the Runt domain, and activate or repress transcription of the target genes. The consensus sequence of the runt protein binding site is 5'-AACCACA-3'. The sequence is similar to a forkhead transcription factor, which is exemplified by FoxH (FAST) with a concensus binding site = 5'-AATCCACA-3' (79). Both proteins can competitively bind at the same site. However, runx family members can bind more strongly at the site, but over-expression of FoxH can compete with any Runx protein and inhibit the transcriptional activity of Runxs (79). Runxs are transcriptional regulators not only through binding to their cognate DNA element, but also by interactions with co-activators or co-repressors (80).

Each of the R-Smads, including Smad1, Smad2, Smad3, and Smad5, interacts with RUNX1, RUNX2, and RUNX3 through the TGFβ family signaling in African monkey kidney fibroblast cells (COS-7) (76, 77). This interaction might occur via phosphorylated Smads because the TGF-β signal induces phosphorylation of R-Smads. The Smad interaction domain (SMID) of RUNXs is embedded in a known nuclear matrix targeting

signal (NMTS) domain that mediates nuclear trafficking. The interaction between Smad and RUNX is clearly diminished by mutation at this site as well as loss of activity, at least for RUNX2 (80, 81). RUNX-Smad complexes appear to be formed in the cytoplasm without TFG-β family stimulation, but they are located in the nucleus after TGF-β stimulation (82). RUNX-Smad complexes require the NMST of RUNX to localize in the nucleus (83). Smads are localized in ROS 17/2.8 cells that express RUNXs, but Smads are not associated with the nuclear matrix of HeLa cells that does not express RUNXs. Smads are localized in the nucleus when RUNX is expressed in HeLa cells with TGFβ signaling. In the absence of TGFβ signaling, Smads are localized in the cytoplasm whereas RUNXs are localized in the nucleus. These results indicate that RUNXs might be one of the factors for transporting Smads into the nucleus. Phosphorylation of Smads might be required to bind RUNXs to enter the nucleus. However, NMST of RUNXs might be sufficient to localize the complex into the nucleus.

RUNX genes are tightly regulated and expressed in a tissue-specific manner (78). Expression of RUNX2 is stimulated by TGFβs, and the signal is transferred through JunB and p38 MAPK in C2C12 cells (84). Expression of JunB is stimulated by GnRH (85), and p38 MAPK is an activin-signaling protein in LβT2 cells (24). These facts suggest that RUNX2 might be a transcription factor important for FSHB expression in response to activin signaling in LβT2 cells. However, TGF-β represses RUNX2 expression in primary mouse calvarian osteoblasts cultures, rat osteosarcoma (ROS) 17/2.8 cells, and caIB 2T3 cells (86). In addition, Smad3 and Smad4 can repress transcriptional activity of RUNX2 through their

physical interaction in ROS 17/2.8 cells. The regulation of RUNX2 by TGF-β might be dependent on cell type.

RUNX1 functions as a repressor or activator for target genes depending on its partner proteins. RUNX1 stimulates myeloperoxidase (MPO) expression with the co-activators p300 and cAMP response element binding (CREB)-binding protein (CBP) that are functionally conserved transcriptional co-activators, although RUNX1 interacts directly with Smad3 (87, 88). CREB was revealed to be an inhibitor for FSHB expression by activin in LβT2 cells (89). Therefore, RUNX1 and CBP might be repressors or recruit other co-repressor(s) for FSHB expression in LβT2 cells if they are critical factors. RUNX1 also acts as a repressor for p21 expression when it interacts with mSin3A in COS-7 cells (90). Therefore, it is clear that the function of RUNX1 as an inducer or repressor is determined by its co-activator(s).

There are several putative RUNX binding sites in the oFSHB promoter, especially two putative RUNX binding sites, at -172/-164 and –66/-58, standout as being important in the critical sequences between –170 and -39bp of the oFSHB promoter (ConSite http:/www.phylofoot.org/consite) (Chapter 1). Interestingly, the -172/-164 site is adjacent to a putative Smad binding site (Fig. 4), and both proteins are known to interact with each other (76, 77). The site was mutated to confirm its importance *in vitro* and *in vivo* (41). The site was important for activin inducing FSHB expression in both studies. The other site, -66/-58bp of the oFSHB promoter, was also shown to be important *in vitro* with LβT2 cells and *in vivo* with transgenic mice (Chapter 2 of this thesis). The core region of the putative RUNX1 binding site was shown to be important for basal and activin-induced oFSHB expression *in*

vitro, but the site was only important for basal expression *in vivo*. Interestingly, mutations in the 5' region of the putative RUNX1 binding site clearly inhibited oFSHB expression by activin *in vitro*, but had little affect on oFSHB expression in the pituitary of transgenic mice. However, the mutants showed increased expression in tissues that normally do not express FSHB in vivo, especially in testis and brain. Expression was also high in the kidney, liver spleen, lung, and heart compared to wild type oFSHBLuc. Although it is not clear yet, the time course of activin inducing FSHB expression suggests a molecule like RUNX1 might be the Smad-partner protein. FSHB induction by activin requires 20-24 h, but smads are phosphorylated within minutes and gradually can activate their target genes within 2-6hrs by activin signaling. However, RUNX1 typically requires a longer time to activate target gene expression by TGF-β family because the TGF-β signal activates Smads, activated Smads express JunB, JunB then stimulates RUNX expression, and RUNX-Smads complexes induce the target genes such as FSHB. The signaling pathways involved in activin action clearly need further study.

IIIE. Forkhead transcription factors

Forkhead family members are transcription activators. Forkhead proteins have been shown to act primarily as transcriptional activators but this is not always the case. Forkhead family members have diverse roles including developmental and metabolic processes as well as mechanisms involving cell-proliferation. Forkhead family memebers have a highly conserved DNA-binding domain, termed the forkhead (FH) or winged helix domain (91, 92).

This binding site is similar to the RUNX binding site described above in the RUNX section. Both RUNX and forkhead proteins can bind competitively at the same DNA sequence. Forkhead proteins can also bind Smads through protein-protein interactions between the MH2 domains of Smad2/3 and the Smad-interaction domain (SID) of forkhead proteins (93, 94).

FoxH1 is also known as "forkhead activin signal transducer1" (FAST1). It was first identified as a protein that bound to the promoter of the gene encoding mesoendodermal homeobox, Mix2 (91). FoxH1 can bind DNA without partnering with Smads, but it only acts as a transcriptional activator when it also binds Smads2/3. Thus, forkhead proteins require partner proteins, such as Smads, to alter expression of their target genes. Although FoxH1 is known to partner with Smads to induce gene expression, it is primarily expressed in early stages of development (94). Therefore, it is unlikely, itself to be involved in induction of FSHB since this is expressed as an adult. There are about 80 Fox family members that might partner with Smads to induce FSHB, however.

FoxL2 is a Fox family member that might help induce FSHB expression. It is expressed in the mouse pituitary during embryogenesis and also throughout adulthood. Its expression has been co-localized with αGSU, LHB, and FSHB in gonadotropes (93, 95). FoxL2 can physically interact with Smad3 (not Smad2) to mediate activin signaling in LBT2 and αT3-1 transformed murine gonadotropes (93). These results indicate that FoxL2 could be a Smad partner to help induce FSHB expression in gonadotropes. A coincident pattern of expression for FoxL2 and FSH also suggests that FSH might be a downstream target of Fox2L during

activin induction. It is already known, for example, that FoxL2 is a transcriptional inducer of the gene encoding the GnRH receptor where it acts through the "GnRH receptor activating sequence" (GRAS) in αT3-1 cells (95). FoxL2 is also an inducer for αGSU expression in gonadotropes. There is no proof, however, that FoxL2 is involved with either FSHB or LHB expression in mouse gonadotrope cell culture (92).

FoxQ1, also known as HFH-1L, is another forkhead transcription factor that might be involved with FSHB induction by activin since it is up-regulated between 5 and 10 hrs after incubation with activin in LβT2 cells (A. Jesse Gore's thesis (2008)). FoxQ1 has a well-conserved amino acid sequence in mammals (96), showing 85% homology between humans and mice. The DNA binding motif is especially well conserved and shows 100% identity in the human, mouse and rat. FoxQ1 is expressed at an early stage in vertebrate development (97), but the role of this protein at the developmental stage is not clear yet. The only known function of FoxL1 during development involves hair differentiation in mice (98, 99). FoxQ1 can also repress smooth muscle differentiation genes, such as telokin and SM22α *in vitro*, but this result has not been report *in vivo*. FoxQ1 is expressed in the stomach from embryogenesis throughout adulthood in mice. Also, a deficiency in FoxQ1 causes impaired gastric acid secretion after birth (100, 101) and increased embryonic morbidity. Linking FoxQ1 to FSHB induction, however, is unlikely since a FoxQ1 knockout showed normal reproductive function (101).

IV. Summary of Dissertation Work

The study presented here scanned 5.5 kb of oFSHB promoter (4741 bp) and intron/exon (759 bp) looking for enhancers and/or silencers needed for activin induction of ovine FSHB transcription. One novel site was identified as being important for induction in LBT2 cells and was then tested further *in vivo* as a transgene. It was ultimately found not to be important for activin induction, but it was needed presumably for basal expression.

Chapter I describes efforts to identify activin sensitive sequences that fall outside the promoter region between -167/-58 bp. Although sequences within this region had been identified as being important for activin induction, researchers still felt that important sequences might reside between -4741/-178 bp or in the TATA box and/or exon/intron areas. The results from this study now show that these regions have no role in activin induction of oFSHB transcription. This study was performed in LBT2 cells, however, and the results should be confirmed *in vivo* to confirm their physiological relevance.

Chapter II describes the identification of an important site between -68/58 bp using transient expression in LBT2 cells. This region contains a known Pitx1 site and a putative Runx1 binding site. When mutations were made from -68 bp through -165 bp plus another at – 62 bp, expression was decreased by 97 – 99% in the pituitary. When the pituitary was dispersed and tested in primary tissue culture, the activity that remained responded at least as well as wild type expression to activin. Mutations in sites not including the Pitx1 site depressed neither basal expression nor activin-induced oFSHB expression, but did have significant effects on pituitary-specific expression.

The results reported in this thesis require further study to identify the transcription factors that actually bind the two critical promoter sites at -167/-158 bp and -68/58 bp. To date, electrophoretic mobility shift assays have been unable to identify any natural protein that binds either of these sites except Pitx1 when expressed at high levels.

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CHAPTER I

Activin induces ovine follicle stimulating hormone beta using -169/-58 bp of its promoter and a simple TATA box

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This manuscript was submitted in *Reproductive Biology and Endocrinology*

Abstract

Background

Activin increases production of follicle stimulating hormone (FSH) by inducing transcription of its beta subunit (FSHB). Induction of ovine FSHB (oFSHB) by activin has been studied in LBT2 gonadotropes using transient expression of oFSHBLuc (-4741 bp of oFSHB promoter plus exon/intron 1 linked to luciferase). Several sequences between -169/-58 bp of the oFSHB proximal promoter are known to be necessary for activin induction, but sequences outside this region also appear important. Deletions between -4741/-752 bp decrease activin induction by more than 70% suggesting important 5' sequences. Induction disappears entirely if a minimal T81 thymidine kinase promoter replaces the oFSHB TATA box promoter and 3' exon/intron. The study reported here was designed to determine which, if any, sequences outside -169/-58 bp are important for activin induction of ovine FSHB.

Methods

Eleven progressively shorter deletion mutants of oFSHBLuc were created between -4741/-195 bp. Also, five deletions were created internal to this region to -175 bp which were replaced with exogenous DNA of equal length. All deletion constructs were analyzed for basal and activin-induced expression. In addition, the minimal TATA box promoter of oFSHBLuc (-40/+3 bp) was replaced by minimal T109 thymidine kinase and rat prolactin promoters to determine the importance of promoter sequences. Finally, substitutions were made in 3' intron/exon sequences to determine their importance.

Results

Successive 5' deletions progressively lowered induction by activin treatment from 9.5-fold to 1 (no induction) and simultaneously increased basal expression. The replacement deletions showed, however, that changes in "spacing" and not loss of specific sequences caused all these changes. Substitution of the oFSHB minimal promoter (TATA box) with the rat prolactin promoter (TATA box) did not alter activin induction, but substitution with the thymidine kinase promoter (no TATA box) destroyed it. Finally, replacement mutations in the 3' region did not significantly decrease activin induction.

Conclusions

These data show that 5' sequences distal to -175 bp in the oFSHB promoter do not contain specific sequences required for activin induction. Likewise, 3' oFSHB sequences are not significantly involved in activin induction of oFSHB. Finally, activin induction of oFSHB transcription seems to require a minimal TATA box promoter.

Background

Follicle stimulating hormone (FSH) is made only in pituitary gonadotropes and stimulates gonads for normal reproductive function in females and males (1-3). Transcription of the beta subunit of FSH (FSHB) is rate limiting for overall hormone production and the most potent and influential direct inducer of FSH production appears to be activin (4, 5). Significant research has focused on classical Smad activation by activin

and its down-stream signals leading to FSHB expression, but the evidence for Smad involvement with ovine FSHB (oFSHB) is not yet clear (6, 7).

A complementary approach to understanding activin signaling is to identify promoter sequences required for induction. The standard approach for these studies is to analyze transient expression of oFSHB promoter/reporter gene constructs in transformed murine gonadotropes (LBT2 cells). The construct used for studying regulation of ovine FSHB is oFSHBLuc (-4741 bp of ovine FSHB promoter plus exon/intron 1 linked to luciferase; see Fig 1). The results from *in vitro* LBT2 studies can be confirmed in transgenic mice to show the physiological relevance of a particular promoter sequence.

Transgenic studies recently confirmed that a Smad-related site between -168/-158 bp of the ovine promoter is required for ≥ 99 % of ovine FSHB expression *in vivo* (7). This site was first discovered using transient expression of oFSHBLuc mutants in LBT2 cells (8). More recently transgenic studies were used to confirm the importance of a Pitx1/2 site between -68/-63 bp required for 99 % of ovine FSHB expression *in vivo* although it seems to have no connection to activin action (Sang-oh Han, manuscript in preparation, our laboratory). This site is conserved in all mammals studied to date and has also been analyzed using rodent constructs analogous to oFSHBLuc (9, 10).

Interestingly, 5' truncations of rodent FSHBLuc constructs are reported to decrease activin induction in LBT2 cell cultures (11, 12). Truncations from -1990 to -304 bp in mouse constructs reduced fold-induction by 60 %. Similar studies with ovine FSHBLuc showed that a deletion from -4741 to -750 bp decreased fold-induction by 70 % (Pei Su; unpublished

observations in our laboratory). One interpretation of these data is that there are specific sequences in the 5' region important for activin action.

Finally, ovine FSHB promoter sequences between -4741/-39 bp have been unable to support activin induction when placed behind the minimal T81 thymidine kinase promoter (Pei Su, unpublished results; our laboratory). By contrast, four copies of the palindromic Smad binding site of the murine FSHB promoter do confer activin induction on a minimal TK promoter/luciferase construct (≥ 10-fold induction) (13). These results suggest differences between activin induction of the rodent and ovine genes. One difference could involve the exon and intron that are included in the oFSBHLuc construct. Alternatively, it could simply reflect a specific need for a TATA box promoter for expression of ovine FSHB or some other reason. To date, the 3' ovine intron has been considered important only for basal gene expression by making mRNA processing more efficient and/or effective, but it might also contain sequences needed for activin induction. Therefore, it is possible that sequences either in the TATA box or 3' region of the ovine FSHB gene play some role in activin induction.

The study reported here examined the 5', 3' and TATA box regions of the ovine promoter to determine which, if any, are important for activin induction of the ovine FSHB subunit.

Methods

Reagents and plasmids

Recombinant human activin A was purchased from R&D systems (Minneapolis, MN). FuGENE)®6 transfection reagent was purchased from Roche Applied Science (Indianapolis, IN) and QuickChange® Site-Directed Mutagenesis kits were obtained from Agilent Technologies Co. (La Jolla, CA). Restriction enzymes including BglII, KpnI, SacI, SacII, Acc65I, AgeI, XhoI, HindIII, and EcoRI as well as dual luciferase assay kits were purchased from Promega (San Luis Obispo, CA). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). All primers for point mutations were obtained from Sigma-Aldrich Co. (St. Louis, MO). Mutated sections for all plasmids were sequenced by SeqWright Technology (Houston, TX) to confirm sequence correctness and all common reagents such as yeast extract, tryptone, agar, Tris and boric acid were purchased from Fisher Scientifc Inc. (Pittsburgh, PA).

The oFSHBLuc plasmid used throughout this study (-4741 bp of 5' promoter plus intron 1 linked to a luciferase reporter gene in a pGL3 plasmid) was the same as that reported earlier (14). Nearly 10 kb of human FSHB sequence (-3511/+5918 bp) was provided by Dr. T. Rajendra Kumar (University of Kansas Medical Center, Kansas City, KS).

Transfections in LBT2 cells and reporter assays

Immortalized murine gonadotropes (LBT2 cells) were obtained from Dr. Pamela Mellon (University of California, San Diego, CA) and maintained in complete DMEM containing

10% (v/v) fetal bovine serum plus 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C under 5 % CO_2: 95 % air. For experiments, cells were plated at a density of 30,000 cells with 50 μl of complete media per well in 96-well tissue culture plates. Cells were transfected 24 hr after plating in quadruplicate by adding 50 μl of serum-free media containing 50 ng plasmid plus 0.15 μl Fugene6. Cells were also co-transfected with 5 ng/well pRL-TK which was used as an internal control since its production of Renilla was not altered by activin. After 24 hr of transfection, media were removed and cells were treatead with or without 25 ng/ml activin for 22 hr prior to harvest with passive lysis buffer. Firefly luciferase and Renilla were quantified sequentially using a Victor-Light micro plate luminometer (PerkinElmer, Waltham, MA).

Deletion constructs in the 5' promoter of oFSHBLuc

A series of KpnI restriction sites was created by making point mutations at -4042, -3387, -2362, -1966, -1440, -1015, -546, -195 bp in oFSHBLuc. Subsequent Kpn1 digestion cut out sequences between these sites and the formerly unique Kpn1 site at -4793 bp of oFSHBLuc. Re-ligation created 8 of 11 total deletion mutants. Two of the remaining deletions utilized ApaI and EcoRI restriction sites already present in oFSHBLuc (ApaI at -4786 and -2932 bp; EcoRI at -4744 and -306 bp)**.** The last deletion mutant was made by creating a SacI site at –4775 bp. Subsequent deletion with SacI cut out the fragment between –4775 bp and an existing SacI site at –748 bp. The plasmid was then re-ligation. All mutants were sequenced to verify their structure.

Replacement constructs in the 5' promoter of oFSHBLuc

Many point mutations were made to create restriction sites used to excise and replace promoter sequences in oFSHBLuc. All restriction site mutants were tested independently to show they had no effect on either expression or activin-mediated induction of the construct. Replacement 1 (Rep1) was made by replacing –4793/-4043 bp with –1965/-1218 bp using the Kpn1 restriction sites at -4793 and -1965 (see above) and newly made AgeI sites at -4043 and -1218. Replacement 2 (Rep2) was made by replacing –4043/-2760 bp with –2028/-744 bp of oFSHBLuc using AgeI and BglII restriction sites. Replacement 3 (Rep3) was made by replacing –2760/-2028 bp with +5/+740 bp using BglII and SacII restriction sites. Replacement 4 (Rep4) was constructed by replacing –2028/-744 bp with –4043/-2760 bp after creating AgeI and BglII sites. Replacement 5 (Rep5) was constructed by replacing –750/-175 bp with –3341/-2760bp after creating SacII and BglII sites. Replacement 6 (Rep6) was constructed by replacing –90/-39bp of oFSHBLuc with a synthetic oligonucleotide of the same size in which A and T were exchanged for C and G. The sequence was inserted using AgeI and BglII sites at -90 bp and -39 bp, respectively. Rep6 lacks the Pitx1 site known to be important for FSHB expression and was included as a negative control expected to show low expression and/or low induction by activin in LBT2 cells. Replacement 7 (Rep7) was constructed by replacing sequences at –24/-3 bp, just downstream of the TATA box. The synthetic replacement sequence (random sequence) had a BglII site at the 5' end (see sequence below, lower case) followed by the wild type TATAAA box (bold); an MluI site

(lower case) was added at the 3' terminus (-39 agatctTG**TATAAA**CAAGAACAAGAAATGCAacgcgt-3).

Replacement constructs in 3' oFSHBLuc (1 to 701 bp)

Replacement 8 (Rep8) removed exon 1 from oFSHBLuc (+7/+58 bp) and replaced it with a sequence containing BglII at +5 bp and SacII at +62 bp; the new sequence was synthetically made by substituting original nucleotides as follows: A to C and T to G. Replacement 9 (Rep 9) removed intron 1 from oFSHBLuc (+83/+660 bp) and replaced it with sequences between –3343 and -2760 bp of the oFSHB promoter using SacII and BglII sites as done with Rep 5 (see above). This strategy preserved sequences normally associated with splicing such as the GU adjacent to exon 1 and the AG and associated adenosine located several nucleotides upstream of the 5' end of exon 2 (15).

Thymidine kinase promoter constructs

The pT109Luc plasmid, containing 109 bp of the herpes simplex thymidine kinase promoter, was purchased from the American Type Culture Collection (Manassa, VA). Production of oFSHB(TK)Luc involved substituting the promoter of pT109Luc (-132/+52 bp; named TK in this report) for the same region in oFSHBLuc. First, AgeI and BglII restiriction sites were created at –40bp and +3, respectively, in the wild type oFSHBLuc plasmid. An AgeI restriction site was also created at -132 bp of the pT109Luc plasmid and then sequences between AgeI and an existing BglII site in the pT109Luc promoter were cut

and the pT109 promoter (-132/+52 bp) was substituted for wild type sequences in oFSHBLuc.

Rat prolactin promoter constructs

Dr. Richard N. Day (University of Virginia, Charlottesville, VA) provided 6XCRE-37PRL-Luc which contains 6 copies of a consensus cyclic AMP response element linked to the minimal rat prolactin promoter. Production of oFSHB(rPRL)Luc involved substituting the minimal promoter of 6XCRE-37PRL-Luc (–44/+3 bp; named rPRL in this report) for the same region of oFSHBLuc. In this case, BglII and AgeI restriction sites were created at -39 bp and +3 bp, respectively, in oFSHBLuc and BglII and AgeI sites were created at -44 bp and +3 bp, respectively, in 6XCRE-37PRL-Luc. This allowed substitution of the minimal rat prolactin promoter for the equivalent wild type promoter sequence in oFSHBLuc. Production of oFSHB Δ-175/-39(rPRL)Luc involved creating a BglII site at -175bp of oFSHB(rPRL)Luc followed by digestion with BglII which excised sequences between -175 and -39 bp. The construct was re-ligated to produce Δ-175/-39oFSHB(rPRL)Luc. Replacement 10 (Rep10) was constructed from oFSHB(rPRL) by replacing sequences between KpnI (-4793 bp) and EcoRI (–207 bp) with human coding sequence between +1049/+5939 bp. An EcoRI site was put into oFSHB(rPRL) at -207 bp and the large segment between KpnI (-4793 bp) and EcoRI was excised from the construct. The human sequence was obtained from hFSHB-GEM3 after creating an EcoRI site at +5939 and using an existing ACC651 site at +1049 bp (compatible with KpnI) of hFSB-GEM3. These sites

were cut with Acc65I and EcoRI and the resulting fragment was ligated into oFSHB(rPRL) to produce Rep 10. All constructs were sequenced for verification of structure.

Statistics: All experiments were repeated 3 times or more with similar results. LBT2 cells respond with significant variation to activin over time, so pooling results from different experiments makes it very difficult if not impossible to show statistical differences from different constructs or treatments. Therefore, results are plotted as representative data from one complete experiment performed with quadruplicate cultures. Statistical comparisons between multiple samples were accomplished using ANOVA followed by either Tukey's multiple comparison test (Figures 2, 4A, 4B) or Student Newman-Kuels analysis (Figure 3). Statistical calculations were performed using Prism version 4 (GraphPad software, Inc., San Diego, CA)

Results and Discussion

Ovine FSHB promoter sequences between -4741/-195 bp were not required for activin induction

Our laboratory observed in 2000 that a deletion between -4741/-750 bp in the 5' region of oFSHBLuc (Fig 1) decreased activin induction of this construct by 70 % in LBT2 cells (Pei Su; unpublished observation our laboratory). This deletion also increased basal expression substantially. Since this could mean that important response elements reside in this region, perhaps a silencer or enhancer, a systematic study was initiated to explore this possibility by

making sequential deletions between -4741/-195 bp ending just upstream of the known response elements between -169/-58 bp. The data in Fig. 2A indicate that "fold-induction" progressively decreased from 9.5- to 1-fold (no induction) as promoter length became shorter. Plotting promoter length opposite "fold-induction" highlighted the direct correlation between these two characteristics. Significantly, there was no single deletion that caused a major decrease in fold-induction suggesting that a non-specific phenomenon was responsible for loss of activin effectiveness. Figure 2A also shows that promoter length was inversely proportional to basal expression and no single deletion caused a major increase in basal expression. Figure 2B depicts the raw data from which Figure 2A were derived. It shows important original data that are not shown when "fold-induction" is plotted. Note that activin treatment caused all constructs to have a firefly/Renilla luciferase ratio between 150-300 and that basal expression reached this level when the promoter length was shortest (-306 and -195 bp). Based on these observations, it was postulated that deletions changed "spacing" in the plasmid which somehow increased basal expression until it equalled activin induction.

To test the above hypothesis and the effect of spacing on activin induction of oFSHBLuc in LBT2 cells, 5 deletions were created and replaced with substitute sequences of equivalent length to preserve original spacing in the plasmid (Fig 3, see WT versus replacements 1-5). Replacements 2 and 4 (Fig 3) were made specifically to test the hypothesis that a silencer might reside between -1966/-748 bp since basal expression increased several fold when this section was deleted. Replacement 2 contained two copies of this region while Replacement 4 lacked it entirely. Because basal expression for these two replacement constructs was

statistically identical when compared to each other, it was concluded that there is no silencer between -1966/-748 bp. This same comparison also showed there was no special sequence between –4043/-2760 bp either. In fact, none of the replacement constructs were different from WT expression (basal or activin-induced) except Replacement 4 which showed slightly higher basal expression when compared to WT. It was concluded, therefore, that the decrease in fold-induction caused by 5' distal deletions was only due to changes in spacing which allowed basal expression to increase and overshadow activin induction.

The above results and discussion of 5' distal ovine promoter sequences are not meant to imply these sequences have no relevance for FSHB expression. Studies with transgenic mice indicate that 5' sequences distal to -750 bp are needed for pituitary expression *in vivo* (14). Kato *et al* also reported that Prop 1 binding sites between -852/-746 bp in the porcine FSHB promoter are required for expression in vivo (16, 17) and the ovine FSHB promoter shares high homology with the porcine promoter in this region. The data in Figures 2-3 of this report were focused on activin-induction specifically and not other factors that LBT2 cells might not detect.

Ovine FSHB 3' sequences (exon/intron 1) were not required for activin induction

Figure 3 also shows that replacement of the first 3' exon (Rep 8) and intron 1 (Rep 9) had little to no effect on activin induction. Replacing intron 1 with neutral sequence from the 5' promoter caused no difference in basal or activin-induction expression compared to WT expression. Likewise, replacing exon 1 with a synthetic oligonucleotide (switching A/C and

T/G) caused no significant difference between induction by activin compared to WT induction. Based on these data, it was concluded that 3' sequences in the first exon and intron (+7/+701 bp) were not required for activin induction of oFSHBLuc.

The importance of a minimal TATA box promoter

Figure 3 shows that changing A/C and T/G in the short sequence between the ovine TATA box and transcription start site had no effect on activin induction indicating there is nothing special about this short stretch of sequence. However, substitution of the entire minimal TK promoter (TK109) destroyed activin induction (Fig 4A) as observed previously when the T81 promoter was used. It may be that this promoter with its GC and CAAT boxes increased basal expression so high (20-fold higher than WT) that activin action was overshadowed by basal expression much like that observed with 5' deletions. Nevertheless, the minimal rat prolactin promoter also increased basal expression significantly (8-fold) and activin induction was quite robust using this promoter.

It could be, however, that a TATA box is needed. The data in Fig 4B show that the minimal rat prolactin promoter (has a TATA box) did support maximal activin induction when the full length ovine FSHB promoter was attached to it. When the distal 5' region was replaced with apparently neutral sequences from the 3' human FSHB coding gene, the rat promoter was still able to support activin induction as long as it contained ovine sequences between -207/-39 bp. Deletion of the ovine promoter between -175/-39 bp did destroy activin induction as predicted since these sequences are known to be necessary for activin

induction of FSHB expression (7-10). Therefore, it was concluded that the -169/-58 bp segment which is known to be essential for activin induction of ovine FSHB promoter plus a minimal TATA box comprise all necessary and sufficient sequences required for activin induction of ovine FSHB expression, at least, in LBT2 cells.

Conclusions

The combined date reported in this and previous studies showed that the critical sequences of oFSHBLuc for its induction by activin comprise sequences located between –169/-58 bp plus the simple generic TATA box found in oFSHB-Luc or the rat prolactin promoter. Neither the distal 5' region (-4741/-175 bp) nor 3' exon/intron sequences are important for activin induction of oFSHBLuc in LBT2 cells. The necessary and sufficient sequences for ovine FSHB expression comprise sequences only between -169/-58 bp plus a minimal TATA box promoter.

Competing interests

The authors declare that they have no competing interests related to this study.

Authors' contributions

SH participated in the design of the study, performed all DNA mutations, transfected the constructs in LβT2 cells, performed luciferase assays, analyzed the data, and wrote the

manuscript. WLM participated in the design of the study, helped interpret the data, and revised the manuscript.

Acknowledgements

The authors thank Dr. T. Rajendra Kumar (University of Kansas Medical Center) for the hFSHB plasmid, Dr. Richard N. Day (University of Virginia) for the rPRL promoter in the 6xCRE plasmid, and Dr. Pamela Mellon (University of California) for LβT2 cells. This study was supported by the North Carolina State University Agricultural Research Service and National Institutes of Health Grant R01-HD-042459.

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Figure legends

Figure 1 - Diagram of the wild type oFSHBLuc promoter/reporter construct

The wild type oFSHBLuc expression plasmid is shown including -4741 bp of 5' promoter, TATA box (–31/-26 bp), exon 1 (1/63 bp), intron 1 (64/702 bp), part of exon 2 (703/765 bp) and firefly luciferase gene. Regions known to be important for ovine FSHB expression are marked: a putative Smad binding site (–163/-159 bp) (7, 8), Pbx1 binding site (–136/-131 bp) (8), Pitx1 binding site (–68/-63 bp) (9, 10).

Figure 2 - Deletion mutations between –4741/–195 bp of oFSHBLuc.

A series of deletions from -4741 bp to -195 bp produced shorter and shorter lengths of 5' ovine promoter. (A) Promoter length in each construct is plotted proportionately. Opposite each deletion is either the "fold-induction" or basal expression associated with the construct presented as the mean ± sem of quadruplicates in one complete and representative experiment. (B) The raw data used to create figure 2A are shown. These data were corrected for co-expression of Renilla, but Renilla expression varied ≤ 10 % across all samples. D-# designates the bp at the 5' terminus of the deletion.

Figure 3 - Replacement mutations between –4741/+701 bp of oFSHBLuc.

oFSHBLuc was divided into 9 sections to determine where activin-responsive sequences might be located. For replacement constructs 1-5, deleted regions are designated with a box

that indicates what sequences were substituted for the deletion. For replacement constructs 6-9, the section deleted and replaced is written above the promoter line. The replacement sequences for 6-9 are identified in Materials and Methods. Fold-induction was calculated as a ratio of luciferase expression ± activin corrected for Renilla co-expression. Basal expression was calculated and normalized to wild type basal expression (wt = 1). Bars represent the mean ± sem of results from quadruplicate cultures from a single but representative complete experiment. Means with different letters are statistically different from wild type induction (fold-induction). Means with different symbols are statistically different from wild type basal expression (relative basal expression). ANOVA and Student Newman-Keuls analyses were used to determine significance between means using $P \leq 0.05$.

Figure 4 – Activin induction of oFSHBLuc with substitute minimal promoters

(A) Induction of wild type oFSHBLuc by activin was compared to induction of oFSHB(TK)Luc with a substitute thymidine kinase promoter (TK) placed between -40/+3 bp of oFSHBLuc. Induction of the parent TK construct is also shown. (B) Induction of wild type oFSHBLuc by activin is compared to induction of oFSHB(rPRL)Luc with a substitute rat prolactin minimal promoter placed between -40/+3 bp of oFSHBLuc. Induction by these constructs was then compared to induction of the rat promoter construct lacking sequences known to be important for activin induction Δ-175/-39 oFSHB(rPRL)Luc. The next construct contained the minimal rat prolactin promoter plus ovine 3'exon/intron 1, but lacked

all 5' ovine sequence except the region between -200/-39 bp known to be important for activin action (Rep 10). Finally, activin induction was measured for the original rat prolactin construct. The data in Fig. 4 are plotted as fold-induction since basal expression with the TK and rPRL promoters was increased 20- and 8-fold, respectively, compared to basal expression with the wild type promoter. Bars represent the mean ± sem of results from quadruplicate cultures from a single and representative experiment. Means with different letters were statistically different from wild type induction (fold-induction). ANOVA and Tukey's multiple comparison analyses were used to determine significance where $P \leq 0.05$.

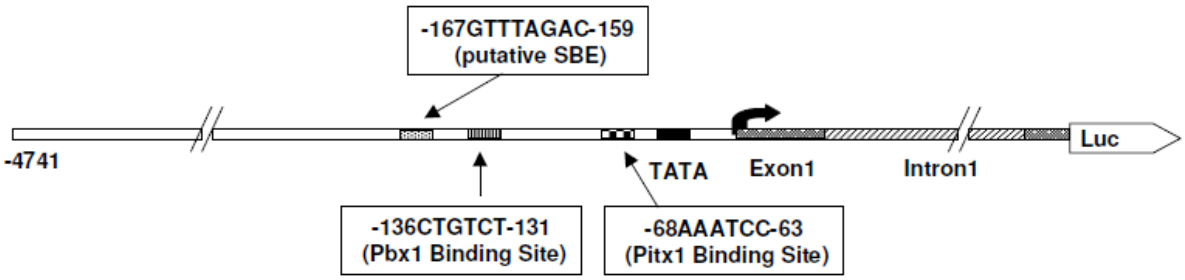


Figure 1. Diagram of the wild type oFSHBLuc promoter/reporter construct.

(A)

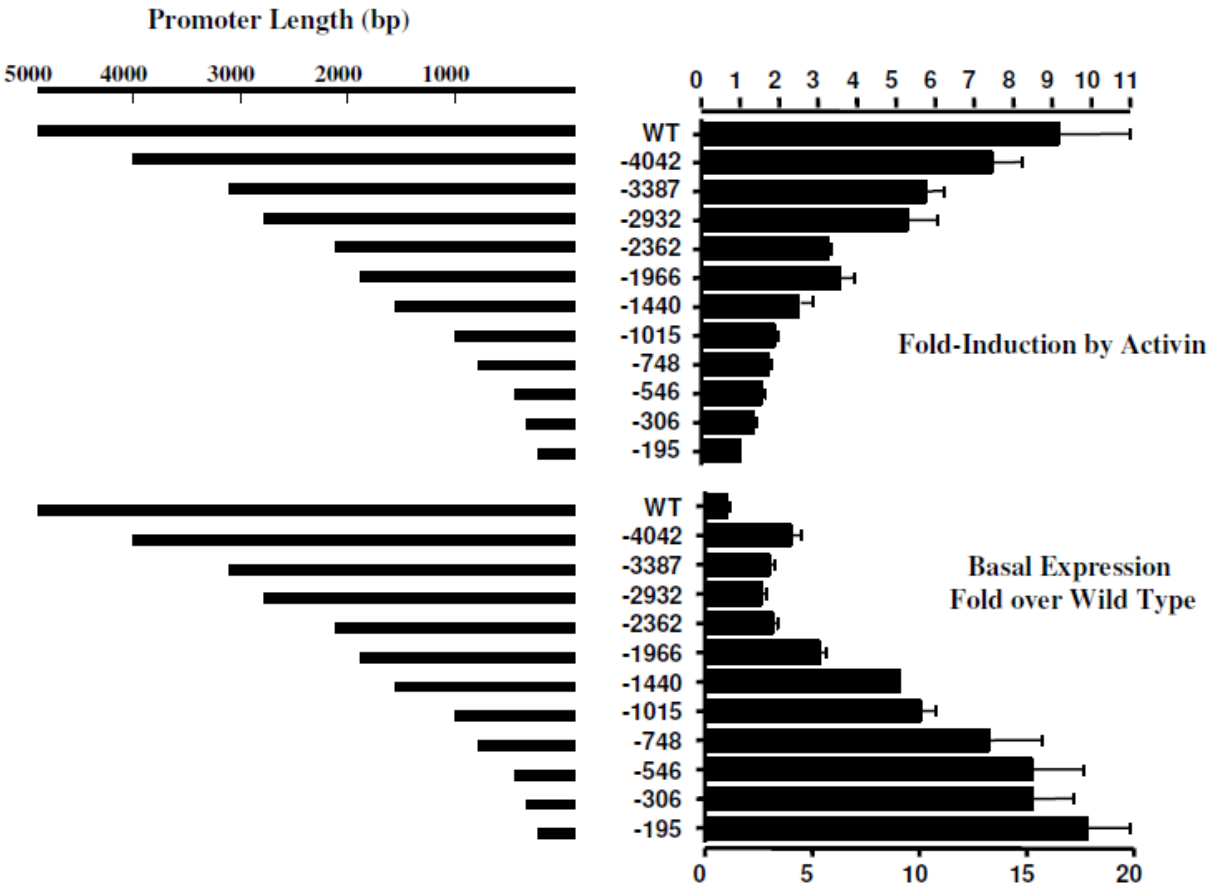
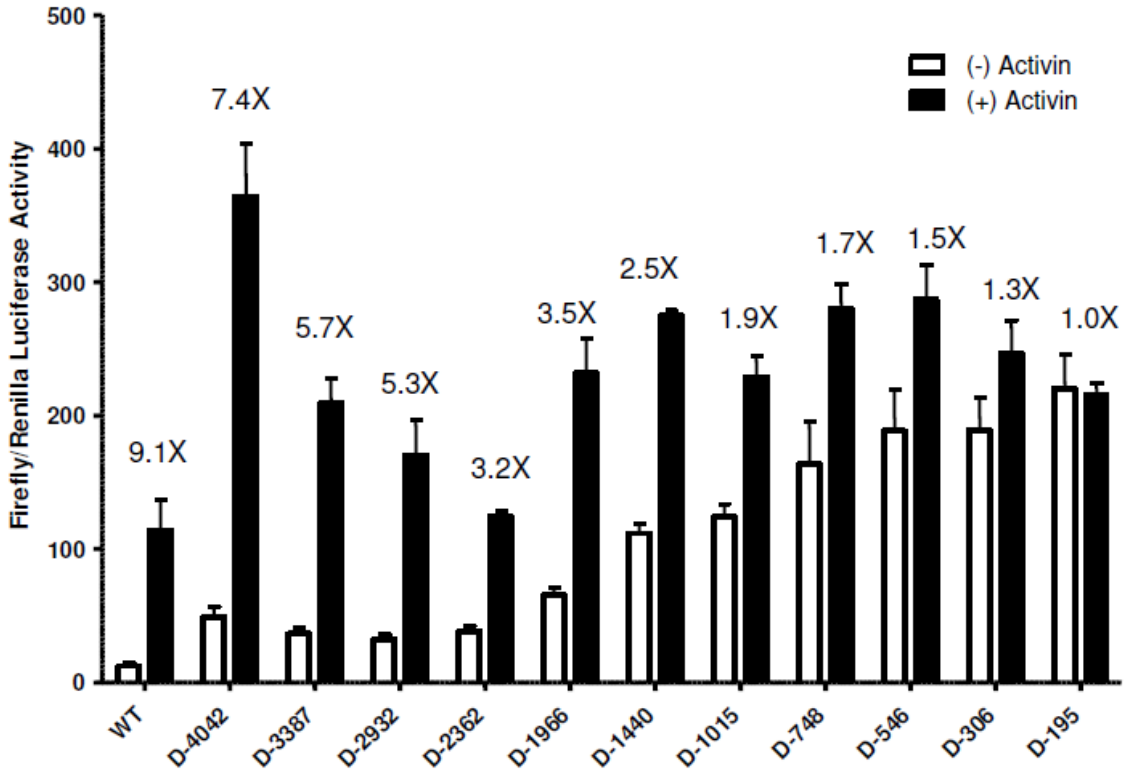


Figure 2. Deletion mutation between –4741/-195 bp of oFSHBLuc.

Figure 2 (Continued)

(B)



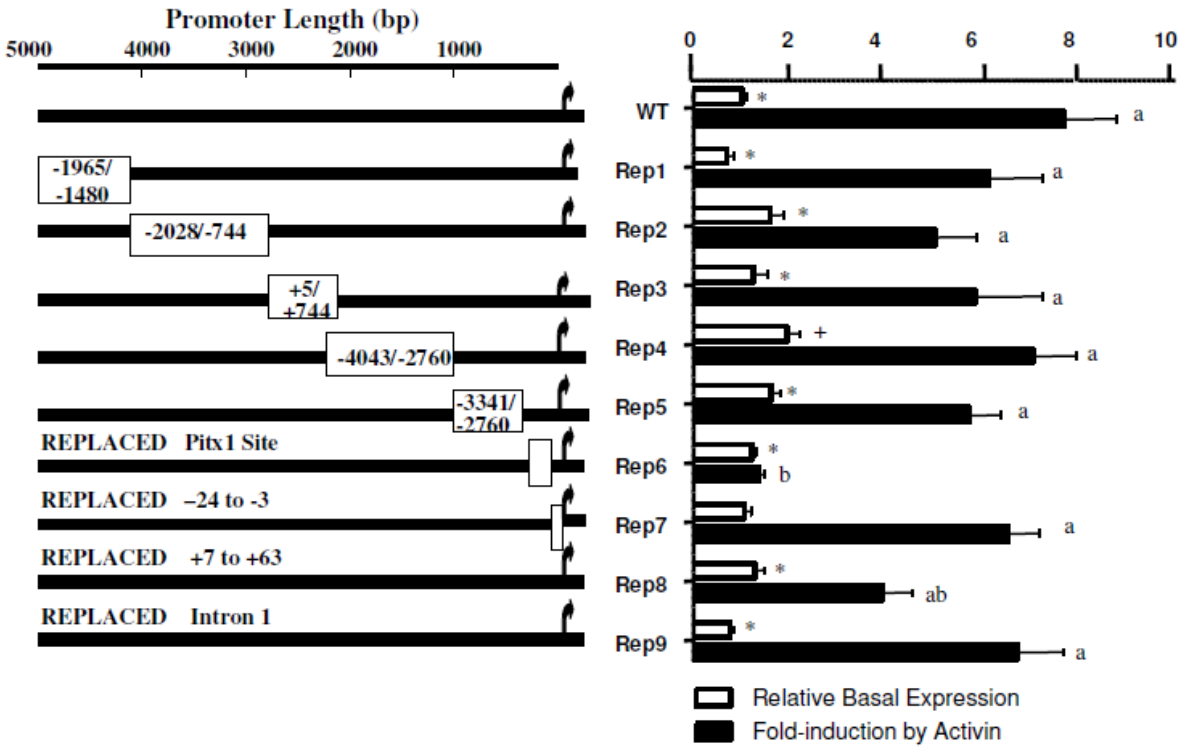


Figure 3. Replacement mutations between –4741/+701nbp of oFSHBLuc.

(A)

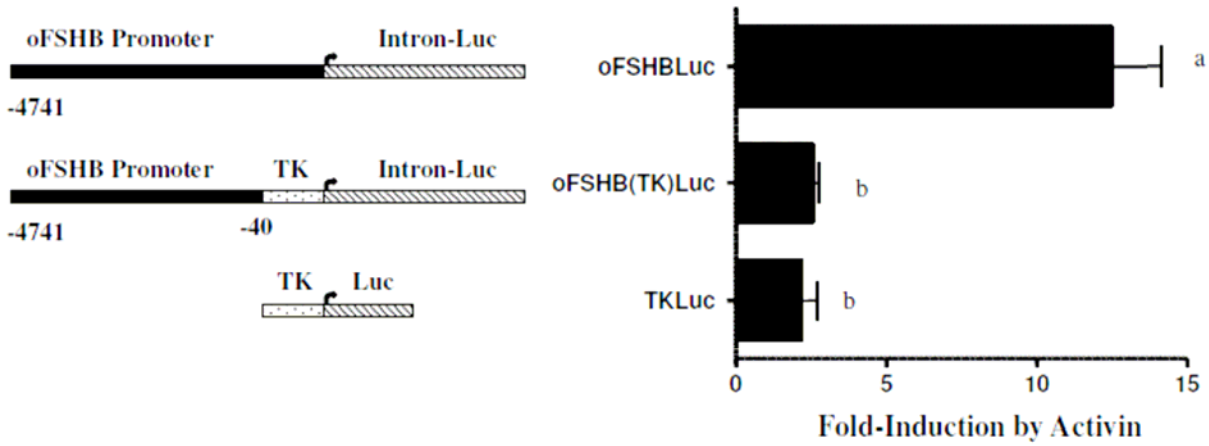
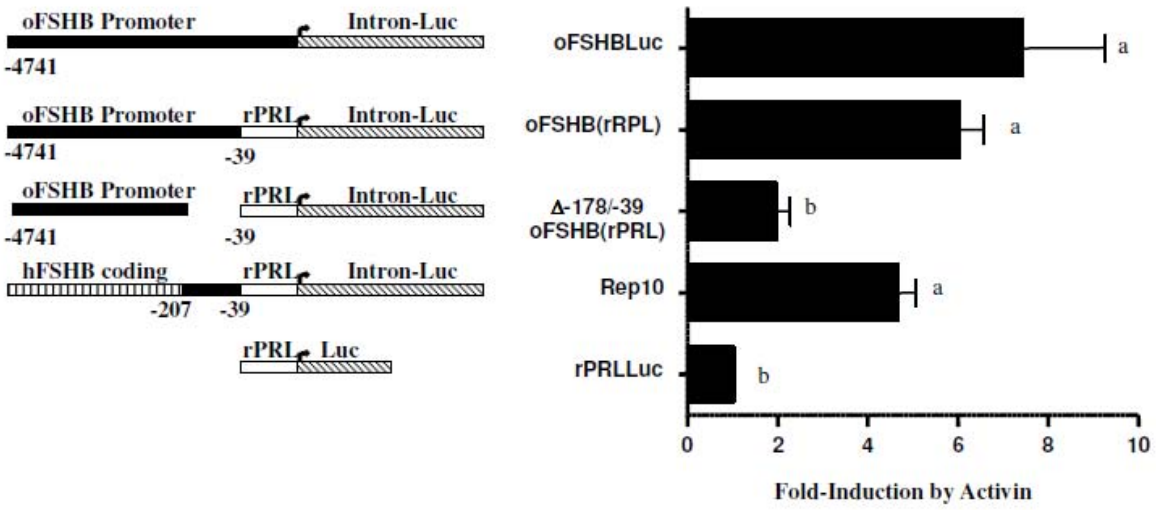


Figure 4. Activin induction of oFSHBLuc with substitute minimal promoters.

Figure 4 (continued)

(B)



CHAPTER II

A conserved Pitx1/2 binding site and adjacent region are important for expression of ovine follicle stimulating hormone beta-subunit *in vivo*.

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Key words: FSH, Pitx1, Runx1, Transgenic, tissue-specific

This work was supported by the North Carolina State University Agricultural Research Service and National Institutes of Health Grant HD 042459

This manuscript will be submitted in *Endocrinology*

ABSTRACT

Follicle stimulating hormone (FSH) stimulates egg and sperm maturation. Its beta subunit (FSHB) is the rate-limiting subunit for production of FSH, and is induced mainly by activin. We recently reported that a site juxtaposed to a putative Smad binding element at -169/-158 bp in the promoter of ovine FSHB (oFSHB) is necessary for activin induction in LBT2 cells and transgenic mice. In transgenic mice this site is required for ≥ 99 % of FSHB expression. The study reported here focuses on another highly conserved site (Pitx1) and adjacent nucleotides between -68/-58 bp in the oFSHB promoter. When oFSHB promoter-reporter constructs were tested in LBT2 cells, this region was as important as the -169/-158 bp region for activin induction, but also for basal expression. Transgenic mice expressing promoter-reporter constructs with the Pitx1 site destroyed by mutation showed 97-99 % less expression than the wild type construct. The response to activin, however, was unaltered in vivo. Adjacent to, and overlapping the Pitx1 site, is another forkhead/runx-like binding site. Mutation of this adjacent region did not significantly affect FSHB expression in vivo, but significantly degraded gonadotrope-specific expression. We conclude that the Pitx1 site between –68/-63 bp of the oFSHB promoter is required for 97-99 % of oFSHB expression *in vivo*, but is unlikely to participate in activin induction of oFSHB. In addition, the region adjacent to Pitx1 is involved in gonadotrope-specific FSHB expression.

Introduction

Follicle stimulating hormone (FSH) is produced in the gonadotropes of all vertebrate pituitaries and is required for egg maturation and optimal sperm performance (1-3). FSH is a α/β heterodimer, and its β-subunit (FSHB) is rate-limiting for overall FSH production. To fully understand the forces that control human fertility, it is important to identify the signaling pathways and factors that regulate FSHB expression.

Activin is the most potent and most probable inducer of FSHB transcription in vivo (4-6). One important element in the promoter of ovine FSHB (oFSHB), which appears in the human FSHB promoter as well, is the sequence between -169/-158 bp (7, 8). This site contains a putative Smad binding site (7) juxtaposed to a second site (8) that might bind a Smad partner, like forkhead or runx family member(s). Transgenic studies indicate this second site is required for induction of oFSHB by activin and ≥ 99 % of oFSHB expression in vivo. This site was originally located by functionally testing many 5' promoter mutants in LBT2 cells (8).

Further analysis of the ovine promoter led to a region between -68/-58 bp. This region is conserved in all mammals studied to date and part of the region has already been investigated as a Pitx1 site (-68/-63 bp) (9, 10). One study focused on this Pitx1 site as being important for robust induction of rat FSHB by GnRH (gonadotropin releasing hormone) (9). Most recently, Pitx1 was shown to interact with Smads and was found to be important for basal and activin-induced expression of murine FSHB (10).

In the studies reported here, the focus is also on Pitx1, but also on a companion site that partly overlaps Pitx1 extending the critical region for oFSHBLuc expression in LBT2 cells to -58 bp. This report shows the importance of this entire region (-68/-58 bp) for expression in LBT2 cells and also in transgenic mice.

Materials and Methods

Reagents

Recombinant human activin A and recombinant mouse follistatin 288 were purchased from R&D systems (Minneapolis, MN). FuGene^{TM}6 transfection reagent was purchased from Roche Applied Science (Indianapolis, IN). QuickChange® Site-Directed Mutagenesis kit was purchased from Agilent Technologies Co. (La Jolla, CA). Taq DNA polymerase, Yeast extract, Tryptone, Agar, Tris, and Boric acid were purchased from Fisher Scientifc Inc. (Pittsburgh, PA). Restriction enzymes including BglII, KpnI, AgeI, XhoI, T4 DNA ligase, Dual luciferase assay kit, Luciferase assay kit, and Passive lysis buffer were purchased from Promega (San Luis Obispo, CA). TaqMan Universal PCR mixer for real-time PCR was purchased from Applied Biosystems (Foster City, CA). Quanti-iT^{M} Protein assay kit, Dulbecco's modified eagle medium (DMEM), Fetal bovine serum, and Hanks' Balanced Salt Solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). Steptomycin, collagenase, and Pancreatin as well as all primers for point mutations in this study were purchased from or synthesized by Sigma-Aldrich Co. (St. Louis, MO). Mutant plasmids and PCR products from transgenic mice were sequenced by SeqWright Technology (Houston, TX).

Immortalized murine gonadotrope LβT2 cells were provided by Dr. Pamela Mellon (University of California, San Diego, CA).

Plasmid constructs

The wild type ovine FSHB promoter/reporter plasmid, oFSHBLuc, was described previously (11, 12). Briefly, oFSHBLuc contains 4741 bp of 5' ovine FSHB promoter plus intron 1 driving expression of a luciferase gene in the pGL3 basic vector. Some plasmids for this study were constructed using simple point mutations and others were mutated by replacing specific promoter regions with synthetic sequences after creating restriction sites of AgeI at –90 bp and BglII at –39 bp of oFSHBLuc. Mutant 1A was constructed by replacing sequences between –90/–39 bp with synthesized sequences where A/C and T/G inter-conversions were made. Mutants 6, 7, 8 and 9 were prepared using direct point mutations at specific sites. Mutants 2-5, 1B and 1C were made by replacing sequences with synthesized oligonucleotides inserted between the AgeI site at –90 and BglII site at –39 (see 1A). M10 was constructed the same way as M 2-5, but the sequences were selected from the human FSHB promoter between -82/-37 bp. All mutations were shown to be correct by sequencing.

Transgenic mice

Transgenic mice were maintained and studied with the approval and oversight of the Institutional Animal Care and Use Committee at the North Carolina State University or the University of North Carolina at Chapel Hill.

Wild type (WT) and 3 different mutants (M7, M8, and M9) were chosen to be used as transgenes. For the generation of transgenic mice, fusion genes consisting of the oFSHB promoter plus luciferase expression gene were cut from –4741 bp to +2738 bp of oFSHBLuc with KpnI and BamHI, and mice containing these transgenes were produced in B6SJL mice as described earlier (8, 12). Transgenic mice were produced at the transgenic mouse facility of the University of North Carolina at Chapel Hill, NC and then bred and cared for at the Biological Resource Facility of North Carolina State University. Testing mice for the presence of a transgene was performed as noted below and measuring luciferase activity in tissues was performed as previously reported (8).

Real-time PCR and PCR

Identification of transgenes was performed using real-time PCR (RT-PCR) and DNA extracted from mouse tails with primers to the luciferase gene: the forward primer = 5'-TGGGCTCACTGAGACTACATCA-3' and reverse primer = 5'-CGCGCCCGGTTTATCATC-3'. To confirm the presence of 5'-oFSHB sequences in transgenic mice, primers were selected for the forward primer between –4644/-4615 bp, 5'-CCTCCATCATCCCCTTCTCCTCCTGCCTTC-3', and reverse primer between –2531/-2504 bp, 5'-TCTCTCCTAACGTCAAGCCTCTTAGAGC-3'. For confirming the presence of 3' sequences of the oFSHB transgene and also producing DNA for sequencing mutated sequences, forward primer was selected at –1740/-1713bp, 5'-CAAAGGAACTTCTCAAGCCAAGGGTGTC-3', and reverse primer +831/+857 bp, 5'-

CGCCGGGCCTTTCTTTATGTTTTTGGCG-3', of the oFSHB promoter. The regions internal to these primers included 1740 bp of the 5'-region, 1st exon, 1st intron, partial 2end exon of oFSHB plus 38 bp of the luciferase coding sequence. The selected regions were amplified by PCR and sequenced to determine the correctness of mutations between –73/-53 bp.

LβT2 cell culture

Immortalized murine gonadotropes (LβT2 cells) were maintained in complete DMEM containing 10% (v/v) fetal bovine serum plus 100 μg/ml streptomycin and 100 U/ml of penicillin at 37°C under 5 % CO_2: 95 % air. For experiments, cells were plated at a density of 30,000 cells with 50 μl of complete media per well in 96-well tissue culture plates. Cells were transfected 24hr after plating in quadruplicate by adding 50 μl of serum-free media containing 50 ng plasmid plus 0.15 μl Fugene6. Cells were also co-transfected with 5 ng of Renilla luciferase expression vector (pRL-TK), as an internal control since its production of Renilla luciferase was not altered by activin. After 24 h of transfection, media were removed and cells were treated with or without 25ng/ml activin for 22 h prior to harvesting with passive lysis buffer. Firefly and Renilla luciferase were quantified sequentially using the Dual Luciferase assay kit following the manufacture's directions in an automated 1420 Victor-Light micro plate luminometer (PerkinElmer, Waltham, MA)

Tissue specific-expression of transgenic mice

Transgenic mice (2-4 months old) were sacrificed, and their pituitary, brain, heart, lung, liver, spleen, kidney and gonads were taken within 5 minutes of death, frozen on dry ice in 0.5 ml tubes and stored at -80 C. Just prior to assay, samples were homogenized in 100 µl of passive lysis buffer, and centrifuged for 10 minutes at 12,000 rpm in a microfuge at 4 C. All samples were assayed within 30 minutes of thawing. Luciferase activity was normalized for protein measured with a Qubet^{TM} Fluorometer (Invitrogen, Carlsbad, CA).

Primary cell Culture

Primary pituitary cells from transgenic mice were prepared as described previously (8, 12). Briefly, transgenic mice (2-4 months old) were sacrificed and pituitaries were dissociated using collagenase for 2hrs at 36°C, followed by Pancreatin for an additional 15 minutes. Cells (50,000) were placed in each well, and incubated for 48 h at 37 C under 95 % air/5 % CO_2 in a humidified incubator. Cells were treated with activin (50 ng/ml) or follistatin (50 ng/ml). After 24 h, the cells were harvested with passive lysis buffer and luciferase activity was measured as with LBT2 cells described above.

Results

The importance of Pitx1 and downstream 5 bp for expression of oFSHBLuc in LβT2 cells

An 11 bp sequence from -68/-58 bp comprises a Pitx1 binding site which overlaps a site that looks as if it might bind a Runx1 transcription factor (Figure 1). Mutation of all nucleotides in this region (mutant 1A) decreased basal expression by 55 % and eliminated activin induction entirely. Mutants 1B, 1C, 2, 3 and 5 all focused on the Pitx1 site. All were equally effective except for mutant 3 which showed activin induction of ~ 200 % whereas the others showed no significant induction. Interestingly, mutant 2 had just one nucleotide mutated, but its basal expression was decreased by 80 % and induction by activin was zero. Mutants 6-8 stretched across the Pitx1 site into the downstream 5 bp associated with a putative Runx1 site. Mutations 6 and 7 showed the lowest basal expression (denoted by the letter "c") while mutation 8 had low basal expression but was induced ~ 100 % by activin. Mutation 9 contained 3 mutations totally outside of the Pitx1 region at the far end of the putative Runx1 region; it had reduced basal expression and was induced by activin only ~ 100 %. Based on LBT2 cell analysis, mutations at any site in this 11 bp region depressed basal expression plus inhibited activin induction of oFSHBLuc.

Expression of WT and mutant oFSHBLuc as transgenes

Mutant 7 contained 4 mutations in the Pitx1 site and 1 downstream of this site. Its expression was reduced by 97 % (females) to 99 % (males) compared to WT expression and

that of the other two mutants. Mutants 8 and 9 showed no difference in pituitary expression in transgenic mice even though the LBT2 data predicted they would be inhibited significantly.

Mutant 7 showed essentially the same level of luciferase expression as WT oFSHBLuc in all tissues except brain where expression was elevated about 4-fold (Table 1). Mutants 8 and 9, however, showed progressively less gonadotrope-specific expression. Expression in the forebrain was 50x higher in the males of mutant 9 compared to WT expression in the brain. Mutants 8 and 9 also were expressed 9x and 18x higher in the testes compared to WT testicular expression. Nevertheless, expression in the mutants was still quite specific for the pituitary (Table 1).

Mutations in the -68/-58bp region did not alter activin induction of any transgenes

Activin maximizes oFSHBLuc expression in mouse pituitary cultures whereas follistatin depresses it to its lowest level (8, 12). Therefore, the comparison between activin and follistatin treatments indicates how much activin can induce oFSHBLuc expression. The data in Figure 2 indicate that WT and all transgenic expression is responsive to activin. In fact, it appears that Mutant 7 which as inhibited the most is actually the most responsive to activin. This is the same mutant that showed absolutely no induction by activin in LBT2 cells (Figure 1).

Discussion.

The Pitx1 site at -68/-63 in the ovine FSHB promoter has been studied before in the rat (9) and mouse (10). In both cases, the Pitx1 site was assumed to be the key, important site so regions upstream or downstream were not investigated. This study shows that nucleotides 5 bp downstream of the Pitx1 site are also important for expression and activin induction, at least, in LBT2 cells which used routinely for understanding FSHB regulation *in vitro*.

Studies by the Bernard laboratory (10) recently showed that Pitx1 can bind to the analogous Pitx1 site and that two nucleotide changes anywhere in this region disrupts binding as detected by electrophoretic mobility shift assays (EMSA) (10). Attempts in our laboratory to bind Runx1 protein (abundant in nuclear extracts from LBT2 cells) to the -68/-58 region were not successful although a consensus Runx1 site bound nicely to runx1 under identical conditions (data not shown). Therefore, Runx1 is unlikely to be the protein partner for Pitx1, but the protein that binds here might be highly related.

The conserved Pix1 site has been studied in the rat and mouse, but other parts of the rodent promoter differ significantly from the ovine (and human) FSHB promoters. Evidence strongly suggests that the important activin-inducible site in sheep resides between -167/-158 bp (12). This site is not conserved in rodents, but rodents have another site that seems significant for them. This site is a palindromic Smad binding element at –269/-263 bp which can be shown to bind Smads (10). No such success has been obtained with the ovine/human

sites so there is the possibility that the Pitx1 site functions somewhat differently in rodents compared to sheep. It seems significant that replacement of the not fully conserved human promoter sequence between -68/-58 bp site did not alter activin induction although it did lower basal expression by half. These data suggest that the human FSHB promoter may work much like the ovine FSHB promoter.

Dr.Bernard laboratory postulated that Pitx1 may work together with Smads binding at the -26/-263 site to induce FSHB expression in the mouse (10). This is an attractive hypothesis, but may not apply to the sheep which has a different Smad-related activin sensitive site. The data presented here show that activin induction is quite robust in mutant transgenes that lack the Pitx1 site. These results indicate that the Pitx1 site is not necessary for activin induction of the ovine FSHB gene.

Finally, it is important to know how FSHB expression is directed so specifically to gonadotropes. The wild type transgene is expressed 100-1,000x more in the pituitary than the next most active tissue (brain) and about 5,000-10,000 more than any other tissue tested. Considering that gonadotropes comprise only 3-5 % of the mouse pituitary and that wild-type oFSHBLuc is expressed only in gonadotropes (2), the specificity of expression is actually 20- to 30-fold higher than noted above. This specificity is very high, and oFSHBLuc contains all the sequences required for this specificity.

It should be noted that a nearly complete mutation of the Pitx1 site decreased oFSHBLuc expression by 97-99 % when it was tested as a transgene, but it did not raise the level of expression in any other tissue except the brain where expression increased only by a factor of 4 compared to the WT construct. Mutant 7 showed even more expression in the brain and Mutant 8 showed expression in the brain that was about 10 % of that in the pituitary. In the testis, expression in Mutants 7 and 8 was > 36-fold and >114-fold higher than in WT oFSHBLuc, respectively. These changes in specificity of expression indicate that sequences between -63 and -58 bp have some influence over gonadotrope-specific expression of ovine FSHB.

It is interesting to compare transgenic and LBT2 results. Studies with LBT2 cells indicated that each transgenic construct should have been expressed very poorly in mice and not induced by activin, but this did not occur. One construct was expressed very poorly, however, and the others showed another kind of malfunction although mutant 7 was expressed almost as well as the WT transgene. These data suggest that LBT2 cells are useful for analyzing FSHB promoter function, but may yield false positives. Another interpretation, however, could be that LBT2 cells are highly sensitive indicators of important promoter sites and that it might take larger mutations to obtain the same results in transgenes. Perhaps the transgene in chromatin form is more forgiving of small mutations than LBT2 cells.

IN SUMMARY, the data presented here show that the Pitx1 site at -68/-63 is required for 97-99 % of ovine FSHB expression in vivo. Furthermore, it shows that sequences 5 bps downstream of the Pitx1 site are required for high level of gonadotrope-specific expression. Finally, the data fail to show any effect of the Pitx1 site on activin induction for the ovine FSHB constructs used in this study.

Acknowledgements

The authors thank Dr. Pamela Mellon (University of California) for her gift of LβT2 cells. This work was supported by North Carolina State University Agricultural Research Service and National Institutes of Health Grant R01-HD-042459.

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Table

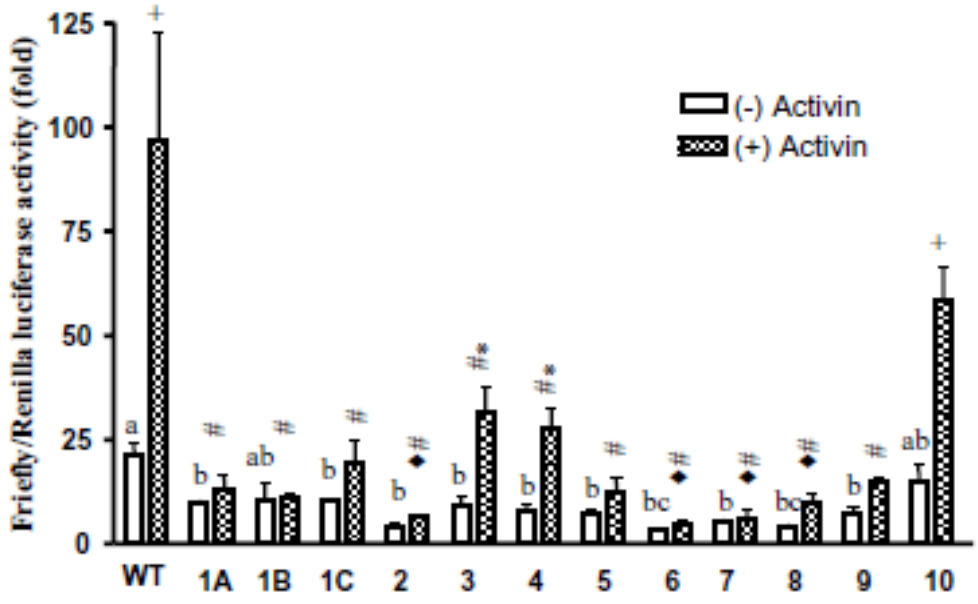
Table-1- Expression of WT and -68/-58 bp mutants of oFSHBLuc transgenes in mouse tissues. Tissues were harvested within 3 minutes of death from mice 2-4 months old, and assayed for luciferase activity. Values represent relative light units of firefly luciferase activity (RLU) times 10^{-5}/mg protein. All values represent the mean ± sem of results from at least five mice.

Figure legends

Figure 1 - Expression of WT and -68/-58 bp mutants of oFSHBLuc in LBT2 cells ± activin. The boundaries and overlap of putative Runx1 and Pitx1 binding sites are shown. Expression of firefly luciferase was normalized to Renilla activity; Renilla activity did not vary more than ± 10 %. Bars represent the mean ± sem of results from quadruplicate cultures from a single but representative complete experiment. Means with identical lower case letters are not significantly different from each other for basal expression ($P \leq 0.05$). Means with different symbols are significantly different from each other at $P \leq 0.05$. ANOVA and Tukey's multiple comparison analyses were used to determine significant differences for basal and activin-induced expression. Fold-induction of WT or mutant 10 (human sequence between -68/-58) was equal ~ 430 %. Activin induction of mutants 3 and 4 was ~ 200 % and

induction for mutants 1C, 8 and 9 was ~ 100 %. Induction for the other constructs was zero (1A, 1B, 2, 5, 6 and 7).

Figure 2 - Activin-induction of WT and mutant oFSHBLuc transgenes in mouse pituitary culture. The differential expression between treatments with follistatin (FS) or activin (Act) represents activin induction. Mouse pituitary cells were incubated 48 h after dispersion, treated with 25 ng/ml activin or 125 ng/ml follistatin for 24h and then assayed for luciferase activity. The bars represent the mean ± sem of results from triplicate cultures. Means with different letters are significantly different. A one-tailed unpaired t-test with Welch's correction was used to determine significance between means using $P \leq 0.05$.



WT	-73CC	AAC	AAA	TCC	ACA	AGG	TTT	A-53
1A	-73**AA**	**CCA**	**CCC**	**GGA**	**CAC**	**CTT**	**GGG**	**C**-53
1B	-73**AA**	**CCA**	**CC** A	TCC	ACA	AGG	TTT	A-53
1C	-73CC	AA**A**	**CCA**	TCC	ACA	AGG	TTT	A-53
2	-73CC	AAC	AA**C**	TCC	ACA	AGG	TTT	A-53
3	-73CC	AAC	AAA	**G**CC	ACA	AGG	TTT	A-53
4	-73CC	AAC	AAA	**A**CC	ACA	**AA**G	TTT	A-53
5	-73CC	AAC	AAA	**TAC**	ACA	AGG	TTT	A-53
6	-73CC	AAC	AAA	**GGT**	AC**C**	AGG	TTT	A-53
7	-73CC	AAC	AA**C**	**GGA**	AC**C**	AGG	TTT	A-53
8	-73CC	AAC	AAA	TC**T**	**AGA**	AGG	TTT	A-53
9	-73CC	AAC	AAA	TCC	AC**G**	**CGT**	TTT	A-53
10	-69CC	AAC	AAA	TCC	ACA	AGG	**TGT**	**T** -49

Figure 1. Expression of WT and –68/-58 bp mutants of oFSHBLuc in LBT2 cells ± activin.

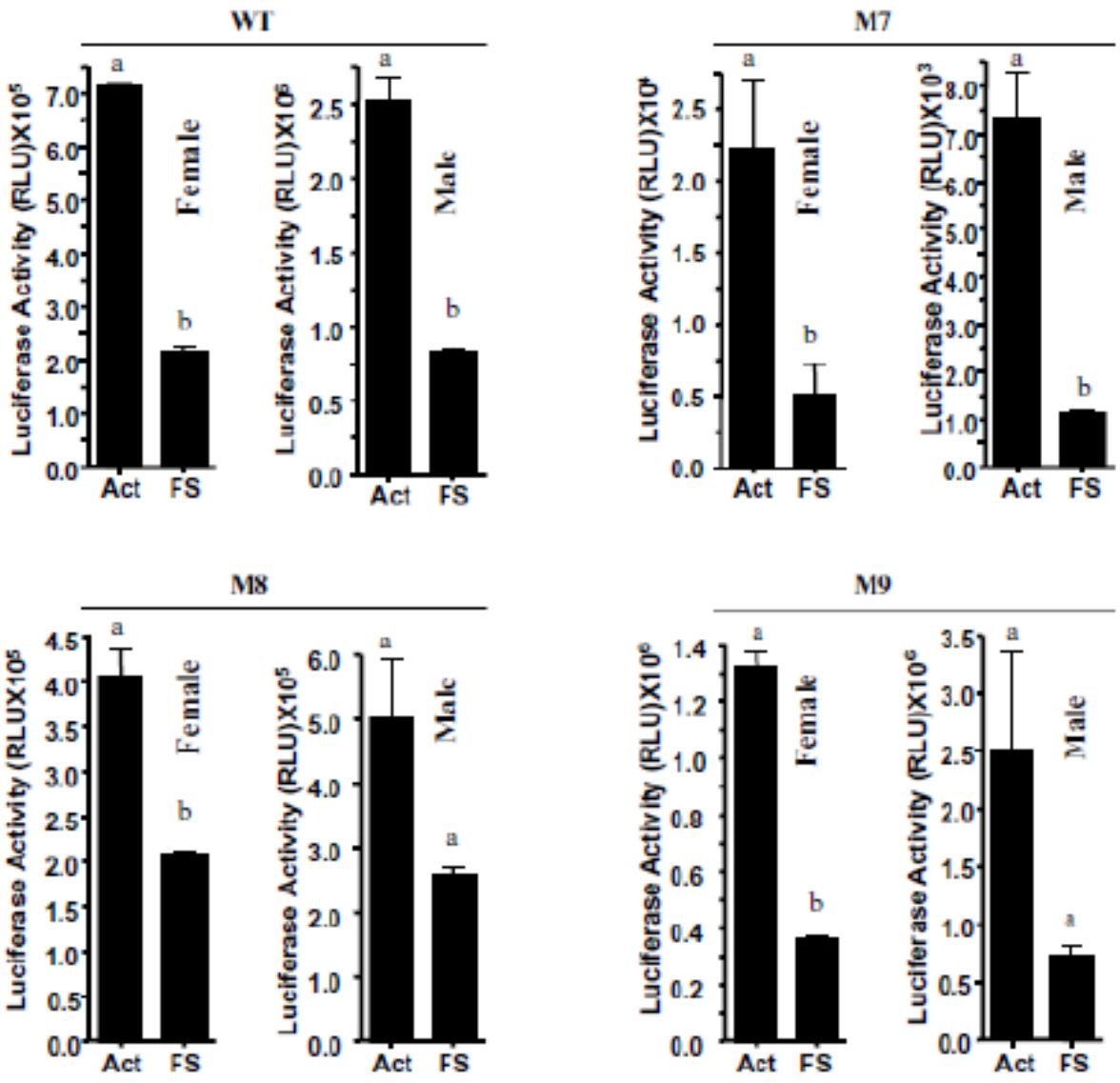


Figure 2. Activin induction of WT and mutant oFSHBLuc transgenes in mouse pituitary culture.

Table 1. Expression of WT and –68/-58 bp mutants of oFSHBLuc transgene in mouse tissues

RLUX10^{-5}/mg protein

WT or Mutants	Sex	Pituitary	Brain	Lung	Heart	Liver	Spleen	Kidney	Gonad
WT	F	348±153	4±2	<0.1	<0.1	<0.1	<0.5	<0.05	<0.5
	M	5485±1896	2±1	<0.5	<0.5	<0.05	<0.5	<0.1	<0.5
M7	F	26±16	16±2	<0.1	<0.1	<0.05	<0.05	<0.05	<0.1
	M	47±20	13±4	<0.1	<0.1	<0.05	<0.05	<0.05	<0.5
M8	F	500±81	132±5	<0.5	<0.5	<0.5	<0.5	<0.1	5±2
	M	2761±600	62±21	<0.5	<0.5	<0.5	<0.5	<0.05	18±5
M9	F	872±326	73±42	1±0	4±2	1±0	2±1	2±1	5±2
	M	729±139	99±40	1±0	3±2	1±0	1±0	2±0	57±12