International Journal of Cell, Animal Biology and Genetics
Vol.1, No.1, pp.9-17, February 2016
Published by European Centre for Research Training and Development UK (www.eajournals.org)

KISS-1 GENE VACCINE IMMUNOCASTRATION OUTCOMES ON GROWTH HORMONE, AND KISS-1 MRNA AND GPR54 MRNA OF RAM LAMBS DIGESTIVE ORGANS.

Birhanu Tesema[1], Yanguo Han[1], Guiqiong Liu[2], Xunping Jiang[2], Jia-yu Zhao, Ijaz Nabeel
[1]Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, College of Animal Science and Technology,
[2]Huazhong Agricultural University, Wuhan 430070, People's Republic of China.

ABSTRACT: *This study was designed to examine the expression of KISS1 gene vaccine immunocastration on growth hormone (GH), insulin like growth factor1 (IGF 1), KISS1 mRNA, GPR54 mRNA and digestive organ. A total of six male Hu lambs of 8 weeks were used for this research and divided into treatment and control group, based on their initial body weight and scrotal circumference. All experimental animals were subjected to the final data collection and analysis. KISS1 gene vaccine pKS-asd was provided for treatment and naked pVAX-asd was for control. The prepared vaccine (1mg/ram lamb) in saline solution was injected into ram lambs at weeks 0, 3 and 6 of experiment. Effects of KISS1 gene vaccine immunocastration on growth hormone, IGF1, KISS1 mRNA and GPR54 mRNA of digestive organ were evaluated. Accordingly, vaccinated animals did not show significant difference compared to controlled group in growth hormone, IGF1 and body growth. However, KISS1 mRNA of pancreas and tongue, and GPR54 mRNA of pancreas in vaccinated animals were significantly lower than control group ($p < 0.05$). Therefore, KISS1 gene vaccine immunocastration has impacts on KISS1 mRNA and GPR54 mRNA of digestive organ, but no changes on growth hormone and IGF1of ram lambs.*

KEYWORDS: KISS1gene, KISS1 Gene Vaccine, KISS1 mRNA, Digestive Organs

INTRODUCTION

Kisspeptin is a group of arginine-phenylalanine (Christine Margaret, 1999), which has been known as potential novel agent for treating reproductive disorders. But its integration with GPR54 signaling system is essential for normal estrus cycle. Neurons of KISS1 have straight targets for the action of sex steroids production through HPG axis. Hypothalamic neuronal inputs are communicated to the GnRH neurons, and do not appear to express many necessary receptors which required for the initiation of the appropriate responses to hormone. The function and concentration of GnRH in the long-term control of hypothalamic-pituitary-axis has been studied and used to express the effects of hormones involved in the regulation of the axis. From the hypothalamus, GnRH travels through the hypophyseal portal blood system to the anterior pituitary release of FSH and LH which are involved in the steroid hormone synthesis. However, the functions of KISS-1 and GPR-54 are not confined to hypothalamic neurones, since both are expressed in various peripheral tissues including pancreas, kidney, placenta, testis and etc (Ohtaki et al, 2001). Recently the site of pancreatic kisspeptin and GPR-54 content were identified as being in the endocrine pancreas, consistent with a peripheral role in whole body fuel homeostasis and stimulates the release of insulin from isolated mouse and human islets. The importance of pancreatic beta cells as regulators of fuel homeostasis,

suggests a potential physiological role for kisspeptin in regulating islet function. The present study was carried out to investigate KISS1 gene vaccine immunocastration outcomes on growth hormone, IGF-1, KISS-1 mRNA, GPR54 mRNA and digestive organ of ram lambs.

MATERIALS AND METHODS

Vaccine Preparation and Vaccination

Sequence of human KISS1 gene encoding the 54-amino acid peptide (corresponding to a a 68-121 of KISS1; GenBankTMaccession number NM_002256) was synthesized chemically by Sangon Biotechnology Co.Ltd (Shanghai, China). pcMV-S vector encoding HBsAg-S gene and pVAX-asd vector without antibiotic resistance gene was provided by Dr. Aixin Liang in Huazhong Agricultural University, x6097 strain (Asd-) was by Professor Ai-zhen Guo in Huazhong Agricultural University (Han et al., 2015). A total of 6 healthy male Hu lambs from Hengtai Sheep Breeding Co., Ltd of Hubei, Huanggang, China, were used at the age of 8 weeks according to the National Institutes of Health Guides for the Care and Use of Laboratory Animals (Hubei district, China 2009). All samples in vaccinated and control ram lambs were came from our previous study (Han et al., 2015), where blood samples collected at different weeks (2, 4, 6, 10 and 14 weeks) and digestive organ tissues were collected in week 30 after primary immunization.

Detection of Hormone Concentrations

From pre and post immunization (weeks 2, 4, 6, 10 and 14) sample was collected and ELISA was used for growth hormone (GH) and insulin-like growth factor 1(IGF-1) analysis in accordance with the kit instruction.

Expression of fluorescence quantitative PCR KISS1 mRNA and GPR54 mRNA in digestive organs

After sheep slaughtered, tissues of digestive organs were collected for detecting the expression of KISS1mRNA and GPR54 mRNA by fluorescence quantitative PCR. Tissue RNA extracted using Trizol method and added to the right amount of liquid N2 into a mortar to grind a powder form. 1ml trizol and polished tissue RNA samples were added to the enzyme free (1.5ml) microcentrifuge tube at room temperature. Then, synthesis of cDNA first strand was conducted in accordance with ferment as reverse transcription kit instructions and KISS1/GPR54 cDNA sequence selection of β- actin as internal control and primers were synthesized (Shanghai Sangon Biological Engineering Technology Co., Ltd). The relative expression was analyzed using 2-ΔΔCt method and average fluorescence quantitative β-actin calculates the amount of each of the wells gene expression at the mRNA level.

Statistical Analysis

The results between two groups were analyzed by unpaired Student's t-test using the SAS 8.1 (SAS Institute, Inc., Cary, NC, USA) and data were expressed as mean ± SD which $p < 0.05$ was considered statistically significant.

RESULTS

KISS-1 gene vaccine immunization on growth hormone and growth

Growth hormone production in ram lambs was demonstrated in the following figure 1. In view of that, growth hormone production on the first immunization week was greater than the rest immunization weeks in both treatment and control groups of experimental animal. The production of hormone was low at fourth immunization weeks and tenth; but statistically there was no significant difference between treatment and control group. Even though there is difference in figure between control and immunized animal at 6th and 14th weeks as well, statistically insignificant difference on the result. The secretion GH is closely from hypothalamic with neurosecretory release and mainly influenced with the production of IGF-1. The intra-muscular injection of KISS-1 gene which inhibits the production of GnRH through GnRH neurons has no linkage with the GH neurosecretory and IGF-1.

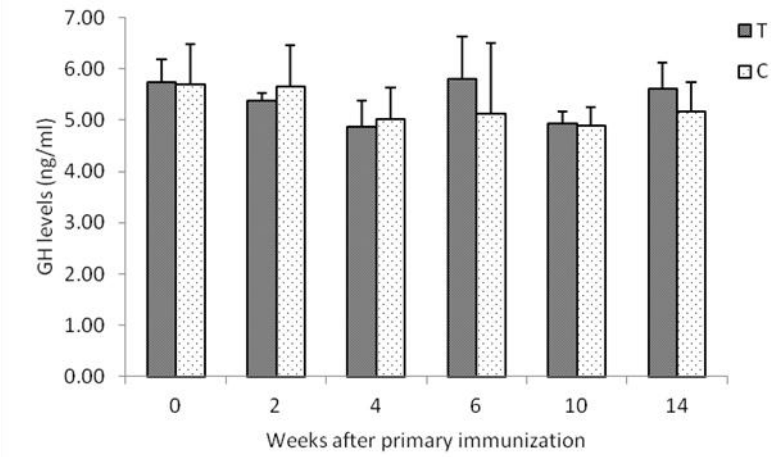

Figure-1 Indicates the relation between KISS1 gene vaccine immunocastration/week and GH in ng/ml.

The result of body weight change in relation to weeks after primary immunization of experimental animals was demonstrated on figure 2, where growth performance criteria were similar in both pKS-asd received and naked pVX-asd groups' sheep on each immunization weeks and shows linear body weight change up to last immunization period. Body weight changes between treatments group (T) and control group (C) animal is might be due to feed and feed conversion efficiency of animal and the constant production level of GH which matter on steroid hormone and metabolism. Besides, the expression of KISS 1 gene vaccine immunocastration through hypothalamus pituitary-axis has no effects on body weight of sheep, and might be also on feeding and feed conversion efficiency of animal.

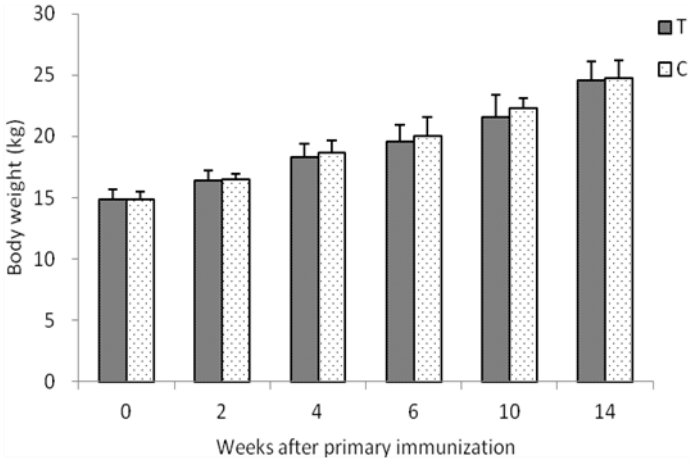

Figure 2. KISS-1gene vaccine immunization/weeks and body weight/kg change.

KISS-1 gene vaccine immunocastration on KISS1 mRNA and GPR54 mRNA of digestive organ

The result of KISS1 gene vaccine immunocastration expression of beta actin on different digestive organ have been demonstrated on figure 3 A and B, which revealed that, the manifestation of KISS1 mRNA in pancreas and tongue of vaccinated was significantly lower than that of control, and the expression of GPR54 mRNA in pancreas of vaccinated ram was significantly lower than that of control, whereas insignificant in the remaining of digestive organs. This result indicated that KISS1gene vaccine immunocastration can affect the exertion of KISS 1 mRNA and GPR54 mRNA in pancreas of ram lambs.

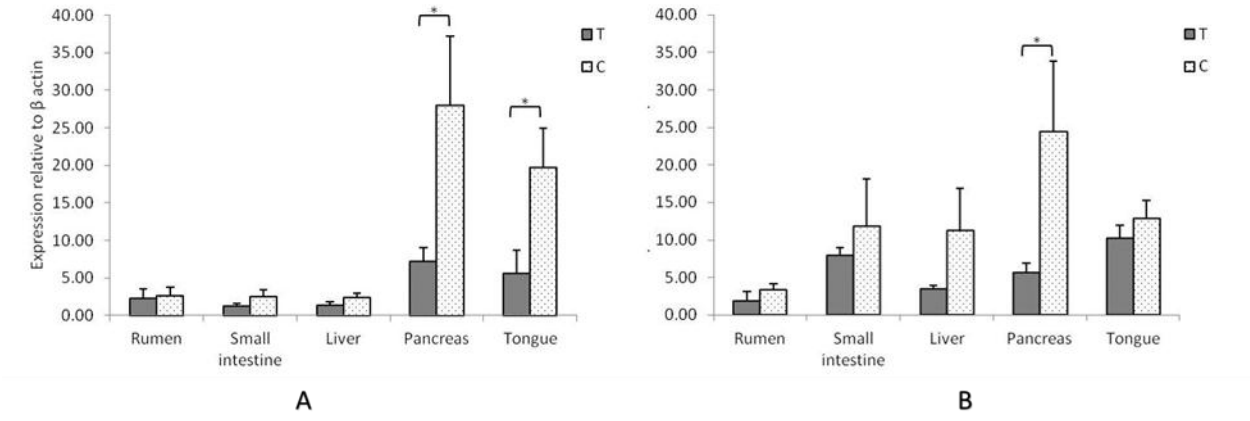

Figure 3. A shows relative expression of beta actin KISS1 mRNA and B shows GPR54 mRNA in digestive organ.

Figure 4A notices digestive tissues of KISS1 RT-PCR analysis and figure 4B denotes GPR-54 RT-PCR. We used design of KISS1 gene (57bp) and GPR54 (71bp) digestive tissues RT-PCR analysis which was expressed specific primer pairs for rams rumen, small intestine, liver, pancreas and tongue tissues. RT-PCR analysis was done by a 3% agarose gel electrophoresis and detects positive of GPR54 mRNA specific primer pairs for rams rumen, small intestine, liver, pancreas and tongue tissues whereas, RT-PCR analysis for KISS1 mRNA showed positive expression in the rumen, small intestine, liver and tongue tissue.

DISCUSSION

Growth hormone is the key hormone acts either directly or indirectly via secretion of insulin-like growth factor (IGF-1) from the liver or target tissues itself. Its major role is to stimulate body growthand other tissues to secrete IGF-1. Growth hormone is secreted by anterior pituitary, and single most important hormone for postnatal growth. In some species like: rate (Murthy C.K, et al., 1994), goldfish (Andries M, et al., 1995) and carp (Murthy C. K, et al., 1994; Li W.S, et al., 2002) GnRH appears to be the primary regulator of GH secretion which is closely associated with hypothalamic, but not somatostatin (Frohman et al, 1990). However, in higher vertebrate gonadotrophic releasing hormone (GnRH) which plays a pivotal role in reproduction control and main path for HPG a xis doesn't stimulate GH. In the current research finding, the effect of GH is the same betwee n control and treatment group throughout the research period regardless of immunization wee ks where the action of GH on tissue is partly mediated by IGF1, which is secreted mainly fro m the liver and also from other non hepatic tissues to act in an endocrine, autocrine and parac rine fashion (Ovesen et al., 1996). Fletcher et al (1995) and Spencer et al. (1991) stated, inject ion of IGF1 into the lateral ventricles of sheep does not inhibit GH release. Secretion of grow th hormone (GH) in the pituitary is regulated by the neurosecretory nuclei of the hypothalamu s which release the peptides Growth hormone-releasing hormone (GHRH or somatocrinin) and Growth hormone-inhibiting hormone (GHIH or somatostatin) into the hypophyseal portal venous blood surrounding the pituitary. The release of GH in the pituitary is primarily determined by the balance of these two peptides, which in turn is affected by many physiological stimulators. KISS1 gene control the release of GnRH through it's neurons and s uppress it's role on reproduction activity. Here the action of KISS1 gene intra muscular vaccination did not show any change on neurosecretory which enervate the synthesis of GH in the hypothalamus, implies KISS1 and KISS1R neurons may not influence the neurosecretory of either GH or IGF-1.

Body weight, nutrition, metabolism and hormone levels may influence the activity of KISS1 neurons (Fernandez-Fernandez R, et al., 2006; Forbes S, et al., 2009). However, animal growth and development is influenced in large part by the gonadal steroids and feeds similarly the expression of the KISS1 gene is regulated by estradiol. This revealed that KISS1/kisspeptin neurons might be involved in integrating estradiol negative feedback and metabolic influences on GnRH neuronal activity. Growth hormone is the factor for the growth and plays crucial role in the steroid hormone synthesis. It indirectly stimulates cell division, maturation and cell division of chrondocytes in the epiphyseal plates, thus widening plates and providing more cartilaginous material for bone formation. In this research, even though there is destruction of gonadal steroid of animal, there was no significance difference observed in the group mean live weight from the day of injection to slaughter (week14) between control and treatment group of animal on body weight gain, this might be compensated due to the regular production of GH production levels appeared in the findings. This finding is comparable with (Amatayakul-Chantler et, al., 2013) that detected in the group mean live weight at either day14 or day91 and the mean body weight increased at a constant rate, reaching a maximum (19.42 ± 1.70 kg) at the age of 8 months. Studies with bulls explore immunizations against GnRH before puberty does not reduce weight gain and feed efficiency (Adams and Adams, 1992; Adams et al., 1993; Finnerty et al., 1996; Huxol et al., 1998; Cook et al., 2000). Kisspeptins, which act via the GPR54 (KISS1R), are essential signals for the central regulation of reproduction by nutritional and metabolic signals which reveal kisspeptin neurons function as

sensors of energy stores and the metabolic state of the organism. The environments of negative energy balance or metabolic stress can inhibit the reproductive axis and coupled to decreased kisspeptin nature at the hypothalamus. Lee et al (1996) identified kisspeptin as an energy sensor. Metabolic stress, like energy insufficiency but also persistent overweight, can change KISS1 expression at the hypothalamus; a phenomenon that seems to be mechanically relevant for the perturbation of the HPG axis (Castellano et al., 2010). Interestingly in the current finding, the longer the time of vaccination the bigger the differences in body weight at slaughter period than the day of immunocastration, which implies kisspeptin neurons in the hypothalamus are an important component to senses nutritional state that bring change on body growth. Different research publicized that, an intermediate effect of weight gain and growth expected in animals would be similar in castrated and GnRH immunized (Aïssat et al. 2002; Huxsoll et al., 1998; Further, Turkstra, 2005). Hence, KISS1 neurons which are essential for fertility would be perfectly suited for transmitting the effects of important metabolic hormones to the brain centers governing energy store and have potential to serve as the central sensors of metabolic factors that signal to the reproductive axis the presence of stored calories (Qiu X. et al., 2015).

KISS-1 could potentially signal to GPR54 expressing neurons via neurotransmitter, neuroendocrine or endocrine routes (Messager et al 2005). The levels of KISS1 mRNA controlled by steroid hormones and the binding of kisspeptin to GPR-54 triggers a few intracellular signaling pathways and markedly inhibit the proliferation rate of cells expressing GPR-54. Both kisspeptin mRNA and GPR54 mRNA were recently demonstrated in both α and β cells in mouse and human pancreatic islets. The expressions of GPR54 mRNA and KISS1 mRNA in ram labs pancreas was significantly lower in treatment than control one. KISS1 caused a stimulation of glucose-induced insulin secretion, but had no effect on the basal rate of secretion at a sub-stimulatory concentration of glucose (Huag-Evance A.C., et al., 2007) and their immunoreactivities were co-localised in both beta and alpha cells within islets. Kisspeptin stimulate GnRH neurons through GPR54 and its critical switches onto

GnRH neurons regulate the expression of GPR-54 mRNA proliferation. In other way, pancreas contains high levels of KISS1 and its receptor was expressed by the majority of islet endocrine cells, and their immuno-reactivates with insulin and glucagon. Islet KISS1and its ability to enhance glucose induced insulin secretion and are consistent with GP R54 being coupled to phospholipase C activation (Kotani et.al, 2001) and infusion of kisspept in-13 inhibits glucose-induced insulin secretion. Furthermore, the addition of kisspeptin to isolated islets potentiates glucose-stimulated insulin secretion, while the peptide has no effect on glucagon secretion (Hauge-Evans et al., 2006). Our observation suggests that KISS1 mRNA and GPR54 mRNA fulfils an important intra islet function in regulating the magnitude of insulin secretory responses to circulate nutrients and showed variation of KISS1 mRNA and GPR54 mRNA in vaccinated and control group, has linkage with glucose production that hinder the expressions of KISS1/GPR54 mRNA. Besides, GH and IGF-1 which secreted by insulin were same throughout the research time in both groups which is an evidence for the variation of KISS1 mRNA and GPR54 mRNA had linkage with/highly dependent on glucose production, but further mechanisms should be needed. GPR54/KISS1 system is expressed in the endocrine pancreas, where it influences beta cell secretory function and suggest an important role for this system in the normal regulation of islet function (Hauge-Evans et al., 2006). Glucagon stimulates via cAMP-PKA-CREB signaling hepatic production of the neuropeptide kisspeptin, which acts on β cells to suppress glucose-stimulated insulin secretion. In short, KISS-1 can suppress the activity of KISS1 mRNA and GPR-54 mRNA through

programmed suppression of GnRH neuron proliferation which appeared in different organ of ram labs.

Lower expression of KISS1mRNA has been observed in tongue of group received pKS-asd vaccine. Tastes are detected by way of G protein-coupled taste receptors (Bachmanov and Beauchamp, 2007) where G-protein linked to stimulative hormone receptor and it's α subunit upon activation which could either stimulate or inhibit the activity of an enzyme. The receptors and G proteins are pre-coupled (Kou et al., 2011) and binding affects the receptor's affinity for ligands. These proteins are cyclic adenosine monophosphate (cAMP) and gamma-aminobutyric acid (GABA) in which, cAMP-dependent pathway is initiated by the binding of GnRH to its receptor and gamma-amino butyric acid (GABA) is acts as a neurotransmitter in the central nervous system. Gamma-aminobutyric acid inhibits nerve transmission in the brain; calming nervous activity depolarizes embryonic GnRH neurons and slows the movement. Kisspeptin derived from KISS-1 gene signaling through the G protein- coupled receptor (GPR54) and its kisspeptin neuron is directly innervate GnRH neurons and stimulates GnRH release. Similarly, G proteins–coupled signaling through cAMP-dependent pathway is binding to GnRH to stimulate hormone receptor and, or through GABA which acts as a neurotransmitter to depolarizes embryonic GnRH neurons. Therefore, KISS-1 gene could potentially signal to GPR54 expressing neurons via neurotransmitter, neuroendocrine or endocrine routes (Messager et al 2005), cAMP and GABA which are the principal pathway for G-protein coupled receptor can serve KISS-1 gene to suppress the expression of KISS-1mRNA in the tongue. However, further mechanisms should be illustrated

CONCLUSION

KISS1 gene vaccine immunocastration did not bring any change on growth hormone and Insu lin like growth factor-1 (IGF-1) between the research groups, but affected the KISS1mRNA a nd GPR54 mRA expression in pancreas of ram lambs.

Acknowledgements

This work was funded by the China National Meat-type Sheep and Goat Industrial Technology System CARS-39-1A.The authors announce no conflict of interest in the research for this study. Contributors: YH: BT: study design, study planning, and data analysis, and manuscript writing; GL, XJ: conception and design, main manuscript authors; ZJ, data analysis and manuscript preparation.

REFERENCES

Adam, T.E. and Adam, B.M., 1992. Feedlot performance of steers and bulls actively immunized against Gonadotropin-Releasing Hormone. J. Anim. Sci. 70, 1691–1698

Adams, T. E., Daley, C. A., Adams, B. M., & Sakurai, H. 1993. Testis function and feedlot performance of bulls actively immunized against gonadotropin-releasing hormone: effect of implants containing progesterone and estradiol benzoate. Journal of Animal Science, 71, 811–817.

Ahmad, N. and Noakes, D.E., 1996. Sexual maturity in British breeds of goat kids. Br. Vet. J. 5, 93–103.

Aissat, D., Sosa, J.M., de Avila D.M., Bertrand K.P., Reeves J.J., 2002. Endocrine, growth, and carcass characteristics of bulls immunized against luteinizing hormone-releasing hormone fusion proteins. J. Anim. Sci. 80, 2209–2213.

Amatayakul-Chantler S, Hoe F, Jackson J.A, Roca R.O, Stegner J.E, King V., Howard R., Lopez E., Walker J., 2013. Effects on performance and carcass and meat quality attributes following immunocastration with the gonadotropin releasing factor vaccine Bopriva or surgical castration of Bos indicus bulls raised on pasture in Brazil. Meat Science 95 (2013) 78–84

Bachmanov AA and Beauchamp GK, 2007. "Taste receptor genes". Annual Review of Nutrition 27: 389–414.

Belibasaki, S. and Kouimtzis S. 2000. Sexual activity and body and testis growth in prepubertal ram lambs of Friesland, Chios, Karagouniki and Serres dairy sheep in Greece.

Bilaspuri G.S and Singh K., 1992. Developmental changes in body weight and testicular characeristics in Malabari goat kids. Theriogenology 37, 507–520.

Braun, W.F., Thompson, J.M., Ross, C.V., 1980. Ram scrotal circumference measurements. Theriogenology 13, 221–229.

Christine Margaret, 1999. Effects of immunocastration on Feedlot Performance in Bulls of Different Breed-types hplanted with Estradiol 17-β. A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Edmonton, Alberta Cook, R. B., Popp, J. D., Kastelic, J. P., Robbins, S., & Harland, R. 2000. The effects of active Immunization against GnRH on testicular development, feedlot performance, and carcass characteristics of beef bulls. Journal of Animal Science, 78, 2778–2783.

Costoff A. 2008. Endocrinology: The Endocrine Pancreas. Medical College of Georgia.Daley, D.A., Adams, T.E., Daley, C.A., Patton, W.R., Evens, J.L., 1995. Effects of immunocastration on growth, carcass characteristics and reproductive development in ram lambs. Sheep Goat Res. J. 11, 31–34

Finnerty J.R, Master V.A, Irvine S, Kourakis M.J, Warriner S, Martindale M.Q. 1996 Homeobox genes in the Ctenophora: Identification of paired-type and Hox homologues in the atentaculatectenophore, Beroe¨ovata. Mol Mar Bio Biotech 5:249–258

Fletcher, P. C., Frith, C. D., Grasby, P. M., Shallice, T., Frackowiak, R. S. J., and Dolan, R. J. 1995. Brain systems for encoding and retrieval of auditory–verbal memory: An in vivo study in humans. Brain 118:401–416.

Frohman, M. A., Boyle, M. and Martin, G. R. 1990. Isolation of the mouse Hox 2.9 gene: analysis of embryonic expression suggests that positional information along the anterior posterior axis is specified by mesoderm. Han Yanguo, Guiqiong Liu, Xunping Jiang, Nabeel Ijaz, BirhanuTesema, Guangyue Xie 2015. KISS1 can be used as a novel target for developing DNA immunocastration vaccine in ram lambs

Hileman S.M., Schillo K.K. & Hall J.B. 1993. Effects of acute, intracerebroventricular administration of insulin on serum concentrations of luteinizing hormone, insulin, and glucose in ovariectomized lambs during restricted and ad libitum feed intake.

Hauge-Evans A. C., Richardson C. C., Milne H. M., Christie M. R., Persaud S. J. and Jones P. M., 2006. Arole for kisspeptin in islet function // Diabetologia.—49, N 9.–Ð. 2131–2135.

Huxsoll, C. C., Price, E. O., & Adams, T. E. 1998. Testis function, carcass traits, and aggressive behavior of beef bulls actively immunized against gonadotropin-releasing hormone. Journal of Animal Science, 76, 1760–1766.

Irwig, M.S., Fraley, G.S., Smith, J.T., Acohido, B.V., Popa, S.M., Cunningham, M.J., Gottsch, M.L., Clifton, D.K. and Steiner, R.A. 2004. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat.

Kou Qin, Chunmin Dong, Guangyu Wu & Nevin A Lambert, 2011. "Inactive-state preassembly of Gq-coupled receptors and Gq heterotrimers". Nature Chemical Biology 7 (11): 740–747.

Kotani M, Detheux M, Vandenbogaerde A et al (2001) The metastasis suppressor gene KiSS-1 encodes kisspeptin, the natural ligand of the orphan G protein-coupled receptor GPR54. J Biol Chem 276:34631–34636

Kovac, J. M.; Leitch, E. M.; Pryke, C.; Carlstrom, J. E.; Halverson, N. W. and Holzapfel, W.L., 2002. Detection of polarization in the cosmic microwave background using DAS. Volume 420,Issue 6917, pp. 772-787

Ohtaki T, Shintani Y. and Honda S. 2001. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 411:613–617

Olster D. H and Foster D. L, 1988. Control of gonadotrophin secretion during the pubertal and seasonal transitions in the male sheep Journal of Reproduction and Fertility 82 179-191

Ovesen P, Jorgensen J. O, Ingerslev J, Ho K. K, Orskov H. and Christiansen J. S, 1996. Growth hormone treatment in subfertile mares. Fertility and Sterility 66, 292–298.

Ozawa A, Inokuma H. and Johke T., 1995. The relationship between plasma insulin-like growth factor I (IGF-1) level and body weight in the horse. Journal of Veterinary Medical Science 57,

Pazos, P., Boveri, M. and Gennari, A., 2004. Culturing cells without serum: lessons learned using molecules of plant origin. ALTEX 21, 67-72.

Salhab S. A., Zarkawi M. F., Wardeh, M. R., AlMasri and Kassem R., 2001. Development of Testicular Dimensions and Size, and Their Relationship to Age, Body Weight and Parental Sizein Growing Awassi Ram Lambs. Small Rum. Res., 40: 187-191.

Schanbacher, B.D., 1982. Response of ram lambs to active immunization against testosterone and luteinizing hormone releasing hormone. Am. J. Physiol. 242, E201-205.

Tilbrook A. J, De Kretser D. M. and Clarke I. J., 1993. Human recombinant inhibin A suppresses plasma follicle-stimulating hormone to intact levels but has no effect on luteinizing hormone in castrated rams. Biology of Reproduction 49 779–788.

Turkstra, J.A., 2005. Active immunisation against gonadotropin-releasing hormone, an effective tool to block the fertility axis in mammals. PhD Thesis, Utrecht University, The Netherlands.

Zhang F., F. Fang, H. Chang B., Peng J., Wu J., Chen H., Wang. and Chen Z., 2013. Comparison of protection against H5N1 influenza virus in mouse offspring provided by maternal vaccination with HA DNA and inactivated vaccine. Arch. Virol. 158: 1253–12