GENE EXPRESSION PROFILING OF BOVINE OVARIAN FOLLICULAR SELECTION

A Dissertation

Presented to

The Faculty of the Graduate School

University of Missouri-Columbia

In Partial Fulfillment

Of the Requirement for the Degree

Doctor of Philosophy

By

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December 2006

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

Gene expression profiling of Bovine Ovarian Follicular Selection

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ACKNOWLEDGEMENTS

First I would like to take this opportunity to express my appreciation to my advisor, Dr. Eric Antoniou, for letting me pursue my doctoral degree under his guidance. For more than six years, he has provided an encouraging environment for me to learn and grow in the research. His consistent guidance and cordial advice on my research have been invaluable.

Many thanks to my committee members: Dr. Allen H. Garverick, Dr. William R. Lamberson, Dr. Gary S. Johnson, Dr. Michael Smith and Dr. Jerry Taylor for their encouragement and mentoring throughout my course of studies and completion of this work. Very special thanks to Dr. H. Allen Garverick and his lab member Malinda N. Burkhart, Dr. Robert S. Youngquist, Dr. Jerry Taylor and his lab member Erin M. Sellner, for their direct collaborations on different parts of this dissertation research. Without their generous help, this project would not be completed as smoothly as today.

I would also like to thank Dr. Leonard B. Hearne for his expertise on microarray analysis and Dr. Dennis B. Lubahn for his concern and help.

Many thanks to my current lab members, Sachin Bhusari, Mallikarjuna S. Basavarajappa, Jaewoo Kim as well as previous lab member, Jarrod J. Robertson for their help with science and lab work, and for creating an enjoyable lab together. I also need to extend my thanks to those who have been there along the way, including Raja S. Settivari, Dr. Kristi M. Cammack, Dr. Tim Safranski, Dr. Robert Schnabel, Jinghua Liu, Yi

Zhuang and many other people in ASRC. Special thanks to Ms. Cynthia L. Glascock and Ms. Doris A. Lyons for their enormous assistance.

Last, and certainly not least, appreciation is expressed to my loving and supporting family. I would like to express my deepest thanks to my parents and my sisters for their constant love and support throughout my life. Finally, and most importantly, none of this could have been completed without the love and support of my wife Wei Zhou. Thanks for all your help on science, for all the understanding and encouragement, for taking care of me and providing a warm home. You are the ones who make me a better person. Thanks.

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3βHSD	3-beta-hydroxy-delta(5)-steroid dehydrogenase
AMH	anti-Mullerian hormone
B2M	beta-2-microglobulin
BMP	bone morphogenic protein
DF	dominant follicle
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
FSH	follicle stimulating hormone
FSHR	FSH receptor
GC	granulosa cells
GDF	growth/differentiation factor
GnRH	gonadotropin-releasing hormone
GnRHa	GnRH agonist
IGF	insulin-like growth factor
IGFBP	IGF binding protein
INHA	Inhibin alpha
INHBA	Inhibin beta A
LH	luteinizing hormone
LHR	LH receptor

MHC	major histocompatibility complex
P450arom	aromatase
P450C17	P450-17α-hydroxylase
P450scc	cholesterol side-chain cleavage enzyme
PAPP-A	pregnancy associated plasma protein-A
SDC4	syndecan-4
SERPINE1	plasminogen activator inhibitor-1
SSC	standard sodium citrate
StAR	steroidogenic acute regulatory protein
TGFß	transforming growth factor-β
THBS2	thrombospondin-2

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ABSTRACT

Lack of, or abnormal, ovarian follicular development is a major factor associated with decreased fertility in mammals. However, development of new treatments is limited by our poor understanding of ovarian follicular development. Understanding the mechanisms by which a cohort of follicles is recruited and a single follicle is selected for further development to a dominant follicle is the key to understanding pre-ovulatory follicular development. The objectives of this study are to identify changes in gene expression profiles during the selection stage of bovine follicular waves.

Follicles of different sizes (8 mm to 12 mm) were collected and intrafollicular concentrations of progesterone, estradiol and androstenedione were measured. Gene expression profiles were obtained using bovine cDNA microarrays.

Of the 17,692 probes on the arrays, 606 unique genes and 32 sequences not matching any known genes were identified as differentially expressed with a cut off false discovery rate (FDR) of 0.05. Seventeen gene expression profiles were confirmed using quantitative RT-PCR.

Five main expression profiles were discovered, in which gene expression patterns change according to follicle size. Genes involved in several biological processes were also identified as significantly over-represented, such as genes involved in immune function or regulation of cell cycle. Annotation of the cellular location of differentially expressed gene products identified the ribonucleoprotein complex and the extracellular matrix as over represented.

This data strongly support the essential role of LH/LHR pathway on follicular selection, the important function of the extracellular matrix and the associated cell receptors, and reveal a potentially important role for the immune system.

Chapter I

Introduction

Cows must reestablish ovarian ovulatory cycles early postpartum and become pregnant within 3 months following parturition to maintain a 12-month calving interval. Lack of, or abnormal, ovarian follicular development during the postpartum period prevents timely reestablishment of pregnancy and is a major factor associated with reduced reproductive efficiency in farm animals. Cellular and endocrine mechanisms regulating the development of ovarian follicles in mammalian species are still poorly understood (Bao and Garverick 1998; Fortune et al. 2001; Ginther et al. 2001; Zeleznik 2001). Understanding the mechanisms by which a cohort of follicles is recruited and a single follicle is selected for further development and becomes dominant in a hormonal milieu suppressive to the development of other large follicles is key to understanding preovulatory follicular development in cattle. As eloquently stated by Greenwald, 1972: **"One of the most intriguing mysteries in ovarian physiology is what factors determine whether one follicle remains quiescent, another begins to develop but becomes atretic, while still a third matures and ovulates."** Understanding the preceding mechanism(s) is relevant to improving methods of estrous synchronization, timed insemination and superovulation in cattle.

Almost 25% of clinical infertility cases are idiopathic, and 15% of couples cannot conceive within the first year (Matzuk and Lamb 2002). A consequence of some fertility treatments is an increase in multiple births resulting from multiple follicle ovulations. Indeed, ovarian hyperstimulation is implicated in 40% of triplet and higher-order birth in the U.S. Multiple births are a serious health problem because perinatal mortality is five times higher in twins and seven times higher in triplets. Multiple births are particularly frequent in patients with normogonadotrophic anovulation that undergo ovarian hyperstimulation with gonadotropin. Even new chronic low-dose protocols still result in around 6% of multiple births (Homburg and Insler 2002). A better understanding of the mechanisms driving follicular selection might provide new solutions for preventing multiple follicles developing and ovulating (Zeleznik 2001).

Although there are more than 100 genes implicated in follicular growth (Ben-Shlomo et al. 2002), only a few are known to play a role in follicular selection and the establishment of a dominant follicle. A genomics approach can be used to study gene expression within dominant follicles in model animals. Recent studies have demonstrated that functional genomic methods can uncover additional genes expressed in the ovary, elucidate potential functions for these genes, and help determine future research directions (Sisco et al. 2003; Evans et al. 2004; Fayad et al. 2004; Mihm et al. 2006).

Cattle provide a good model for understanding the human follicular development process, as both species are monovular and stages of follicular development can easily be followed using real-time ultrasonography.

Objectives

The overall objective of this research was to elucidate the genetic control of bovine ovarian follicle selection. The specific objectives were to 1) construct a high content bovine cDNA microarray, and 2) identify genes differentially expressed between bovine antral follicles at the time of follicular selection.

Chapter II

Literature review

Introduction

Folliculogenesis in the mammalian ovary can be defined as the formation of Graafian (mature, preovulatory) follicles from a pool of primordial (non-growing) follicles (Spicer and Echternkamp 1986). It is widely accepted that the number of primordial follicles in the ovary is fixed at the time of birth in most mammals. In cows, there are approximately 150,000 primordial follicles present at birth and the fate of greater than 99% of them is atresia (Erickson 1966; Ireland 1987; Webb et al. 1992). The development of follicles at all stages is of great interesting with numerous studies focusing on the regulation of steroidogenesis as well as change of gene expression (Bao and Garverick 1998).

The understanding of bovine ovarian follicular development was greatly enhanced since the introduction of real-time ultrasonography into cattle (Pierson and Ginther 1984), which permits investigators to monitor dynamics of follicular development in the living animal without interfering with the ovarian and endocrine status of the cow. Thus the

growth and demise of antral follicles can be reliably followed during the bovine estrous cycle (for reviews, see Fortune 1994; Ginther et al. 1996; Fortune 2003; Ginther et al. 2003; Mihm and Bleach 2003). The development of associated sample-collecting techniques (reviewed by Driancourt 2001), such as follicle aspiration, ovum pick-up (Pieterse et al. 1991), and follicular fluid micropuncture (Kohram et al. 1998), plus the advance of molecular biology, have greatly expanded our understanding of mechanisms regulating follicular maturation through different stages.

Follicular development

Preantral and antral follicle dynamics

Follicles form when primary oocytes are surrounded by a single layer of flattened granulosa cells (also referred as pre-granulosa cells) and become enclosed in a basement membrane. Follicles at this stage are called primordial follicles. As the primordial follicles progress through primary, early pre-antral (secondary), later pre-antral (tertiary) and antral follicles, the granulosa cells (GC) become cuboidal and more layers of GC form (Braw-Tal and Yossefi 1997). The thecal layer starts to form when the follicles reach the mid or late pre-antral stages (see Table 1 for further details).

Antral follicle development in cattle shows two stages of development, a slow growth phase and a fast growth phase. The slow phase takes more than 30 days for the follicle to grow from antrum acquisition at 300 μm to the small follicle stage at 3-5 mm in diameter (Lussier et al. 1987). During this phase oocytes achieve their final size and full developmental competence (Fair et al. 1997). The second phase describes the growth

of follicles from 3 mm to larger sizes, usually in 5-7 days. This is the growth phase that can be routinely monitored by ultrasound. It is usually described as the follicle wave.

During each estrous cycle, there are usually 2 to 3 waves of follicular growth (for reviews, see Fortune 1994; Ginther et al. 1996). Each wave of follicular growth is characterized by the sequential events of recruitment, selection and dominance. The initiation of each follicular wave (recruitment) is characterized by the emergence of a cohort of follicles (usually 2 to 6) 4 to 5 mm in diameter. This cohort of recruited follicles can grow to 8-9 mm in diameter over the next 36 to 48 hours. Then usually one of the follicles continues growth beyond 9mm (selection), while the others stop growth. During the follicular dominance phase, the selected follicle will eventually reach a preovulatory size (~16 mm) and mature while the other follicles of the cohort undergo atresia. If the dominant follicle reaches its maximum size during the luteal phase, it will maintain the size for 3 to 6 days and then undergo atresia. If luteal regression occurs during the growing phase of the dominant follicle, the dominant follicle will ovulate. The ovulated follicle is usually from the second or third follicular wave (reviewed by Bao and Garverick 1998).

In cattle, follicular waves can be detected throughout different physiological stages, including pre-pubertal period, estrous cycle, pregnancy and post-partum anestrus (reviewed by Driancourt 2001). There are reports that the follicular waves can be extended to the growth of even smaller follicles (1 mm in diameter) (Jaiswal et al. 2004), but the monitoring of such small follicles is quite difficult and thus the results should be

viewed with caution. Most people still regard recruitment as the phase of initiation of the follicular wave.

Deviation and size advantage

As described above, in each wave a cohort of follicles is recruited. However, these follicles are not recruited at the same time. The largest follicle (usually the future dominant follicle) appears (at size ~4-5 mm) usually 6 (Ginther et al. 1997a) or 7 hours (Kulick et al. 1999) earlier than the future largest subordinate follicles in heifer. This time advantage is equal to an average of 0.5 mm in diameter size advantage of the largest follicle over the 2nd largest follicle (Kulick et al. 1999; Beg et al. 2003; Ginther et al. 2003). In another study, it has been shown that the emergence of the dominant follicle at 1 mm occurs about 6-12 hours earlier than subordinate follicles in the same wave (Jaiswal et al. 2004), possibly explaining the advance of the largest follicle at recruitment.

After emergence the cohort of follicles will have a common-growth stage. During this stage the largest and 2nd largest follicles will grow at similar rates. At the end of the common-growth stage, the future dominant follicle (most of the time, the largest one) will grow at a continuing rate while the subordinate follicles will regress or temporarily grow at a reduced rate and then regress (reviewed by Ginther et al. 2001a). The consequence of this difference in growth rate is a significant difference in diameter between the dominant and subordinate follicles, an obvious sign that one follicle has been selected (see illustration in Figure 1).

As selection of the dominant follicle is likely a progressive process that occurs before there is a perceptible difference in size, it is difficult to define when selection

occurs. Thus, follicular selection usually refers to the morphological selection and diameter deviation reflects the change (Fortune et al. 2001; Ginther et al. 2003). As defined by Ginther et al. (2003), deviation in individual waves was "beginning at the examination before the first examination with an apparent change in the differences in diameter between the two largest follicles".

Based on several studies (Ginther et al. 1997a; Ginther et al. 1998; Ginther et al. 1999; Kulick et al. 1999), Ginther et al. (2000b) reported that the average observed deviation for the first anovulatory wave in heifers began when the largest follicle was a mean of 8.5 mm. This was also supported by later observations by Beg et al. (2003). **Thus, on average, deviation begins when the largest follicles are at a mean diameter of 8.5 mm.**

Gonadotropin regulation of follicular recruitment and selection

Experiments with GnRH (gonadotropin-releasing hormone) immunization or GnRHa (GnRH agonist) demonstrate that follicle growth before recruitment can occur in an environment characterized by basal FSH (follicle stimulating hormone) and no LH (luteinizing hormone) pulses (Gong et al. 1995; Gong et al. 1996; Crowe et al. 2001). This period of growth is thought to be gonadotropin-independent. However, the recruitment and following growth stages are absolutely dependent on FSH and LH.

Two to three day before the emergence of a follicular wave in cattle, there is a transient increase in FSH concentration (Adams et al. 1992). FSH rises are associated with new follicle waves occurring during the estrous cycle (Adams et al. 1992;

Sunderland et al. 1994), in the postpartum period (Crowe et al. 1998), during pregnancy (Ginther et al. 1996) and before puberty in cattle (Evans et al. 1994). Several lines of evidence support the dependency of the emergence of follicle waves on elevated FSH (reviewed by Mihm and Bleach 2003):

1) Emergence of the first follicle wave is blocked when the periovulatory FSH rise is suppressed using steroid-free bovine follicular fluid (Turzillo and Fortune 1990; Bleach et al. 2001).

2) When the FSH rise is blocked by GnRH immunization or long lasting GnRHa, follicles are arrested at 2-4 mm (Prendiville et al. 1995; Gong et al. 1996; Prendiville et al. 1996; Crowe et al. 2001).

3) When the FSH rise during the cycle is delayed by exogenous estradiol benzoate, emergence of the second follicle wave is also delayed (Bo 1995; O'Rourke et al. 2000).

4) Follicular waves emerge when exogenous FSH is concurrently administered with steroid-free bovine follicular fluid (Bergfelt et al. 1994).

These data strongly indicate that FSH is required for follicular recruitment.

Recruitment of the cohort of follicles in cattle has previously been associated with the acquisition of expression of mRNAs for P450scc and P450arom in granulosa cells (Bao et al. 1997a). These genes give follicles the ability to produce estradiol. Garverick et al. (2002) have also shown that FSH treatment in GnRH agonist-treated heifers could induce the expression of mRNAs for P450scc and P450arom in granulosa cells from small follicles and could markedly upregulate the expression of these enzymes

in granulosa cells from recruited follicles, indicating the key role of FSH in follicular recruitment.

Follicular growth and steroidogenesis are dependent on the coordinated actions of FSH and LH with their receptors on granulosa cells and thecal cells. A well-accepted model is the two-cell/two-gonadotropin model (Fortune and Quirk 1988). In this model, granulosa cells express the FSH receptor and thecal cells express the LH receptor before selection. Both cell types have the P450scc enzyme which is required to convert cholesterol to progestin. The progestins, pregnenolone and progesterone, are precursors for the synthesis of androstenedione in thecal cells and LH stimulates the activity of the enzyme P450C17 to produce androstenedione. Androgens are transferred to granulosa cells and are converted to estradiol-17β by the P450arom enzyme.

These data clearly show that **FSH plays an important role in the recruitment of follicles and their growth before selection.**

With the growth of the recruited follicles, FSH concentration will decrease and the acquisition of the LH receptor on granulosa cells was proposed to differentiate from other cohort members the follicle destined to become dominant (Bao et al. 1997a). That is, there is a shift of dependence from FSH to LH for the selected follicles. Though there are reports that the differences of LH receptor mRNA and protein between the dominant and subordinate follicles are not obvious until after selection, (Evans and Fortune 1997), the shift of dependence is thought to be critical, as without shifting (by supplying exogenous

FSH), multiple follicles of the cohort can be maintained and no selected DF can be identified (Mihm et al. 1997; 2000). On the other hand, using a GnRHa model in which pulsatile LH secretion is suppressed, follicles cannot grow beyond 9 mm (Gong et al. 1996). Thus, LH pulses are indispensable for follicle development beyond 9 mm in diameter.

Along with the acquisition of LH receptor by the granulosa cells at, or after, selection there is also an acquisition of 3βHSD in granulosa cells in the same follicles that acquired expression of LH receptor (Bao et al. 1997b). This is possibly necessary for the great steroid production ability of the dominant follicles.

Following its selection, DF growth, estrogen activity and lifespan are controlled by the LH pulse pattern. Infusion of exogenous LH either prolonged dominance or induced ovulation (Duffy et al. 2000; Hampton et al. 2003). Increased LH pulse frequency (by treatment of low level of progestin) will prolong dominance (Sirois and Fortune 1990; Stock and Fortune 1993; Mihm et al. 1999). Abolishment of LH pulses by administration of a GnRH antagonist reduced maximum sizes and dominance periods of the first and second DF (Fike et al. 1997). Thus, the selected DF may not depend much on FSH, but were extremely sensitive to LH pulsatility.

Final maturation of the ovulatory follicles is initiated by the preovulatory LH surge. After the LH surge, oocytes will resume meiotic division and the follicles are prepared for ovulation.

Mechanisms of follicle selection

FSH: follicle-coupling hypothesis

It was proposed by Ginther et al. (2000b) that the essence of selection of a dominant follicle is a close two-way functional coupling between changing FSH concentrations and follicular growth. The hypothesis is described and reviewed in detail by Ginther et al. (2000b; 2001a). In short, FSH concentration modulates the growth of follicle(s) and follicle(s) also mediate the concentration of FSH.

Briefly, the rise of blood FSH that caused the recruitment of the follicle wave peaked when the largest follicle was 4-5 mm in diameter in heifers, then FSH declined. This decline continued until 10-20 hours after follicular deviation. Multiple recruited follicles were likely contributing to the decline, as they produced increasing amounts of estradiol and inhibin (Martin et al. 1991). While these follicles imposed an FSH decline, they still needed FSH for their normal growth. This was supported by a study showed that experimental reduction of FSH at the middle of the FSH decline was associated with a decrease in diameter of the three largest follicles (Ginther et al. 2000b).

At the beginning of the diameter deviation, the FSH:follicle relationship changed from multiple- to single-follicle coupling. That was, the decline of FSH was now mainly caused by the future dominant follicle. This was supported by the fact that FSH concentration increased soon after the ablation of the largest follicle at the expected beginning of deviation (largest follicles at 8 to 9 mm in size). While the future DF was primarily responsible for FSH decline, FSH is still needed as its growth was impaired if FSH was experimentally depressed at the expected beginning of deviation. So, the

hypothesis is that the future DF advantage is that it can withstand a lower FSH concentration than smaller follicles (Ginther et al. 2001a).

It was shown that when the one, two or three of the largest follicles were ablated at the expected end of the common-growth phase (deviation point), the subsequent dominant follicles formed from the largest retained >7.0 mm follicles in 14 of 15 heifers. When all retained follicles were <7.0 mm, the first follicles to reach 7.0 mm became dominant in 7 of 8 heifers (Ginther et al. 2001b). In some heifers, follicles became dominant even though they were as small as 4.0 mm on the day of ablation of the larger follicles (Ginther et al. 2001b). This indicates that **all follicles of the common-growth phase have the potential for future dominance** (Ginther et al. 2001b). Actually, an injection of exogenous FSH to delay the decline of FSH resulted in a delay in the selection process in cattle (Mihm et al. 1997; 2000) and prolonged exposure to FSH alone could stimulate and support multiple follicles to grow beyond >10 mm in diameter (Hampton et al. 2004).

These finding indicate that while all follicles have the capability to develop into the dominant follicle, **the drop in FSH concentration is a key mechanism in the selection of only one follicle for growth and future ovulation**. A developmental changes occurs in the largest follicle that enables it to continue growth with a declined FSH concentration that is inadequate to support growth of the smaller follicles (Ginther et al. 2001a).

However, there are data that suggest that the FSH-operated mechanism is not the only mechanism involved in selection, as reviewed by Driancourt (2001). First, it was

difficult to obtain a single ovulation by only manipulating FSH and LH concentration in GnRH immunized cows (Crowe et al. 2001). This indicates the involvement of other regulatory factors. Second, in super-ovulation trials, the presence of dominant follicles can interfere with ovarian responses to gonadotropin injection (Guilbault et al. 1991; Wolfsdorf et al. 1997). This indicates that large follicles can reduce the sensitivity of smaller follicles to gonadotropin stimulation, inhibiting their growth and maturation. The inhibitory effect is not just through inhibin as the effect can be observed with either the injection of inhibin-free follicular fluid (Law et al. 1992) or injection of follicular fluid in inhibin immunized cows (Wood et al. 1993). Thus, factors other than inhibin, e.g., estradiol, are likely involved in the inhibitory effect of the largest follicles on the growth of smaller follicles.

Therefore, the drop in FSH concentration is a key mechanism in the selection process, but it is possibly not the only regulatory factor.

LH and LH receptors

Although LH pulses are indispensable for follicle development beyond 9 mm in diameter (Gong et al. 1996), the role of LH receptor in follicular selection is still debated. It is observed that around the time of selection, LH receptor mRNA expression is initiated in granulosa cells (Xu et al. 1995a; Bao et al. 1997a). This leads to the hypothesis that acquisition of LH receptor on granulosa cells is the (or a) primary determinant of selection for dominance. However, additional studies have suggested there is no LH receptor mRNA and protein level differences between the largest and second

largest follicles until after DF selection (Stewart et al. 1996; Evans and Fortune 1997). A recent study by Hampton et al. (2004) also found that expression of LH receptor in granulosa cells was detected in all >10 mm follicles from different treatment groups (GnRHa/FSH, GnRHa/FSH+LH, and control), indicating that the role of LH/LH receptor in selection can be overridden by FSH. Thus, though the acquisition of LHR on granulosa cells is important for the survival of the dominant follicles, there seems to be a short time period, from the start of deviation to the expression of LHR on GC, which survival difference cannot be fully explained by the acquisition of LHR in granulosa cells.

Estradiol

Shortly before, or at the beginning, of deviation in heifers, the future dominant follicles begin to produce significantly higher amounts of estradiol compared to subordinate follicles (Ginther et al. 1997b; Beg et al. 2001; Beg et al. 2002). High estrogen production and high estrogen/progesterone (E:P) ratios are regarded as the typical characteristics of healthy follicles. Follicles undergoing atresia are characterized by the loss of ability to produce estradiol and a decrease of E:P ratio (for reviews, see Kobayashi et al. 2006). Both anovulatory dominant follicles (Xu et al. 1995b) and subordinate follicles (Austin et al. 2001) lose their capacity to produce estradiol before the onset of atresia.

The effect of estradiol on the hypothalamus-pituitary-ovary axis has been extensively studied and involves another important hormone, progesterone. Briefly, estradiol and progesterone regulate the synthesis and release of gonadotropins through negative and positive feedback effects on the hypothalamus and pituitary. When estradiol

levels are low and progesterone is high (luteal phase), there is a negative feedback of estradiol on the hypothalamus, thus preventing high amplitude pulse of GnRH, which in turn, will lead to the lower releasing levels of LH. When estradiol is high and progesterone is low (follicular phase), it will have a positive feedback on GnRH, causing a GnRH surge, which, in turn, leads to an LH surge. At the pituitary level, estradiol can stimulate synthesis and secretion of LH, increase the concentration of GnRH receptor and thus enhancing pituitary's response to GnRH (Senger 2003).

The local effect of estradiol on follicular cell proliferation and differentiation has also been extensively studied. Estradiol increases sizes of in vitro cultured bovine granulosa cells (Hulshof et al. 1995) and enhances the steroidogenesis in granulosa and thecal cells in cattle (Fortune and Quirk 1988).

Estradiol also has important roles in induction of LHR in granulosa cells. It has been shown that the induction of granulosa LHR in rodents is dependent upon both FSH and estradiol concentrations (Segaloff et al. 1990). In cattle, the increase of estradiol concentration also precedes LHR expression in granulosa cells (Bodensteiner et al. 1996; Evans and Fortune 1997). The secretion of estradiol from the dominant follicles is also believed to decrease the FSH concentration. Ginther et al. (2000a) have demonstrated that in heifers, following ablation of the largest follicle at the beginning of deviation, there is a decrease of estradiol concentration and an increase of FSH in blood. The increase in FSH concentration is delayed by injections of exogenous estradiol, which supports the hypothesis that "the largest follicle releases increased estradiol into the blood at the beginning of follicular deviation, and that the released estradiol is involved in the continuing depression of FSH concentrations to below the requirement of the smaller

follicles" (Ginther et al. 2000a). This is further supported by the observation that in heifers treated with antiserum against estradiol, FSH concentration increases and the beginning of deviation is delayed (Beg et al. 2003).

Though there are proposals that estradiol has a functional local role in the deviation process independent of the systemic negative effect on FSH (Beg et al. 2003), Driancourt (2001) suggests that estradiol production is possibly not a pre-requisite for follicle dominance as:

1) When estradiol output is greatly reduced by treatment with an aromatase inhibitor, the development of the dominant follicle is not blocked.
2) When the steroid output of large follicles is blocked by epostane, a steroid synthesis inhibitor, the development of the dominant follicle is not blocked.
3) Dominant follicles produce variable amounts of estradiol at different physiological stages, though the difference between the diameters of the dominant and the largest subordinate follicle is similar.

The GnRHa experiments by Hampton et al. (2004) also questioned the essential role of estradiol in selection as both in cows with low level (GnRHa/FSH treated) and in cows with high level (GnRHa/FSH+LH treated) of follicular fluid estradiol, multiple follicles could grow to >10 mm in diameter and all of them had LHR expression in granulosa cells.

In summary, **the role of estradiol to negatively regulate the FSH concentration is clear at the time of deviation**.

Key steroidogenic enzymes

A series of investigations have studied changes in mRNA expression of gonadotropin receptors (FSHR and LHR), and key steroidogenic enzymes, such as P450SCC, P450C17, P450arom, 3βHSD and StAR in the association of follicular recruitment and selection (Xu et al. 1995a; Xu et al. 1995b; Bao et al. 1997a; Bao et al. 1997b). As summarized by Bao and Garverick (1998), there is an increase of expression or activity of LHR, P450scc, P450C17, 3βHSD, and StAR in thecal cells and an increase of expression of FSHR, P450scc, P450arom, 3βHSD, and LHR in granulosa cells during various stages of the follicular wave. However, except for the 3βHSD and LHR in granulosa cells, which are predominantly detected in the ≥8 mm healthy follicles, all the others are probably not related to follicular selection as similar profiles are also found in similar size follicles of the same cohort (Bao and Garverick 1998). The role of LHR expressed in granulosa cells in dominant follicular selection has been previously discussed. The role of 3βHSD is still not clear. It could potentially be related to the dominant follicle selection (Bao and Garverick 1998).

Although all 6 to 9 mm follicles have expressions of P450arom mRNA (Bao et al. 1997a), there may be a difference of aromatase activity as the future dominant follicles usually have the highest estradiol production (see previous discussion). Granulosa cells collected from different size follicles also show different estradiol production abilities in vitro (Rhodes et al. 2001), leading to the hypothesis that increased granulosa cell aromatase activity may be associated with dominant follicle selection.

TGFß superfamily members

Members of the transforming growth factor-β (TGFß) superfamily have been shown to play important roles in folliculogenesis in a stage dependant manner. There are more than 30 members of this family, as summarized by Knight and Glister (2003), including 3 TGFß, 1 AMH (anti-mullerian hormone), 2 inhibins, 3 activins, 20 BMP (bone morphogenic proteins1-20), and 9 GDF (growth/differentiation factors1-9). Among them, TGFß, AMH, inhibins, activins, BMP 2, 4, 6, 7 15, and GDF9 have been shown to be expressed in ovary (Knight and Glister 2003).

Most of the TGFß superfamily members exert their function through membrane receptors. There are 7 type I receptors and 5 type II receptors (Knight and Glister 2003). Besides these receptors, there are a few binding proteins that can regulate the ligand activities though they don't have intracellular signaling capabilities. These include β-glycan, follistatin, α2-macroglobulin and inhibin binding protein (inhBP 120) (Knight and Glister 2003).

Activins and inhibins were primarily known for their abilities to stimulate (by activin) or suppress (by inhibin) pituitary FSH secretion, respectively (Knight and Glister 2001). Activin exerts its effect through the membrane type I and type II receptors (both are needed) and inhibin can interfere its function by first binding to β-glycan to increase affinity for activin type II receptor, then forming inhibin-β-glycan-type II activin receptor complex to prevent activin binding to the type II receptor, thus leading to the inhibition of activin activity (see a review by Kobayashi et al. 2006).

While ovary-origin inhibin could suppress FSH through a long loop negative feedback, activins are believed to be a local regulator (Knight and Glister 2001). This is supported by the fact that activins are expressed in multiple tissues (Meunier et al. 1988) and all the circulating activins are bound to follistatin, with no activity (Woodruff 1998). While the pituitary origin activins can stimulate FSH secretion, the ovary origin activins are believed to stimulate granulosa cell estradiol production (see a review by Kobayashi et al. 2006). Inhibin is reported to inhibit granulosa cell estradiol production, possibly through antagonism of activin action (Kobayashi et al. 2006).

The increase of circulating inhibin starts with the emergence of a follicular wave. In mares, it is believed the first two days of FSH depression is due to inhibin (Ginther et al. 2001a). The dominant follicles secrete an increasing amount of inhibin A (as well as estradiol) to cause the further decrease of FSH support (Ginther et al. 2001a). The increased inhibin A can also enhance LH-induced androgen production in thecal cells, which in turn supply the increasing production of estradiol by granulosa cells in the selected dominant follicles (Knight and Glister 2003). Activin, on the other hand, can attenuate the LH-induced androgen production in thecal cells (Knight and Glister 2003). As for the role of activin in dominant follicular selection, it is observed that there is a transient elevation of follicular fluid activin A, estradiol and free IGF1 in the new future dominant follicles when the largest follicles are ablated at the expected time of deviation (Ginther et al. 2002). There is also evidence that the stimulatory actions of FSH and IGF I on granulosa cell estradiol production is likely mediated by activin A (Glister et al. 2001). Thus, FSH induces an increase of activin in the future dominant follicles, which could

lead to higher aromatase activity and estradiol production, prevent atresia and promote survival (selection) (Knight and Glister 2003).

GDF9 and BMP15, both secreted from oocytes, were shown to be important for ovarian follicular development (for a review, see Juengel et al. 2004). GDF9 seems to be essential as female GDF9 knock out mice are infertile with follicular growth arrested at the primary stage (Dong et al. 1996). Both GDF9 and BMP15 can affect ovulation rate in sheep since heterozygous mutant sheep have an increased ovulation rate and homozygous mutants are infertile (Juengel et al. 2004). In cattle, GDF9 has been shown to promote granulosa cell proliferation, and inhibit estradiol and progesterone production induced by FSH and IGF-I together, but not the production induced by FSH alone (Spicer et al. 2006). Though GDF9 and BMP15 were shown to affect the expression/activity of FSH receptor in granulosa cells (Otsuka et al. 2000; Knight and Glister 2006), the role of these two proteins in dominant follicular selection is not clear.

BMP4 and BMP7 are selectively expressed in thecal cells and BMP6 is expressed in both granulosa cells and oocytes (Glister et al. 2004). All three has been shown to enhance basal and FSH or IGF-I stimulated secretion of estradiol, inhibin-A, activin-A and follistatin, promoting granulosa cell proliferation, while suppressing progesterone secretion (Glister et al. 2004). In cultured primary thecal internal cells, they were shown to suppress basal and LH-induced androgen production, similar to the effect of activin (Glister et al. 2005). Thus, BMP4, 6, and 7 could potentially affect the signaling

pathways initiated by FSH and IGF-I and contribute to the dominant follicle selection process (Knight and Glister 2006).

In summary, the TGF-β superfamily members may play important roles in follicular development, as they can potentially mediate the functions of FSH, IGF-I, and/or LH systems. The depression of FSH level by inhibin is a major part of the selection process. The increased steroidogenesis and granulosa cell proliferation, mediated by activin and other family members, could also contribute to selection.

IGF-system

IGF-I and IGF-II in association with gonadotropins can stimulate the growth and steroidogenesis of ovarian follicles. IGF-I and IGF-II are reported to stimulate steroidogenic acute regulatory protein gene expression (Bao and Garverick 1998; Le Roy et al. 2000), increase production of P450-17α-hydroxylase (Magoffin and Weitsman 1993), increase production of progesterone (Veldhuis and Furlanetto 1985), and synergize with FSH to enhance aromatase activity (Adashi et al. 1985). IGF-I and IGF-II also have the potential to prevent ovarian follicular cell apoptosis (for a review, see Quirk et al. 2004). The ovarian IGF system consists of different elements including (Mazerbourg et al. 2003; Fortune et al. 2004; Spicer 2004):

1) Two ligands, IGF-I and –II.
2) Two receptors (type I and II). Type I receptor mediates most of the somatomedin-like actions of both IGF-1 and IGF-II.
3) Six IGF binding proteins (IGFBP-1, -2, -3, -4, -5 and -6)

4) At least one IGF binding protein protease (PAPP-A, pregnancy associated plasma protein-A).

The expression of IGF-I and –II in bovine follicles is not in agreement among different studies. For example, Armstrong et al. (2000) have reported the detection of IGF-II only in thecal, not in granulosa, and no IGF-I in either thecal or granulosa cells by in situ hybridization. However, cultured granulosa cells show expression of IGF-II (Armstrong et al. 2000). Another lab has reported the detection of both IGF-I and –II in granulosa and thecal cells (Spicer et al. 1993; Spicer and Echternkamp 1995). A third study showed IGF-I in granulosa cells and IGF-II in thecal cells by in situ hybridization (Yuan et al. 1998). Despite the discrepancies at the mRNA level, there is no doubt that IGF proteins are present in the ovary. It was found that there are no differences in concentrations of total IGF (i.e. free IGF plus IGFBP-bound IGF) in the follicular fluid of dominant versus subordinate bovine follicles (de la Sota et al. 1996; Stewart et al. 1996). As local IGFBP regulate the bioavailability of free IGF, there could be fluctuations of the concentrations of IGFBP leading to changes in free IGF concentrations. This is supported by several studies which demonstrated that dominant follicles had lower binding activity of IGFBP-2, -4 and -5 than subordinate follicles (see the above mentioned reviews). These changes in binding activity or concentrations of follicular fluid IGFBPs could be due to locally decreased transcription, increase degradation (proteolytic activity), or both, or could be due to lower amounts of blood supply.

Changes of mRNA and protein levels of IGFBPS:

1) IGFBP2

By in situ hybridization, IGFBP-2 mRNA was nearly undetectable in the granulosa cells of dominant follicles but was expressed in subordinate follicles (Yuan et al. 1998; Canty et al. 2006). These results were supported by others using RT-PCR or real-time RT-PCR (Schams et al. 1999; Santiago et al. 2005), where IGFBP-2 mRNA was reported to be several fold lower in dominant follicles. When follicles were pooled into groups based on size, large (≥8 mm) healthy follicles had less IGFBP-2 mRNA in granulosa cells than small follicles or large atretic follicles (Roberts and Echternkamp 2003). These reports support that the IGFBP-2 mRNA decreases in granulosa cells of dominant follicles. The expression of IGFBP-2 in thecal cell is rare (Canty et al. 2006).

IGFBP-2 can be proteolytically degraded by PAPP-A, but IGFBP-2 is less sensitive than IGFBP-4 to cleavage by PAPP-A (Monget et al. 2003). The lesser sensitivity to degradation and the observed significant positive relationship between IGFBP-2 protein and its mRNA led Santiago et al. (2005) to suggest that **the decrease of IGFBP-2 protein level is likely caused mainly by the decrease of IGFBP-2 mRNA expression in dominant follicles of cattle**.

2) IGFBP3

The expression of IGFBP-3 was mainly localized to thecal cells, with low or no expression in granulosa cells (Yuan et al. 1998; Schams et al. 1999; Canty et al. 2006). In all studies, there were no differences of IGFBP-3 protein in follicular fluid or mRNA in thecal cells or granulosa cells (Santiago et al. 2005) between dominant and subordinate follicles or between different size groups. One

possibility that no change of IGFBP-3 was observed was because of its low expression even in thecal cells (Canty et al. 2006).

3) IGFBP4

For the expression of IGFBP-4, some reports show that there was no difference between the mRNA levels in granulosa cells of dominant and subordinate follicles, whereas there were several fold less protein levels in dominant follicles (Stewart et al. 1996; Mihm et al. 2000; Austin et al. 2001; Rivera and Fortune 2001; Spicer et al. 2001; Rivera and Fortune 2003a; Santiago et al. 2005; Canty et al. 2006). Others reported increased IGFBP-4 mRNA in large healthy follicles compared to small follicles or large atretic follicles (Schams et al. 2002; Roberts and Echternkamp 2003). Recent studies focusing on the regulation of IGFBP-4 reported high proteolysis activity of IGFBP-4 in dominant follicles by PAPP-A. Thus, despite the discrepancy at the mRNA level, it is generally agreed that **decreased levels of IGFBP-4 protein in the follicular fluid in dominant follicles of cattle may be due mainly to increased proteolysis of IGFBP-4**.

4) IGFBP5

IGFBP-5 protein levels were greater in the follicular fluid of subordinate follicles than in dominant follicles (Spicer et al. 2001; Schams et al. 2002; Rivera and Fortune 2003a; Santiago et al. 2005; Canty et al. 2006). The greater IGFBP-5 proteolytic activity and the lower levels of mRNA lead Santiago et al. (2005) to

suggest that **the lower levels of IGFBP-5 in dominant follicles may be due to both increased proteolysis and decreased gene expression**.

The changes in these IGFBP concentrations between dominant follicles and subordinate follicles, due to transcription or proteolysis, or both, strongly indicate that the IGF system plays a critical role in follicular selection. This idea was also supported by the observation that in GH-deficient cattle, which displayed a limited IGF1 production, follicular dominance did not occur and follicles were arrested at 8mm in diameter (Chase et al. 1998). The evidence for a critical role of the IGF system in follicular selection is reviewed by Rivera and Fortune (2003a) and Fortune et al. (2004). When follicular fluid from the two to three largest follicles in a cohort before divergence in size were examined using a surgical in vivo sampling technique, the follicles with the lowest concentration of IGFBP-4 always became dominant even though they don't always have the highest amounts of follicular fluid estradiol (Mihm et al. 2000). Based on the results of a series of experiments, Rivera and Fortune (2003a) have found that an increase in PAPP-A is the earliest biochemical difference in the future dominant follicles. They suggest that selection is the result of a progressive series of changes beginning with the acquisition of PAPP-A, which leads to a decrease of IGFBP-4 and -5 and an increase of free IGF. The increased free IGF synergizes with FSH to increase follicular estradiol production, which exerts negative feedback to decrease FSH secretion, preventing further development of the other follicles of the cohort (Fortune et al. 2004) (Figure 2).

In summary, there are great changes of IGFBP-2, -4 and -5 concentrations around the time of ovarian follicular selection. Among them, the most critical marker could be

the low IGFBP-4 concentration in the future dominant follicles, which is likely a consequence of increased PAPP-A in the future dominant follicles. This process could be a critical part of the selection mechanism.

Role of macrophages

Macrophages are immune cells derived from bone-marrow precursors, which when mature, enter the bloodstream as monocytes. They can then adhere to endothelial cells and subsequently migrate into tissues (Butcher 1992). As reviewed by Wu et al. (2004), macrophages in the ovary have multiple functions (Figure 3), including removing atretic cells, presenting antigens, releasing cytokines, growth factors and chemokines and secreting proteases.

Macrophages have efficient phagocytosis abilities. However ovarian macrophages phagocytose exogenous particles less efficiently than macrophages from other tissues (Itoh et al. 1999). Thus, ovarian macrophages possibly use phagocytosis primarily to remove apoptotic cells rather than foreign debris. Indeed, it has been shown that ovarian macrophages can phagocytose atretic granulosa cells and apoptotic luteal cells in multiple species (for a review, see Wu et al. 2004).

Macrophage involvement in ovarian follicular development is supported by several lines of evidence:

1) Ovarian macrophages start to localize to the thecal cell layer of healthy follicles and increase in numbers as follicles grow.
2) Ovarian macrophages secrete many factors that are known to regulate follicular development, such as cytokines (e.g. Interleukin-1, -6, -10,

and -12, interferon alpha, tumor necrosis factor α), chemokines (e.g. monocyte chemo-attractant protein-1 and -3, Interleukin-8), growth factors (e.g. epidermal growth factor, IGF, vascular endothelial growth factor, TGF α and β) and proteolytic enzymes (e.g. cathepsins, urokinase-type plasminogen activator, and matrix metalloproteinase). Although other cell types such as granulosa cells and thecal cells also secrete some of these factors, some of them are known to be preferentially expressed in macrophage-rich regions of the ovary such as the thecal layer, corpus luteum and atretic cells (for detailed information, see Wu et al., 2004).

3) Reduced numbers of ovarian macrophages are associated with reduced follicle growth and impaired fertility in two rodent models (Cohen et al. 2002; Duggal et al. 2002).

4) Co-culture of rat granulosa cells with peritoneal macrophages results in proliferation of the granulosa cells (Fukumatsu et al. 1992), and macrophage-like cells proliferate in co-culture with granulosa cells when cells from bovine antral follicles are cultured with their natural follicular fluid (Spanel-Borowski and Ricken 1997).

Combining the knowledge of the changes of location and function of macrophages during folliculogenesis, it was hypothesized by Wu et al. (2004) that **macrophages located in the theca of growing follicles can promote cell proliferation, stimulate follicle growth, and prevent apoptosis, while macrophages located in the**

granulosa cell layer (only found at advanced stages of atresia) are likely to phagocytize the apoptotic granulosa cells.

A role for macrophages in follicular selection is proposed by Bukovsky (2006). During selection of dominant follicles in human, the pericytes in the theca interna and in the vascular layer were highly activated in non-dominant follicles while they were activated only in the vascular layers in dominant follicles. What's more, the monocyte-derived cells (MDC) were non-activated in the vascular layer adjacent to the follicular basement membrane, activated in theca interna and invade the granulosa layer in non-dominant follicles while they were activated in vascular layer, non-activated in theca internal, and absent from granulosa layer in dominant follicles. Based on these observations, Bukovsky (2006) suggests that the theca interna compartment could play a critical role in follicular selection. If more studies can reveal the time sequences of the activation of those pericytes and MDC and their regulations along with the follicular diameter and endocrine changes, it would be much easier to explain the process of dominant follicular selection.

Apoptosis

In mammalian ovaries, once the growth of primordial follicles is initiated, a majority will go atretic at some time point with less than one percent proceeding to ovulation. Atresia is a process that exhibits both biochemical (including endocrinal) and morphological features of programmed cell death (apoptosis) (for a review, see Johnson 2003). Atretic follicles can usually be identified by features such as cell membrane

blobbing, chromatin condensation and DNA degradation, as well as changes of hormone levels in follicular fluid. Morphological criteria can be used on both small and large follicles while endocrinal criteria are usually used on large follicles since measurement of follicular fluid concentrations of estradiol and progesterone is needed.

The most comprehensive morphological classification of atretic follicles in cattle was carried out by Irving-Rodgers et al. (2001). Based on a complete study combining histological, ultrastructural and immunohistochemical data, the authors simplified the classification of atresia into two types, **antral atretic** follicles and **basal atretic** follicles, based on where the granulosa cells first die. In antral atretic follicles, the membranal granulosa cells close to the antrum die first, while in basal atretic follicles, the basal layer granulosa cells die first, followed by the cells closer to the antrum. The two types of atresia have quite different behaviors indicating different mechanisms of apoptosis. However, basal atresia is only seen in follicles < 5mm while antral atresia is common in all follicle sizes (Irving-Rodgers et al. 2001).

Atresia is major player in follicular selection as the death of subordinate follicles ensures the selection of only one dominant follicle. The survival and death of different individual follicles are likely determined by the crosstalk among endocrine, paracrine and autocrine factors. The proto-oncogenes, tumor suppressor genes, survival genes and death genes are likely working together to modulate the process (for reviews, see Amsterdam et al. 2003; Sasson and Amsterdam 2003; Quirk et al. 2004). In fact, the earliest two detectable differences between the healthy dominant follicles and subordinate follicles which will undergo atresia are the greater availability of IGF1 in (future) dominant follicles and its greater ability to produce estradiol (see discussion about estradiol and

IGF). Both IGF1 and estradiol have been shown to protect granulosa cells against apoptosis and to stimulate granulosa cell proliferation in vitro. Their growth promoting effects are likely due to their ability to promote the cell cycle G1-to-S progression as that is the time when granulosa cells predominantly undergo apoptosis (Quirk et al. 2004).

It has been suggested that survival factors derived from oocytes, such as IGF-1 or GDF-9, are the major factors that determine the fate of primordial and primary follicles, while in late pre-antral and larger follicles, granulosa cell products are the major determining factors (Johnson 2003). Among them, the gonadotropin initiated Protein kinase A (PKA) signaling pathway is the major survival pathway. This pathway can be activated by FSH, LH, VIP (vasoactive intestinal peptide), or PACAP (pituitary adenylate cyclase-activating polypeptide). Downstream of PKA there is up-regulated expression of Bcl-2, Bcl-xL, Mcl-1, IAPs, etc. These either directly promote survival, or suppress the expression of proapoptotic factors (such as Bax, Apaf1, Caspase 3), or inhibit the activities of the proapoptotic factors (Johnson 2003).

Besides the PKA pathway, there is also evidence that FSH activates other pathways, including PKB, PKC, etc. PKB (protein kinase B, also known as Akt) is activated through phosphorylation by PDK1. Besides FSH, IGF-1 and TGFα (transforming growth factor α) are known to activate PKB and to promote granulosa cell survival (Westfall et al. 2000; Johnson et al. 2001). The whole pathway involves a series of actions, including:

1) Binding of ligands (e.g., IGF1, TGFα) to the receptor,
2) Activation of PI3K,
3) Phosphorylation of PIP2 to PIP3 by PI3K,

4) Recruitment of PKB by PIP3 to the membrane,

5) Phosphorylation of PKB by PDK1.

Once PKB is phosphorylated, it is fully activated and can act on downstream targets. A Inhibitor of PI3K, which blocks the PKB pathway, can act against the survival promoting effect of IGF1 and TGFα, and promote apoptosis (Asselin et al. 2001).

Downstream of PKB, there is potentially activation of IAP, Bcl2, and Bcl-xl (through IKK activated Nf-kB) to promote survival, or inhibition of Bad, Caspase 9, and Forkhead family proteins to prevent apoptosis (Johnson, 2003; KEGG pathway, http://www.genome.jp/kegg/kegg2.html). Interestingly, the activation of Nf-kB by PKB can be either anti-apoptosis or pro-apoptosis, depending on how the signal initiates. For example, TNF signals will activate Nf-kB through PKB to induce c-myc and apoptosis (Ozes et al. 1999).

More importantly, when granulosa cells are treated with inhibitors of the PKB pathway (PI 3-kinase-inhibitor LY294006) and with activators of the PKA pathway (such as 8-bromo-cAMP, LH) (Johnson et al. 2001), apoptosis is inhibited. When granulosa cells are treated with inhibitors of the PKA pathway and activators of the PKB pathway (such as IGF1), apoptosis is induced (Johnson 2003). This indicates that **the PKA pathway is the dominant survival pathway, and the PKB pathway is likely an important auxiliary survival pathway**.

The PKC pathway, as well as other signaling pathways, such as the Sgk (serum and glucocorticoid-induced kinase) pathway and the ERK (extracellular signal-regulated kinase) pathway, have been related to promoting cell survival. However, they may not be essential as blocking these pathways doesn't promote apoptosis (Johnson 2003).

Besides the above mentioned FSH, LH, IGF1, and TGFα, other main survival factors include EGF (epidermal growth factor), FGF (fibroblastic growth factor), prolactin, laminin, leptin, glucocorticoid and estradiol (Amsterdam et al. 2003). FGF can active PKC delta, which in turn, modulates calcium homeostasis and promotes granulosa cell survival (Peluso et al. 2001). Glucocorticoid can upregulate the expression of Bcl-2 and/or attenuate its degradation, providing a protective effect on apoptosis induced by serum deprivation, cAMP, P53 or TNFα (reviewed by Amsterdam et al. 2003). Glucocorticoid can also increase the level of phosphorylated PKB, leading to cell survival (Sasson and Amsterdam 2003).

As for apoptotic pathways, there are usually multiple redundant pathways that can respond to internal and external signals. Internal signals, such as the withdrawal of growth factors or activation of P53, usually induce the perturbation of mitochondria to release cytochrome c, activate proapoptotic proteins such as Bad and Bax, and inactivate antiapoptotic factors such as IAP. These will lead to the activation of a caspase cascade and cause cell death (Bridgham et al. 2003). This caspase cascade starts from the release of cytochrome c from mitochondria, followed by the formation of apoptosome (cytochrome c, Apaf1, procaspase9), the activation of Caspase 9, then Caspase 3 and 7, and finally Caspase 6. There are also apoptotic signals, such as activation of granzyme B, that could bypass the mitochondrial signals for apoptosis (Amsterdam et al. 2003).

External signals, such Fas ligand, TRAIL, TNFα, IL-1, will bind to the corresponding death receptors, and initiate another caspase cascade (from Caspase 8 and 10 to Caspase 3 and 7, then Caspase 6), leading to cell death (Bridgham et al. 2003). While this caspase 8-initiated caspase cascade bypasses cytochrome c, caspase 8 can also

activate Bid which will trigger the above mentioned mitochondrial pathways, leading to cell death (Hengartner 2000).

There are many reports of the change of expression levels of apoptosis genes between the dominant and subordinate follicles. For example, Fas antigen mRNA levels in granulosa cells are much higher in subordinate than in dominant follicles (Porter et al. 2000). By applying microarray technology, a number of apoptosis genes was identified to be differentially expressed between dominant and subordinate follicles (Evans et al. 2004) or among follicles from different days of the estrous cycle (Mihm et al. 2006). These include anti-apoptosis genes such as BCL2L2 and MCL1 and apoptosis genes such as FADD, TNF, Apaf1, and Death receptor 6 (for a more detailed list, please refer to the two references). The changes in these gene expression levels seem to coordinate with changes in other genes, such as LH receptor, FSH receptor, etc, to determine the final differentiation stages of the follicles and to initiate apoptosis in the subordinate follicles, leading to selection of the dominant follicles.

Summary

Dominant follicle selection is a complex process involving the co-action of many factors, including gonadotropins (FSH and LH) and receptors, steroids (e.g. estradiol), insulin-like growth factor system (e.g. IGF-1), transforming growth factor-β superfamily, etc. It is likely that there is a delicate balance between the survival and apoptosis signaling pathways, involving the cooperation of different cell types, including theca, granulosa, oocyte, and possibly even macrophage cells. The core of this process is the

decline of FSH concentration so that survival signals (mainly through the PKA signaling pathway) eventually become weak. Inhibin and estradiol are the major factors causing the decline of FSH, primarily from the future dominant follicles at the time of deviation. The growth of the future dominant follicles, however, is not affected, possibly because of the higher free IGF and estradiol. The higher free IGF is the result of the decreased IGFBP caused by their lower transcription levels and/or higher proteolytic activities of PAPP-A. This higher IGF could result in the activation of the survival signaling pathway through PKB. The higher estradiol is likely a result of many effects including FSH and IGF actions mediated by activin and other BMP proteins. The higher estradiol could potentially increase the granulosa cell's sensitivity to FSH to maintain the survival signal even at the lower FSH concentration. Then, when LH receptor is expressed in the granulosa cells, the LH activated survival signal (the PKA signaling pathway) is maintained even though FSH is low, and the selected dominant follicle can now depend on LH for later survival.

Chapter III

Construction of a Bovine cDNA Array

Abstract

The microarray has been a useful tool for investigating changes in gene expression profiles between different samples. It has successfully been used in species including the human and the mouse and has many interesting discoveries. However, for the bovine, the application of microarrays has been limited by the lack of high content arrays. To use microarray technology in our lab, we constructed a bovine cDNA microarray for gene expression studies. There are many factors affecting the construction of the array and the hybridization results, such as the amplification of the cDNA clones, coating of the slides, printing buffers, probe labeling, and hybridization conditions. After testing different conditions, we first created a relative low content bovine cDNA array with 1,536 clones (Bovine 1.6K cDNA array) from a bovine ovary cDNA library. Then, a high content cDNA microarray with 17,692 bovine cDNA clones (Bovine 18K cDNA array) was constructed. Re-sequencing of PCR products from the plates used to print the slides was used to confirm clone identities. Quality checks indicate that these arrays (Bovine 18K cDNA array) are of high quality and will be a useful tool for gene expression investigation.

Introduction

The last decade was a booming period for new technologies for the massively parallel assessment of gene expression, including differential display (Liang and Pardee 1992), serial analysis of gene expression (SAGE, (Velculescu et al. 1995)), cDNA microarray (Schena et al. 1995), oligonucleotide arrays (Lockhart et al. 1996), and massively parallel signature sequencing (Brenner et al. 2000). Among these, microarrays (both cDNA and oligo arrays, homemade or commercial ones) have been the most widely used due to the large amount of information produced in a single experiment and the advantage of being relatively less time consuming.

The principles of the different microarray platforms are quite similar, though specific details differ (Affymetrix Chips will not be discussed here). Probes (cDNA or oligonucleotide (50-mer to 70-mer)) representing the genes of interest are immobilized on specifically coated microscope slides. To investigate the expression patterns of these genes in target cells or tissues, mRNAs are extracted from target sample tissues or cells. Then cDNAs are synthesized from the mRNAs and labeled with different fluorescent dyes. Two cDNA samples labeled with different dyes (e.g., cy3 and cy5) can be co-hybridized onto one slide and the signals of both cy3 and cy5 can be determined for each probe present on the array. The intensities of these signals reflect the relative abundance of the mRNA copies. Analysis of these data reveals the differences of gene expression profiles between different samples.

The cDNA microarrays are usually constructed by mechanically spotting tens of thousands of DNA fragments at high density on the surface of glass microscope slides coated with appropriate materials that help attach the DNA fragment to the slides. cDNA

fragments can be identified by expressed sequence tag (EST) data. ESTs are single-pass sequence readings (usually ~600 bases) of the cDNA clones that usually identify partial sequences from one end of the cDNA.

In our lab, we have first constructed a small scale bovine cDNA array with 1,536 clones from a bovine ovary cDNA library (Takasuga et al. 2001). When much higher content cDNA libraries (the USDA BOV1-5 libraries (including ovary) (Smith et al. 2001; Sonstegard et al. 2002)) became available, we combined the two libraries and created a bovine cDNA array containing a total of 17,692 bovine cDNA clones.

Results

The whole process of array construction and hybridization contains several steps, as illustrated in Figure 4. The construction of the array starts with cDNA amplification and purification. The purified cDNAs are re-suspended in printing buffer and are printed on specifically coated slides. Once the arrays has passed a quality check, they are ready to be used in hybridization experiments.

Hybridization includes the extraction of RNA and labeling of cDNA. The labeled cDNAs are hybridized to the array and the signals can be extracted by scanning the array. The results from the scanned picture can be stored for further analysis.

A. Construction of the array

1. cDNA amplification and purification

The clones from the bovine ovary cDNA library were sequenced from both ends. They include the target DNA fragment inserted in the pZL1 vector (Figure 5). This vector contains the M13 forward and reverse primer sequences and T7/SP6 primer sequences. Thus, we can amplify the cDNA inserts with M13 primer pairs (from IDT DNA, Coralville, IA).

M13 forward: 5'-CCC AGT CAC GAC GTT GTA AAA CG

M13 reverse: 5'-AGC GGA TAA CAA TTT CAC ACA GG

The USDA BOV library cDNA clones are selected from the original library to represent as many unique sequences as possible. All of them were sequenced from the 5' end and many were also sequenced from the 3' end. These clones have the target DNA fragment inserted in the pCMV·SPORT6 vector (Figure 6). This vector also contains the M13 forward and reverse primer sequences and T7/SP6 primer sequences.

The cDNA clone inserts can be amplified by PCR either directly from the cultured clones or from plasmid DNA prepared from the clone. For high-throughput applications, it is too time-consuming and costly to prepare plasmid DNA for all of the clones. The huge number of plasmid preparations also risks cross-contamination. Direct amplification from cultured clones avoids all these problems and still has a relatively high success rate (~87.5% by Hegde et al. 2000). Thus, we have chosen to amplify the cDNA directly from the culture clones.

The problem of using culture medium directly in a PCR reaction was that the PCR efficiency was affected. Testing with different amounts of culture medium (0.5, 1, 1.5, and 3 µl) in a 50 µl reaction volume indicated that the smaller the concentration of the template medium, the stronger the product band. We also found that if the medium was first boiled, we obtained a much cleaner product with less primer dimers. Combining these findings, a protocol was developed that was used to amplify the forty-six 384-well plates from the USDA BOV library and the sixteen 96-well plates containing the bovine ovary cDNA library.

Briefly, each 384-well plate was divided into four 96-well plates for PCR amplification. A small aliquot (0.75 µl) of culture medium was taken from each well and put into 19.25 µl of water in 96-well PCR plate and boiled for 20 minutes. Then 30 µl PCR master mixture containing all other PCR contents except the DNA template was added into each well. The reaction was run for 35 cycles.

PCR reaction: (50 µl per reaction) (for 4 plates)

Total	**10µl**	**×5×100×4**	**20ml**
DNA Template	4µl		
H_2O	3.5 µl		7 ml
10Xbuffer	1 µl		2 ml
dNTP (3mM each)	1 µl		2 ml
Primer For. (20µM)	0.2 µl		0.4 ml
Primer Rev. (20µM)	0.2 µl		0.4 ml
TaqE	0.1µl		0.2ml

PCR program: (B55EST)

1. 95°C 1 min
2. Pause press enter to continue
3. 95°C 2 min
4. 95°C 30 sec
5. 55°C 30 sec
6. 72°C 1.5 min
7. Go to step 2, 35 cycle
8. 72°C 10 min
9. 4°C Hold

After PCR, 3 µl of PCR reaction was taken from each well and checked for amplification status by gel electrophoresis (1% agarose gel) with ethidium bromide staining. If a clear amplification band was seen, the status was recorded as successful; otherwise, it was recorded as failed.

If the plate had an over 80% amplification success rate, the PCR products were purified. Several different precipitation methods, including ethanol precipitation, isopropanol precipitation, 3M sodium acetate (pH5.2), and 10M ammonium acetate were tested. All of them worked well for 1.5 ml tubes. They also occasionally worked for 96-well plates, but were not reliable, due to the low centrifuge force used. Most of the DNA samples were not centrifuged and were lost in the supernatant. Later the 384-well

MultiScreen PCR purification plates (Millipore, Billerica, MA) were used and good DNA recovery rates were obtained.

With the modified method, most of the cDNA clones were successfully amplified and purified for the two libraries. Most of the plates had an amplification rate of around 80% (see Figure 7). The average amplification rate was 79.6%.

Small aliquots of the purified DNA in the ready-to-print plates were taken out and sequenced to check for possible plate mismanagement problems. We had a total of 65 good quality sequencing reads. Among these, 26 individual clones from the USDA BOV 1-5 library had good matches with their originally assigned EST ID sequence (Table 2).

For the 39 individual clones (Table 3) from the bovine ovary cDNA library, most of them matched clones different from their assigned positions, with a few clones having no match to ESTs in the library. There was no pattern indicating any misplacement by our PCR or purification process. It was likely that these differences had their origins in how the master plates were made by the original authors. Nevertheless, the identity of the clones from this library needs to be confirmed by re-sequencing before further analysis.

2. cDNA re-suspension in printing buffer and array printing

We tested poly-L-lysine coated slides (home-made) and GAPS II slides (Corning Incorporated, Corning, NY). Both worked well using our protocol.

For printing buffers, we tested 3×SSC (standard sodium citrate, 1×SSC: 0.15 M NaCI, 0.015 M sodium citrate) (Shalon et al. 1996) and DMSO with CHAPS (50% DMSO with 1% CHAPS). The later buffer was shown to produce the most regular spots and decreased evaporation during printing (Rickman et al. 2003). DMSO can also denature DNA (Hegde et al. 2000).

The machine (Figure 8A) we used to print the array is a high-speed robotic system originally described by the Brown Lab (http://cmgm.stanford.edu/pbrown/mguide/index.html). It was assembled in our lab by Dr. Eric Antoniou. The print head on the array can hold up to 32 pins (Figure 8B), arranged in a 4×8 format. It is a contact printing system. The pins use capillary action to draw fluid into the pin chamber and when the pins make contact with the slide surface, the surface tension drags a small aliquot of solution onto the slide. The pins (MicroQuill DNA Array Pin, Majer Precision Engineering, Tempe, AZ) are tested thoroughly and those that can print high-quality uniform spots are assembled into the machine (see Figure 9 for a test printing). The printing humidity was controlled at 50% and the temperature was set at room temperature (~22°C), which is very close to the best condition (45% humidity, 22°C) described by Hegde et al. (2000).

The arrays printed on the GAPS II slides had a uniformly low background. The background signal under most experimental conditions was lower than 60. The arrays printed with the poly-L-lysine slides also showed good uniform low background, although higher than for the GAPS II arrays (<300). The printed poly-L-lysine arrays still

show good spot morphologies and signal-to-background ratio without serious damage after 19 months of storage (Figure 10), far longer than the 6 months storage limitation recommended by other studies.

3. Array re-hydration

Following printing, the slides need to be treated before they can be used for hybridization. It is a re-hydration process with the aim of increasing the spot size and spreading DNA more uniformly within each spot instead of aggregating in the center when dried slowly. The time of re-hydration is important which directly affects the size of the spots. Different times (ranging from 30s to 90s) should be tested for different batches. The treatment also differs for different slide coatings.

For cDNA printed with 3×SSC on poly-L-lysine coated slides, the slides are first re-hydrated at 55°C for 45 seconds. Then the slides are snap-dried on a 100°C hot plate surface for 5 seconds. These slides are then ready for hybridization treatments.

For cDNA printed with 3×SSC on GAPS II slides, the slides are first re-hydrated and snap-dried as are the poly-L-lysine slides. Then the slides undergo UV cross-linking by using a UV cross-linker (300 mJoules). The slides are ready for hybridization after UV treatment.

For cDNA samples printed with DMSO, there is usually no need to run the re-hydration. Slides can be directly cross-linked by UV treatment (also with 300 mJoules).

4. Array pre-hybridization

Before hybridization, slides must be treated to decrease background and increase specificity. As pointed out by Hedge et al. (2000), the slides must be blocked to prevent the labeled DNA from binding to the slides. It also has the advantage of washing off the un-stabilized DNA probes printed on the array.

We have found that blocking the slides with 0.2% I-block (Tropix/Serva, Heidelberg, Germany) in 1×PBS produces a really clean background. Briefly, the slides are blocked in the I-block buffer for at least one hour at 42°C. Then, the slides are washed with 0.2% SDS once and with water twice (2 minutes each). The slides are dried by centrifugation and should be used as soon as possible (within one week). **This pre-hybridization step must be done for all slides whether used for quality checking or for real experimental hybridization.**

B. Sample labeling and hybridization

In a typical cDNA microarray gene expression hybridization, two samples are labeled with different fluorescence dyes and hybridized together on the same slides. There are different ways to label samples (for examples, see Richter et al. 2002). The amount of RNA needed per reaction is usually the major concern.

1. direct labeling and aminoallyl labeling

cDNA can be labeled either directly by including a certain amount of cy3 or cy5 labeled dUTP (Amersham Cat#PA53022 and PA55022) in the reverse transcription reaction, or the cDNA can be labeled indirectly by including a certain amount of aminoallyl labeled dUTP (Ambion, Cat#8439) in the reaction. The aminoallyl labeled cDNA can be conjugated with either cy3 or cy5 (mono-functional Cy3 or Cy5 reactive pack, Amersham Cat#PA23001 and PA25001) after the reverse transcription reaction. The indirect labeling method usually has higher reverse transcription efficiency and labeling efficiency.

We first tested the protocol with indirect labeling through aminoallyl dUTP incorporation (Figure 11A). The protocol is based on a procedure from the Brown Lab with modifications (http://cmgm.stanford.edu/pbrown/protocols/aadUTPCouplingProcedure.htm). Briefly, the reverse transcription reaction, hydrolysis, cDNA cleanup and dye coupling are the same. After dye coupling, each sample is individually cleaned by using a Qia-Quick PCR Purification Kit (Cat#28104, Qiagen, Valencia, CA) and the concentrations are measured to determine yield.

This method usually requires 10-15 µg of total RNA per reaction. Thus, for a typical experiment in which one sample should have at least three replicates, **it usually requires a total RNA yield of 50 µg or more from the tissue.**

2. T7 RNA polymerase amplification

As mentioned above, the requirement of more than 50 μg of total RNA has limited the application of microarray on many precious tissues or cells. Thus, people have tried different ways to overcome this problem. One of the methods is to amplify the antisense RNA (aRNA) (Phillips and Eberwine 1996) (Figure 11B). One round of amplification could result in a 2,000 fold amplification of antisense RNA (Phillips and Eberwine 1996). It has been successfully used in microarray gene expression studies (Wang et al. 2000).

The protocol used in our lab is based on that of Wang et al. (2000) (http://cmgm.stanford.edu/pbrown/protocols/index.html).

Briefly, double stranded cDNA is synthesized from 2 μg of total RNA by using the Promega Universal RiboClone cDNA Synthesis System (Cat#C4360, Promega) with Eberwine oligo-dT/T7 primer in the first strand reaction.

Eberwine Oligo-dT/T7 primer:

5'AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC TTT TTT TTT TTT TTT-3'

The purified double stranded cDNA is used as a template to amplify the antisense RNA using the MegaScript T7 kit (Cat#1334, Ambion). The aRNA are DNase I treated and purified. The purified aRNA can then be used to make labeled cDNA using random hexamer priming and indirect labeling (see above).

3. 3DNA Array 350 Kit labeling system

The aRNA strategy is a good method when sample RNA yield is really low. However, it is very time consuming and costly. The 3DNA array 350 Kit labeling system (Cy3 kit, Cat#W300130; Cy5 kit, Cat# W300140) is an easy-to-use, high sensitivity, and consistent quality labeling system. It gives good hybridization results with relatively small quantities of RNA samples (3-5 μg total RNA per reaction).

The working principle is illustrated in Figure 11C. Briefly, the reverse transcription is done with the poly-dT primer attached to a specific 3DNA capture sequence specific for Cy3 or Cy5. Then, the purified cDNA is hybridized to the array overnight. After washing off the unbound cDNA, the 3DNA capture reagent, which contains 375 fluorescent dyes per molecule, is hybridized to the array and binds to the capture sequence. After washing off unbound 3DNA reagent, the signal can be captured by a scanner.

In summary, all three methods give good hybridization results. A suitable method can be chosen based the amount of available RNA.

The actual hybridization procedures are usually related to the labeling method and have been covered in the protocols provided with the labeling methods. Thus, they will not be described again.

The aminoallyl labeling method gives very good signal intensities and reproducibility. However, we had to switch to the aRNA method or 3DNA labeling system due to limited RNA quantities (a few of the follicles yielded less than 50μg RNA). A comparison between the two methods indicates that the 3DNA labeling system has a

much higher reproducibility than the aRNA method (Figure 12). Thus, the 3DNA labeling system (with 3DNA 350 kits) was used in our experiments.

Self-self hybridization with the bovine 1.6K array indicated that the mean ± 2SD fold-change for all the genes on these arrays is 1±0.40.

Summary

In this study, we have successfully tested or optimized the critical conditions to run microarray gene expression studies, including PCR amplification of cDNA clones, array printing, sample labeling and hybridization. We have printed several batches of arrays including the bovine 1.6K array and the bovine 18K array. These arrays have been tested and proven to be useful for gene expression studies.

Chapter IV

Data Storage and Bioinformatics Analysis

Abstract

With the wide utilization of microarray technology, the demand to store and analyze the huge amount of data created in the microarray experiments has dramatically increased. There are many commercial or free software packages available to help achieve different data storage and analysis goals. Several different softwares were explored and selected from a set of free programs which are suitable for a small lab to independently achieve the data management requirements. These include BASE for data storage (laboratory information management system) and normalization, and MAANOVA for ANOVA analysis. Several different ways of getting gene annotations for an EST sequence are also discussed.

Introduction

In microarray experiments, differential gene expression is assessed by utilizing the signal intensities from both the cy3 and cy5 channels from all of the genes and arrays. There is much information related to each spot on each array, including spot ID, spot location, diameter, foreground and background intensities (mean, median and standard

deviation) for both channels, spot status (good or bad), and gene information etc. Thus, **there are 14-15 essential information points collected for each spot, more than a quarter of a million data points for one hybridization on a 20K array**. It is impossible to collect and manage this huge amount of data manually. A microarray database is needed.

Results

A. BASE for data storage and normalization

BASE (Bioarray Software Environment) (Saal et al. 2002) is designed for the comprehensive management and analysis of microarray data. BASE provides a LIMS (laboratory information management system) environment. All information related to the microarray experiment is stored and linked, from the sample collection, RNA extraction and labeling, array printing, hybridization, to raw image, resulting file and data analysis. Therefore, anyone can know exactly how the whole experiment was carried out. It also lets the users view and extract data easily with many flexible criteria.

BASE runs on a Linux platform and needs Apache (web server), MySQL (database) and many other libraries. **A main stream desktop equivalent computer (Dual-core, 2 gigabytes of memory) should be enough for a lab with 100 gigabytes of data (about 1,000 hybridizations)**.

The current version (BASE1.2.17) running in our lab is located at http://amadea.asrc.agri.missouri.edu/index.phtml. The installation and maintenance of BASE 1.2.17 is in Appendices.

A simple schematic overview of BASE database is presented in Figure 13.

B. MAANOVA for statistical analysis

After data normalization, the values need to be modeled to remove the effects of array, dye, etc, before an unbiased meaningful comparison can be made. MAANOVA (MicroArray ANalysis of VAriance) (Wu et al. 2002) is one of the software packages designed for this purpose. Statistical analysis of gene expression data from two-color cDNA microarray experiments can be completed and is based on the 2-stage ANOVA model of Wolfinger et al. (2001).

The first stage is the normalization model to remove the effect of Array and Dye.

$$\mathbf{Y}_{ijkgr} = \mu + \mathbf{A}_i + \mathbf{D}_j + \mathbf{AD}_{ij} + \mathbf{r}_{ijkgr}$$

Here Y is the log intensity reading for a particular gene on a certain array, e.g. array "i", dye "j" of RNA variety "k"; μ is the overall mean; A_i is the effect of array i, D_j is the effect of dye j; and AD is the effect of array by dye interactions. The last term, $\mathbf{r}_{ijkgr}$ is the residual and will be used as the dependent variable in the subsequent model.

The second stage is a gene specific model.

$$\mathbf{r}_{ijkgr} = \mathbf{G} + \mathbf{AG}_i + \mathbf{DG}_j + \mathbf{VG}_k + \boldsymbol{\varepsilon}_{ijkr}$$

Here G is the average effect of the gene; AG is the array-by-gene variation; DG is the dye-by-gene variation. VG is the effects of the treatment on the expression of the gene of interest. The error "ε" is the residual.

The second stage model is flexible. Factors can be added or removed from the model, according to the experimental design.

After modeling, a permutation F-test (Cui et al. 2005) can be applied by MAANOVA, and P values adjusted for multiple tests will be calculated for every gene to indicate if expression is changed by treatment/sample.

C. TMEV for clustering

TMEV (TIGR MultiExperiment Viewer) (Saeed et al. 2003) is a versatile microarray data analysis system. It has implemented many sophisticated algorithms for clustering, visualization, classification, statistical analysis and biological theme discovery (http://www.tm4.org/mev.html).

D. DAVID for gene list functional annotation

DAVID (The Database for Annotation, Visualization and Integrated Discovery) (Dennis et al. 2003) is a web accessible service (http://niaid.abcc.ncifcrf.gov/) that provides a set of functional annotation tools to facilitate the extraction of biological meanings of a list of genes.

The combination of these four software/database/website-services provides a suite of tools for management and analysis of microarray data.

E. Gene annotation

In data collection, organization, normalization, ANOVA modeling, and gene clustering, we only need a unique identifier for each probe/gene on the array. Further functional analysis of the selected subset of genes requires that gene annotations are known in order to identify interesting pathways, biological processes, molecular functions, and cellular localization, etc. This is not a problem for commercial arrays as they usually come with annotation information. However, for our bovine arrays, we only have the 5' and/or 3' EST GenBank accession numbers for the cDNA clones printed on the array.

Several methods have been applied to assign gene annotation to the ESTs.

1. Annotation of all EST on arrays

1.1. TIGR annotation

TIGR (The Institute for Genomic Research) has a rich resource on the bovine EST gene annotation, called the TIGR Cattle Gene Index.

http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=cattle

The way TIGR assigns a gene identity to an EST sequence is as follows: good quality EST sequences are compared and clustered together if they meet the following criteria:

a) a minimum of 40 base pair match

b) greater than 94% identity in the overlap region

c) a maximum unmatched overhang of 30 base pairs.

These clusters are then assembled into Tentative Consensus (TC) sequences using The Paracel Transcript Assembler. The TC sequences are then searched against a non-redundant protein database and the results are linked together. (For more details, refer to the TIGR website:

http://www.tigr.org/tdb/tgi/faq2.shtml).

The newest available version of the TIGR Cattle Gene Index is Release 11.0, released on September 28, 2004. The website supports a Batch search. However, the output contains sequence information which makes it not suitable to annotate 17, 692 ESTs through web queries. Thus, we have downloaded the database and have written a Perl program to extract the TC number, Gene annotation, and Gene Ontology terms.

The files downloaded from the TIGR database are:

BTGI (the file contains TC number, corresponding sequence, and Gene Annotation)

BTGI.GO (the file contains TC number, Gene Ontology term)

BTGI.TC_EST (the file contains TC number, corresponding EST number)

BTGI.TCs (the file contains different version of TC numbers)

By combining the TC number, Gene Annotation, and Gene Ontology together and linking to the EST sequences, we obtained annotation for 86.5% (15,307) of the cDNA probes, as shown below:

CloneID	1BOV100B3
5' read Accession	AW654745
3' read Accession	CK770542
TC	TC290553

GO term	[GO:0007498(mesoderm development)()(P)] [GO:0006366(transcription from Pol II promoter)()(P)] [GO:0003677(DNA binding)()(F)] [GO:0003702(RNA polymerase II transcription factor activity)()(F)] [GO:0003714(transcription corepressor activity)()(F)] [GO:0009790(embryonic development)()(P)] [GO:0005634(nucleus)()(C)][GO:0006355(regulation of transcription, DNA-dependent)()(P)] [GO:0003700(transcription factor activity)()(F)]
Annotation	homologue to UP|TF21_HUMAN (O43680) Transcription factor 21 (Podocyte-expressed 1) (Pod-1) (Epicardin) (Capsulin), complete

1.2. DRAGON annotation

The second method we used to annotate of the ESTs was through the DRAGON (Database Referencing of Array Genes Online) (Bouton and Pevsner 2000) website:

http://pevsnerlab.kennedykrieger.org/annotate.htm

DRAGON uses the UniGene ID as the link to extract information from different databases, such as UniGene, Swiss-Prot, Pfam and the Kyoto Encyclopedia of Genes and Genomes (KEGG). It takes the GenBank accession numbers of the ESTs and finds the corresponding UniGene IDs, then extracts related information from all the databases according to the user's query. It is very convenient and can handle large gene lists.

By using DRAGON, we obtained the annotation of about 85% of the cDNA samples presented on the array, as shown below:

CloneID	1BOV100B3
5' read Accession	AW654745
3' read Accession	CK770542
UniGene-ID	Bt.5219
Annotation	Transcription factor 21

However, due to the different ways TIGR and DRAGON compute the annotations, there are about 20% discrepancies between the annotation results from these two databases. TIGR annotation was used in the case of discrepancies. About 9% of the probes on the array are not annotated by neither method.

2. Further annotation of differentially expressed probes

2.1. BLAT annotation

BLAT (BLAST-like alignment tool) (Kent 2002) on DNA is designed to quickly find sequences of 95% and greater similarity over a length of 40 bases or more. This tool is available on the UCSC Genome Bioinformatics website (http://genome.ucsc.edu/), together with the rich resource of the genome sequences of different species.

As the human genome sequence is the most complete sequence, and human gene ID usually links to the most detailed information about a gene, we chose to search the differentially expressed bovine EST sequences against the human genome.

BLAT can reveal to which part of the gene the EST sequence matches, whether it is in the untranslated region, or if it includes the translated region. The problem with BLAT is that in order to make sure that the match corresponds to a gene and not to some genomic region where no known gene is located, one has to run the search one EST clone at a time. Consequently, the annotation process is very time consuming.

The steps to run a BLAT search are illustrated in Figure 14. Using BLAT, we manually checked about 450 probes. An output example is given below:

Description transcription factor 21

Representative Refseq	NM_198392
Protein	O43680 (aka TCF21_HUMAN or TF21_HUMAN)
RefSeq Summary	TCF21 encodes a transcription factor of the basic helix-loop-helix family. The TCF21 product is mesoderm specific, and expressed in embryonic epicardium, mesenchyme-derived tissues of lung, gut, gonad, and both mesenchymal and glomerular epithelial cells in the kidney. Two transcript variants encoding the same protein have been found for this gene.

2.2. BLAST annotation

BLAST (the Basic Local Alignment Search Tool) compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches if local similarities are found between sequences (http://www.ncbi.nlm.nih.gov/blast/index.shtml and references therein).

There are several advantages running a BLAST search. First, BLAST will return the results with an Expected (E) value which describes the number of hits one can "expect" to see just by chance when searching a database of a particular size. Thus, not only does it find the matching sequence, it also tells you whether the matching is unique or not. Basically, the lower the E value, the more likely the match is genuine and unique. Second, one can set up a server to run the search locally or use a network client to submit a list of queries to NCBI, which means a huge list can be run automatically.

We chose to set up the network client to run the search.

First we downloaded the Netblast program and set it up according to the instruction on the website

(http://www.ncbi.nlm.nih.gov/blast/docs/netblast.html).

Then, the search through the following example command line was conducted:

```
blastcl3 –p tblastx –d hs_genome/hs_refm –v 5 –b 1 –e 1e-5 –i myquery.txt –o output.txt
```

In this command, there are combinations of different options.

"blastcl3": activate the Netblast program

"-p tblastx": using the alignment program "tblastx", that is, translate the input DNA into protein, translate the DNA sequence in the specified database into protein, then align the resulted protein sequences and return the record if a match is found

"-d hs_genome/hs_refm": using the human Reference mRNA database

"-v 5": Number of database sequences to show one-line descriptions (5 here)

"-b 1": Number of database sequences to show alignments (1 here)

"-e 1e-5": Expected value (1e-5 here)

"-i myquery.txt": Name of input file

"-o output.txt": Name of output file

We have run a BLAST for about 600 probes, including all of the differentially expressed probes not annotated by BLAT.

In summary, by using the TIGR and DRAGON annotation methods, annotations for about 85% of the probes on the array were found. By using BLAT or BLAST, the human orthologous genes were found for about 1000 differentially expressed bovine ESTs.

Chapter V

Molecular Mechanisms Regulating of Bovine Ovarian Follicular Selection

Abstract

Lack of, or abnormal, ovarian follicular development is a major factor associated with impaired fertility in mammals. However, development of new treatments is limited by our poor understanding of ovarian follicular development. Understanding the mechanisms by which a cohort of follicles is recruited and a single follicle is selected for further development to a dominant follicle is the key to understanding pre-ovulatory follicular development. The objectives of this study are to identify changes in gene expression profiles during the selection stage of bovine follicular waves.

Follicles of different sizes (8 mm to 12 mm) were collected and intrafollicular concentrations of progesterone, estradiol and androstenedione were measured. Gene expression profiles were obtained using bovine cDNA microarrays.

Of the 17,692 probes on the arrays, 606 unique genes and 32 sequences not matching any known genes were identified as differentially expressed with a cut off false discovery rate (FDR) of 0.05. Seventeen gene expression profiles were confirmed using quantitative RT-PCR.

Five main expression profiles were discovered, in which gene expression patterns change according to follicle size. Genes involved in several biological processes were also identified as significantly over-represented, such as genes involved in immune function or regulation of cell cycle. Annotation of the cellular location of differentially expressed gene products identified the ribonucleoprotein complex and the extracellular matrix as over represented. More than one hundred genes were found to have a strong positive or negative association with LHR.

These data strongly support the essential role of LH/LHR pathway on follicular selection, the important function of the extracellular matrix and the associated cell receptors, and reveal a potentially important role for the immune system.

Introduction

Mammals ovulate either one or multiple oocytes per reproductive cycle. The beginning of follicular growth is similar across species, with multiple follicles (cohort) growing to the antral stage. In cattle, humans and other mono-ovulatory species, one follicle is selected from the cohort and continues its growth (Hodgen 1982; Fortune 1994; Zeleznik 2001). The other follicles from the cohort will ultimately undergo atresia. Understanding the mechanisms by which a single follicle is selected for further development and dominance in a hormonal milieu suppressive to the development of other large follicles is the key to understanding preovulatory follicular development. As eloquently stated by Greenwald, (1972): "One of the most intriguing mysteries in ovarian physiology is what factors determine whether one follicle remains quiescent, another begins to develop but becomes atretic, while still a third matures and ovulates."

Although there many genes implicated in follicular growth (Ben-Shlomo et al. 2002), only a few are known to play a role in follicular selection and establishment of a dominant follicle.

Around the time of follicular deviation, expression of mRNA for the LHR in granulosa cells of a single follicle is detected suggesting that acquisition of the LHR on granulosa cells is associated with follicular selection and dominance (Xu et al. 1995; Bao et al. 1997). Furthermore, all dominant follicles express mRNA for the LHR in granulosa cells (Xu et al. 1995; Bao et al. 1997). IGF I is essential for in vivo follicular development in mice (Baker et al. 1996), horses (Ginther et al. 2004) and cattle (Beg et al. 2002; Ginther et al. 2004). As the follicles grow, the bioavailability of IGF I increases in the follicular fluid (Webb et al. 1999; Fortune et al. 2001; Mazerbourg et al. 2003; Webb et al. 2003; Spicer 2004). This small but potent increase is facilitated by a decrease in IGF binding protein (IGFBP) -2, -4, and -5 activities in follicular fluid (Besnard et al. 1996; Besnard et al. 1997; Cwyfan Hughes et al. 1997; Rivera and Fortune 2003; Roberts and Echternkamp 2003). The decrease in IGFBP-2 activity is partly driven by a corresponding decrease in its mRNA expression in pig, sheep and cattle follicles (Besnard et al. 1996; Roberts and Echternkamp 2003; Voge et al. 2004) but not in the primate ovary (Arraztoa et al. 2002). The decrease in IGFBP -4 and -5 binding activity is controlled by an increase in follicular fluid activity of an IGFBP protease, the pregnancy-associated plasma protein-A (PAPP-A) (Conover et al. 1999; Conover et al. 2001; Mazerbourg et al. 2001; Rivera and Fortune 2003). BMP15 and GDF9 proteins are present in the follicular fluid of follicles. The genes are expressed almost exclusively in

oocytes and play an important role in oocyte initiated regulation of granulosa and cumulus cells functions (Findlay et al. 2002; Juengel et al. 2004; Su et al. 2004; McNatty et al. 2005). Different mutations in BMP15 and GDF9 were each found to have a major effect on ovulation rate in sheep (Galloway et al. 2000; Hanrahan et al. 2004).

We have used cows as a model animal to study gene expression within dominant follicles around the time of selection. Like humans, cattle are monovular. The growth of follicles can be accurately monitored by real-time ultrasonographic examination (Hamilton et al. 1995; Bao et al. 1997). Finally, bovine follicles are large structures that provide adequate amounts of total RNA so that multiple measurements can be made on a single follicle (Xu et al. 1995; Xu et al. 1995; Bao et al. 1997).

cDNA microarray technology was used to identify genes differentially expressed in growing follicles sampled before, during and after the expected time of follicular selection as indicated by size of follicle.

Materials and methods

Animals and treatments

This study was conducted under an approved animal protocol granted by the University of Missouri Institutional Animal Care and Use Committee in accordance with all federal, state, and local regulations. Twenty-three Angus heifers with normal estrous

cycles received a luteolytic dose of prostaglandin (PG) F2α (Lutalyse, The Upjohn Company, Kalamazoo, MI, USA) during the luteal phase. Heifers were checked every 8 hours for estrus. Ovarian structures were monitored by transrectal ultrasonography twice daily starting 12 hours following observation of estrus until the largest follicles reached the desired size (8 mm to 12 mm). Heifers were then transported to a surgical suite where ovaries were removed via laparotomy through the paralumbar fossa (Youngquist et al. 1995).

Tissue processing

Following removal, ovaries were immediately placed in sterile saline and cooled on ice. Follicles greater than 5 mm in diameter were dissected free from other ovarian tissue, measured and frozen over liquid nitrogen within 30 min of removal and stored at -80°C. For follicles ≥7 mm in diameter, 10 to 20 μl of follicular fluid were aspirated using a tuberculin syringe and stored at -20°C. Dissected follicles were cut into half and stored separately at -80°C.

Hormone analysis

Concentrations of progesterone (P4), Δ^4-androstenedione (A4), and estradiol-17β (E2) in follicular fluid were determined using a direct, solid-phase radioimmunoassay (RIA) (COAT-A-COUNT; Diagnostic Products Corporation, Los Angeles, California) (Calder et al. 2001) after dilution (1:10 to 1:1000) in PBS. In the P4, A4, and E2 assays, intra-assay coefficients of variation were 6.6, 10.3, and 10.9% respectively.

RNA Extraction

Total RNA was extracted from each follicle using the RNAqueous kit according to the manufacturer's instructions (Ambion Inc., Austin, Texas). The RNA was then treated with DNase I to remove potential contaminating DNA by using a DNA-Free kit (Ambion, Inc.). Following DNase I treatment, the RNA was phenol-chloroform extracted through phase-lock tubes (Eppendorf, Hamburg, Germany) and purified using Microcon-30 columns (Millipore, Billerica, MA). The quality and integrity of the purified RNA was checked through agarose gel electrophoresis and the quantity was determined by taking optical density readings on an ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). The purified RNA samples were kept at −80°C until used.

Microarray preparation

The cDNA libraries used to make the microarrays consisted of 17,692 bovine cDNA samples, including 1,536 bovine ovary cDNA (Takasuga et al. 2001) and 16,156 bovine cDNA clones from USDA BOV1-5 libraries (including ovary) (Smith et al. 2001; Sonstegard et al. 2002), representing 15,634 unique genes. The cDNA inserts were amplified in 96-well PCR plates with M13 forward (5'-CCC AGT CAC GAC GTT GTA AAA CG) and reverse (5'-AGC GGA TAA CAA TTT CAC ACA GG) primers (IDT DNA, Coralville, IA). PCR products were visualized in 1% agarose gels using ethidium bromide staining. All products were then purified with 384-well MultiScreen PCR purification plates (Millipore, Billerica, MA). The purified DNA products were dried and

stored at -80°C until used. Before printing, the dried PCR products were re-suspended in 3×SSC for 48 hours. Arrays were printed on poly-L-lysine (Sigma, St. Louis, MO) coated Gold Seal micro slides (Gold Seal Products, Portsmouth, NH) at the MU animal science microarray core. As external controls, the arrays also contained 10 Arabidopsis genes, each of them printed 10 times across the array (Spot report oligo, Stratagene, Cedar Creek, TX). Blank spots and 3× SSC were used as negative controls. A robotic microarray printer with 32 printing tips was used to spot the DNA. The bovine cDNA arrays had a total of 19,200 spots and spots were organized in 32 blocks arrayed in 8 rows and 4 columns. Each block had 600 spots arranged in 24 rows and 25 columns. Slides were stored in the dark until used.

Microarray hybridization

RNA samples from the 14 largest follicles of 14 heifers with follicle size of 8mm to 11.5 mm were used in the hybridization. A loop design was used with two samples hybridized on each array and each samples repeated 3 times (swap dye) on different arrays. The hybridization was carried out using the 3DNA Array 350 kit (Genisphere Inc., Hatfield, PA) according to the manufacturer's instructions. For each hybridization, 5 µg of total RNA of each follicle sample were used. After hybridization, the slides were scanned using an Axon GenePix 4000B scanner (Axon Instruments Inc., Union City, CA) at 5 µm resolution, and the image was analyzed using GenePix Pro 4.0.1.12 software (Axon Instruments Inc., Union City, CA). The resulting files and the images were linked together and stored in the local BioArray Software Environment (BASE) database (Saal et al. 2002). The raw data files on which this paper is based have been deposited at the

National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/ geo/), with series number GSE5209 and platform number GPL3962.

Microarray statistical analyses

Data were first filtered to remove all control spots, 3× SSC spots, and blank spots. The background corrected median intensities were normalized using pin-based LOWESS normalization within BASE. The normalized intensities were then input into the software package MAANOVA (Wu et al. 2002) to model the data and run statistical analyses. Briefly, the intensities were first log2 transformed and then a 2-stage ANOVA model (Wolfinger et al. 2001) was applied. The first stage was the normalization model to remove the effects of Array and Dye. The second stage was application of a gene specific model where the effect of sample-gene interaction and array-gene interaction were modeled with both effects set as random. Here each follicle is regarded as an individual sample. An F_s test (Cui et al. 2005) was used to test the significance of sample effect for each probe (1000 permutations). This model was called the Sample model.

Probes with an adjusted permutation F_s test (False Discovery Rate) value less than 0.05 were regarded as significantly differentially expressed across samples. This list was then filtered to remove probes in which no sample had an expression value that differed by more than 1.4 fold from the mean expression. The 1.4 fold criteria is based on the results of self-self hybridizations in which equal amounts of RNA were labeled with Cy3 and Cy5 fluorescent-dyes from the same sample and co-hybridized on the same array. The mean ± 2SD fold-change was calculated for all the genes on these arrays (1±1.40).

The filtered list was then input into TIGR multi-experiment viewer software (TM4-MeV) (Saeed et al. 2003) to run hierarchical clustering (Eisen et al. 1998) and self organization map (SOM) (Kohonen 1982; Tamayo et al. 1999) analyses.

For each differentially expressed bovine EST sequence, the TIGR Cattle Gene Index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=cattle) was used to obtain the gene annotation. BLAT (http://genome.ucsc.edu/) (Kent 2002) and TBLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) were used to find the human homolog genes. Gene ontology (GO) terms were obtained using DAVID (http://apps1.niaid.nih.gov/david/) (Dennis et al. 2003). DAVID was also used to detect biological processes that were significantly up or down regulated across all samples.

Since divergence in the growth rates of follicles (dominant and subordinates) starts when the dominant follicles were around 8.5 mm in diameter (Ginther et al. 2000b), a new model (Group model) in which the follicles were grouped by size were used to compare the group effect. There were four groups in the Group model, with two 8 mm follicles in group 1 (regarded as before deviation), four 8.5 mm follicles in group 2 (beginning of deviation), four 9 mm follicles in group3 (shortly after deviation), and four >9 mm follicles in group 4 (dominant group). In the second stage gene specific ANOVA model, the effect of group-gene interaction and array-gene interaction were modeled with array effect set as random and group effect set as fixed. An F_s test (Cui et al. 2005) was used to test the significance of group effect for each probe (500 permutations). Probes with adjusted permutation F_s test (False Discovery Rate) value less than 0.05 were

regarded as significantly differentially expressed across groups. This list was then filtered to remove probes in which no group had an expression value that differed by more than 1.4 fold from the other group expression values. The filtered list was then annotated as described for the list from the Sample model.

For the Sample model, the difference between two sample (follicle) could be due to the physiological status or due to the different genetic background. Thus, the list of probes from the Group model was compared to the list of probes from the Sample model.

Quantitative real-time PCR (qRT-PCR)

Two-step qRT-PCR was used to confirm expression patterns of target genes in the study. First, 4 μg total RNA of each follicle sample was reverse transcribed using Stratascript RT (Stratagene, La Jolla, CA) with oligo dT and random hexamer primers. Then, 2.5 ng of cDNA were used in a 25 μL PCR reaction to get a final concentration of 0.1 ng/μL of cDNA in a SYBR green assay (Platinum® SYBR® Green qPCR SuperMix-UDG, Invitrogen, Carlsbad, California). Primers for each gene were designed using Primer Express Software (Applied Biosystems, Foster City, CA) and ordered from Invitrogen. The qPCR reaction was done in an ABI prism 7500 sequence detection system (Applied Biosystems). The relative quantification of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB) were compared to two genes (phosphofructokinase (PFKM) and cingulin-like 1 (CGNL1)) showing stable expression levels across samples on the microarray. Beta-actin was chosen as the internal control gene as it showed a parallel expression profile in qRT-PCR with PFKM and CGNL1 while being expressed at a higher level in the samples. For the other genes, relative

quantification of gene expression changes were recorded after normalizing for β-actin expression, computed by using the $2^{-\Delta\Delta CT}$ method (user manual #2, ABI Prism 7700 SDS).

Results

Follicle growth and hormone profiles

Follicular growth was monitored in all cows twice daily by rectal ultrasound. Only follicles that could be seen as growing in diameter were included in the experiment.

The largest follicles (F1) were on average 0.5 mm bigger than the subordinate follicles (F2 and F3) in most cows until F1s reached on average of 8.5mm in diameter. Then, the difference in size between F1s and subordinate follicles increased (Figure 15A). This is in agreement with a previous report (Ginther et al. 2001) showing that deviation in size difference starts when the largest follicles (F1s) are about 8.5mm in diameter, a point at which F1s and subordinate follicles start to show different growth rates. Since our aim was to identify changes in gene expression that accompany selection of the future dominant ovarian follicle, only the 14 largest healthy follicles from 14 cows with a diameter of 8mm or larger were included in the microarray experiment. The diameter size and E2/P4 ratios of the largest 3 follicles (F1, F2 and F3) of these cows are shown in Figure 15.

Besides the size advantage, F1 follicles also have a higher E2/P4 ratio in almost all animals (Figure 15B). As shown in Figure 16, follicle growth was associated with a rapid increase of estradiol (E2) concentration in follicular fluid once follicles reached a diameter of 8 mm or larger. The concentrations of progesterone (P4) and androgen (A4)

did not change significantly in follicular fluid, regardless of the increase of the follicle sizes. This is in agreement with a previous study (Xu et al. 1995). These differences in size and E2/P4 ratios clearly show that the largest follicles (F1s) were the ones most likely destined to continue growth and ovulate.

Microarray Results—Sample model

Differentially expressed probes

Of the 17,692 probes on the array, 1,628 probes were identified as differentially expressed in at least one follicle with a false discovery rate (FDR) of 0.05 (Cui et al. 2005). Probes for which no follicle had an expression value that differed by more than 1.4 fold from the mean expression were removed (see materials and methods section for justification of this threshold). Nine hundred and seventy-six probes remained after this filtering step and were further classified. Human orthologs of these bovine probes were identified using TBLASTN and BLAT. The probes represent a total of six hundred and six unique genes, plus thirty two sequences not matching any known genes.

Over represented biological processes and cellular locations

Functional categorization of these six hundred and six genes using the NIH-DAVID database and bioinformatics tools (Dennis et al. 2003) revealed that genes involved in several biological processes were significantly over-represented in the list, such as immune-related genes (MHCI and II, Beta-microglobulin, Immunoglobulins, Interferon induced transmembrane proteins 1 and 8), regulation of cell cycle (i.e.; G1 to S phase transition 1, Cyclin B1) and control of apoptosis (i.e.; Bl2-like 1, Myxovirus resistance 1, P21, Bcl2-associated x protein, Survivin) genes (Table 4). In addition, the

NIH-DAVID functional annotation tool identified over-represented cellular components containing proteins produced by the differentially expressed genes, such as the ribonucleoprotein complex and the extracellular matrix (ECM) (Table 5). Proteins localized to the ECM include Glypican 6, Glypican 5, Fibrilin 1, Fibulin 1, Fibronectin 1, Fibronectin type III domain containing 1, Collagen type I alpha 1, Collagen type I alpha 2, Collagen type II alpha 1, Collagen type IV alpha 3, Collagen type VI alpha 3, Collagen type XVII alpha 1, Laminin alpha 4, Tenascin C, Integrin beta 5, Thrombospondin 2, CD44 antigen and von Willebrand factor.

Identification of clusters of gene with similar expression patterns

A subset of genes, many coding for structural proteins of the extracellular matrix, displays a unique pattern of expression (Table 6, Figure 17A). These genes are up-regulated in 8.5mm follicles, then gradually down regulated in 9 and 10 mm follicles. In addition, the genes tend to be up-regulated again in two 10.5-11 mm follicles. The interesting pattern of expression of these ECM genes led us to explore more expression patterns based on follicle size.

Hierarchical clustering and self organizing map (SOM) analyses were used to identify group of probes with similar expression profiles (cluster). The five major clusters are shown in Figure 17B. Cluster 1, 2 and 3 represent groups of probes that are mainly up-regulated in the 8.5 mm follicles. Cluster 4 contains probes that are mainly down-regulated in the 8.5 mm and ≥10 mm follicles, while up-regulated in 8 and 9mm follicles. Cluster 5 corresponds to a group of probes that are down-regulated in follicles larger than 8mm in diameter.. These five clusters strongly indicate that there are changes in gene

expression when the largest follicles reaches 8.5 mm in diameter, a time point where selection is believed to happen (Ginther et al. 2001).

Annotation of the 225 probes in clusters 1 to 3 indicates that they represent 67 unique genes, (Table 7). One interesting characteristic of cluster 1 is that it contains 143 probes for different immunoglobulin chains. Furthermore, there are 17 probes (out of 22 totals) in cluster 2 for major histocompatibility antigen genes. The 107 probes in clusters 4 and 5 represent 104 unique genes. A large number of these genes are involved in cell organization and biogenesis, cell proliferation and cell cycle (Table 8).

Microarray Results—Group model

Differentially expressed probes

The follicles were sorted in groups according to their size. Four groups were created, 8mm (n=2), 8.5mm (n=4), 9mm (n=4) and >9mm (n=4). Based on FDR of 0.05, a total of four hundred and fifty-eight probes were identified to be differentially expressed between any of these four groups. Two hundred and seventy-one probes (Table 9) remained after removing probes that did not show a change of more than 40% between any of the groups (see material and methods section for justification of this threshold). Annotation of these probes indicates that they represent 169 unique genes and 7 unknown sequences.

Over represented biological processes and cellular locations

Functional categorization of these one hundred and sixty-nine genes using the NIH-DAVID database and bioinformatics tools (Dennis et al. 2003) revealed that genes involved in several biological processes were significantly over-represented in the list, such as immune-related genes and mitotic cell cycle genes (Table 10). In addition, annotation of the cellular location of the products from these differentially expressed genes identified various cell components as over represented, such as the non-membrane-bound organelle and the extracellular matrix (Table 10).

Expression profiles of the differentially expressed probes

As shown in Figure 18A, there are three major expression patterns (HC1, 2 and 3) present among these 271 probes. Genes belonging to the HC1 pattern are up-regulated in 8.5 mm follicles, while HC2 genes are up-regulated in both 8.5 mm and >9mm follicle groups, and HC3 genes are down-regulated in follicles larger than 8mm in diameters.

Real-time PCR Results

Quantitative real-time PCR (qRT-PCR) was used to verify the results of the microarray analysis. Four genes (beta-actin, GAPDH, PFKM, CGNL1) were compared to select the internal control of the qRT-PCR experiment. As beta-actin had a similar expression profile as PFKM and CGNL1, which showed stable expression levels across samples on the microarray, it was chosen as the internal control. GAPDH had variable expression levels relative to the other three genes in some of the samples.

Fifteen genes were analyzed by qRT-PCR using beta-actin as the internal control (Table 11, Figure 19). Included were 2 genes showing large expression changes on the array (metallothionein 1 (MT1) and 2A (MT2A)), 6 genes involved in follicle development (CYP21A2, 3-beta-hydroxy-delta(5)-steroid dehydrogenase (3-beta-HSD, HSD3B2), Inhibin alpha (INHA), Inhibin beta A (INHBA), Follistatin (FST), IGFBP2), and 7 genes from individual clusters shown in Figure 17B (Cluster1: beta-2-microglobulin (B2M), Cluster 4: pyruvate kinase 3 isoform 1 (PKM2), stearoyl-CoA desaturase (SCD), deaminase domain containing 1 (DEADC1); Cluster 5: thrombospondin 2 (THSB2), syndecan 4 precursor (SDC4) and plasminogen activator inhibitor-1 (SERPINE1)). Among them, 13 showed similar expression profiles as the array results, as shown in Figure 19.

Finally, three genes not present on the array (LH receptor (LHR), Aromatase (CYP19A1), pregnancy-associated plasma protein A (PAPP-A)) and 3 genes with intensities too close to background for a reliable measurement of gene expression (anti-Mullerian hormone (AMH), IGF1, IGF2) were analyzed by qRT-PCR as they play important roles in follicle development (Table 11, Figure 20). Among them, LHR and Aromatase showed relative high expression levels in the larger follicles.

Discussion

In agreement with previous reports (Kulick et al. 1999; Beg et al. 2003), the future dominant follicles (F1s) have on average a 0.5 mm size advantage over the second

largest follicles (F2s). This size advantage starts to become larger when F1 are 8.5 mm or larger, reflecting differences in growth rates (Ginther et al. 2001). This supports the concept that deviation happens when F1s are on average 8.0 to 8.5 mm in size. There is also an E2/P4 ratio advantage of F1s over F2s (Figure 15) and the advantage increases when F1s are 10mm or larger (by then, F1s are usually 2 mm larger than F2s), indicating the establishment of dominance.

Known genes involved in follicular growth

Inhibins are well known for their role in inhibiting pituitary FSH synthesis and secretion (Bernard et al. 2001; Ginther et al. 2001). In our microarray and qRT-PCR data, there is a large increase of INHA mRNA level from 8.0 mm to 9.0 mm follicles (Figure 19). There is also a slight increase of INHBA mRNA level from 8.0 mm to 9.0 mm (Figure 19). Observed increases are in agreement with previous work describing that inhibin A concentration increases near or after the beginning of presumptive deviation (Ginther et al. 2001) and confirm the reliability of our microarray experiments.

Anti-Mullerian hormone gene (AMH) expression decreased in follicles larger than 9 mm (Figure 20). This is consistent with a previous report describing a decrease of AMH protein expression in human follicles (Weenen et al. 2004).

IGF and related proteins were shown to play important roles in follicle development (Fortune et al. 2004; Webb et al. 2004; Kobayashi et al. 2006). In our qRT-PCR data (Figure 20), a transient increase of IGF-1 mRNA level was detected in the 8.5 mm follicles, a pattern similar to the probes identified in Cluster 1 of Figure 17B. Though this is not necessarily an indication of protein level or free IGF-1 level, it is interesting as

IGF1 is known to promote synergistic enhancement of FSH action in antral follicles. Furthermore, IGF2 mRNA levels tended to be higher in 9mm follicles (Figure 20). Increased IGF1 or 2 expressions could promote cellular growth if the additional IGF1 or 2 produced are not bound to IGF binding proteins (IGFBPs). There are reports that an increased expression of a protease of IGFBPs (Fortune et al. 2004; Webb et al. 2004), the associated plasma protein A (PAPPA), is detected prior to selection. Our measurements of mRNA from PAPP by qRT-PCR showed great variability in the level of expression (Figure 20) between follicles, but no clear trend at any specific stage of follicle size. An increase in IGFBP2 was also detected in follicles larger than 9mm (Figure 19), but PAPP expression was also elevated in some of these large follicles (Figure 20). Thus, the potential effect of the increase in IGFBP2 expression on concentrations of free IGF1and IGF2 is unclear.

Aromatase (CYP19A1) is expressed in granulosa cells of recruited follicles and the level of expression increases with follicles size (Bao et al. 1997). Another report (Mihm et al. 2006) indicated that there is a slight but not statistically significant increase of Aromatase mRNA level with time. We also observed by qRT-PCR an increase of aromatase mRNA level from 8mm to 9mm, but not in larger follicles (Figure 20). A previous report (Sisco et al. 2003) indicated that while there are large variances of aromatase level with either size or days in different heifers, the differences between dominant and subordinate follicles are dramatic, even slightly preceding follicle deviation.

Genes involved in immune function

A particularly interesting cluster contains genes involved in response to biotic stimulus and immune response (Tables 4 and 8, and Figure 17B; Clusters 1 and 2), including immunoglobulin heavy and light chains, and major histocompatibility class I and II antigens. These genes were up-regulated transiently in the 8.5mm follicles. The expression of these genes exposes the existence of immune cells in the follicles. Indeed, Bukovsky et al. (1995) have reported macrophages residing under the basement membrane in growing large antral follicles in the human ovary. Those macrophages are MHC II positive, and the dendritic shape macrophages have physical contact with granulosa cells. Combining the changes of location and function of macrophages during folliculogenesis, it is hypothesized by Wu et al. (2004) that macrophages located in the theca can promote cell proliferation, stimulate follicle growth, and prevent apoptosis, while macrophages located in granulosa cell layer (only found at advanced stages of atresia) are likely to phagocytose the apoptotic granulosa cells. It was recently reviewed (Condeelis and Pollard 2006) that macrophages within the tumor microenvironment can facilitate angiogenesis and extracellular-matrix breakdown and remodeling and promote tumor cell motility, thus helping the metastasis of tumor cells. It is tempting to think that the cells expressing differentially expressed immune-related genes, such as macrophages, could function in a similar way to help the future-dominant follicles. The up-regulation of immune-related genes in 8.5 mm follicles strongly points toward the involvement of the immune system (possibly macrophages in thecal layer) in the follicle selection process.

Beta-2-microglobulin (B2M) gene expression was confirmed by qRT-PCR (Figure 19). Beta-2-microglobulin is a serum protein found in association with the major

histocompatibility complex (MHC) class I heavy chain on the surface of nearly all nucleated cells (Gussow et al. 1987). It is essential to the expression of MHC class I antigens (Arce-Gomez et al. 1978).

Extracellular matrix genes

A number of genes involved in the extra cellular matrix (ECM) structure and ECM-cell receptor interactions were identified to be differentially expressed across the follicles. These include Glypican 6, Glypican 5, Fibrilin 1, Fibulin 1, Fibronectin 1, Fibronectin type III domain containing 1, Collagen type I alpha 1, Collagen type I alpha 2, Collagen type II alpha 1, Collagen type IV alpha 3, Collagen type VI alpha 3, Collagen type XVII alpha 1, Laminin alpha 4, Tenascin C, Integrin beta 5, Thrombospondin 2, CD44 antigen and von Willebrand factor (Figure 17A, Table 6). The differential expression of these genes suggests that remodeling of extra cellular matrix and the related ECM-cell receptor interactions are involved in the growth and development of ovarian follicles.

As reviewed by Irving-Rodgers and Rodgers (2005), type IV collagen and laminin form the backbone of the basal lamina matrices of ovarian follicles. A recent study (Berkholtz et al. 2006) also indicates that fibronectin, collagen type I and IV , and laminin are all found in mouse ovarian follicles and their contents change during follicle development. A study by Yasuda et al. (2005) suggests that tenascin is critical in controlling the degenerative changes of tissues in mouse ovaries, and fibronectin is possibly indispensable for the function of tenascin as tenascin is always co-localized with fibronectin. CD44 is the receptor of hyaluronan and the hyaluronan-CD44 system plays

an important role during cumulus expansion (Sato and Yokoo 2005). It has also been reported that hyaluronic acid inhibits apoptosis in granulosa cells via CD44 (Kaneko et al. 2000). The von Willebrand factor mediates adhesion of platelets to the subendothelium at the sites of vascular injury and disorders (Weiss 1991) and is suggested to be associated with follicle atresia in Philippine swamp buffaloes, in reverse expression pattern of vascular endothelial growth factor expression (VEGF) (Feranil et al. 2005). Thrombospondin 2 is reported to be a potent endogenous inhibitor of tumor growth and angiogenesis (Streit et al. 1999). In cattle, THBS2 mRNA level is significantly higher in small follicles (<5 mm) than either medium (5-10mm) or large (>10 mm) follicles, in an inverse pattern of vascular endothelial growth factor (Greenaway et al. 2005). The expression pattern of Thrombospondin 2 was confirmed by qRT-PCR. We also measured the expression of SerpinE1 using microarrays and qRT-PCR. SerpinE1 (plasminogen activator inhibitor I) modulates the remodeling of extracellular matrix (ECM) by plasminogen activator (Liu 2004). In cattle there is a sharp transient increase of SerpinE1 mRNA at ~6 hours after GnRH (Dow et al. 2002) or hCG (Cao et al. 2006) injection in the periovulatory follicles, then up again at ~24 hours with GnRH but not with hCG. When these data are combined with our data, it suggests that SerpinE1 is down-regulated as follicle size increases, and is then up-regulated at the LH surge, possibly to increase plasminogen activator activity and facilitate the process of ECM remodeling during the ovulatory process.

A large number of genes have expression profiles correlated with LH receptor expression

In cattle, divergence of the future dominant follicle is associated with the initiation of expression of LH receptor and 3β-HSD in granulosa cells (Xu et al. 1995; Bao et al. 1997). Since the granulosa and thecal cells were not separated in the current study, when the LHR started to be expressed in granulosa cells could not be determined. Nevertheless, using qRT-PCR, we observed a clear trend for an increase in LH receptor mRNA as follicle size increases (Figure 20). A pattern matching algorithm (Pavlidis Template Matching) (Pavlidis and Noble 2001) was used to find genes with a pattern of expression similar to LHR. Fifty five probes were identified by Pavlidis Template Matching with a P-value of 0.05 or less (Table 12). These included 4 genes involved in the biosynthesis of steroids (farnesyl diphosphate synthase, farnesyl-diphosphate farnesyltransferase 1, isopentenyl-diphosphate delta isomerase and squalene epoxidase) and 2 genes involved in C-21 steroid hormone metabolism (CYP21A2 and HSD3B2 (3β-HSD)). The strong correlations between LHR, CYP21A2 and 3β-HSD are also supported by their qRT-PCR data (Pearson r= 0.88 between LHR and CYP21A2 expression levels and r=0.69 between LHR and 3β-HSD). Using the same method, 98 probes were identified that have a pattern of expression negatively correlated to LHR (data not shown). These include syndecan-4 (SDC4) and SerpinE1. The relationships are also supported by the qRT-PCR data (Pearson r=-0.67 between LHR and SDC4 and r=-0.70 between LHR and SerpinE1). Together, there are 153 probes (15.7% of the differentially expressed genes) showing expression patterns similar or opposite to LHR, strongly suggesting that

the LH/LHR axis has a deep effect on gene expression in these follicles, as also discussed by Mihm et al. (2006).

One 9.0 mm follicle has a peculiar expression profile

One follicle (C8; 9.0 mm in diameter, see Figure 15) displayed much lower LHR mRNA level than other 9.0 mm follicles (Figure 20). This indicates that C8 was either close to the developmental stage of an 8.0 mm follicle or that it was not a healthy follicle. Unfortunately the follicular fluid for C8 was lost during dissection so that we could not measure E2 and P4 concentrations in the follicular fluid. Nevertheless, the possibility that C8 is not like other large follicles is supported by the high expression levels of syndecan-4 (SDC4), thrombospondin-2 (THBS2) and SerpineE1 (see Figure 19). All three genes had a much higher expression levels in C8 than in other follicles with similar diameters (Figure 19). SDC4 expression is reported to be associated with follicular atresia in mouse ovary (Ishiguro et al. 1999). THBS2 and SerpinE1 genes function as growth inhibitors (see discussion above). Together, these three genes (SDC4, THBS2 and SerpinE1) are involved in ECM structure, angiogenesis and ECM remodeling. Their peculiar levels of expression in C8 strongly suggest that this follicle is not at of the same developmental stage as the other 9 mm follicles.

Comparison with other gene expression studies

Several recent articles were published describing attempts to identify genes involved in bovine dominant follicle development (Sisco et al. 2003; Evans et al. 2004;

Fayad et al. 2004; Mihm et al. 2006). These studies used either suppression subtractive hybridization to compare dominant and small follicles (Sisco et al. 2003; Fayad et al. 2004) or microarrays to compare dominant and subordinate follicles (Evans et al. 2004) or different stages of dominant follicles (Mihm et al. 2006). Although there are differences between follicle samples, developmental stages or methods between our study and the previous studies, many common genes were identified as being differentially expressed. For example, among the twenty-two clones identified by Fayad et al. (2004) which have gene annotations, six were also identified by our method, Connexin 43, Inhibin beta A, 17kDa myosin light chain, SerpinE2, Splicing factor praline/glutamine rich and Tumor necrosis factor α-induced protein 6. We have also identified several glutathione-S-transferase subunits with high homologies to two clones (GSTA1 and 2) identified by Fayad et al. (2004). When comparing our results with the gene list from Mihm et al. (2006), besides the dramatic increase of LH receptor expression, we identified another eleven common genes that show differential expression, such as Cyclin D2, Connective tissue growth factor, and some chemokines ligands. The identification of these common genes strongly validates the samples and methods used herein.

Comparison between the Sample model and the Group model

Among the 271 probes identified by the Group model, 238 probes were also present in the Sample model filtered probe list. Twelve probes are not in the filtered probe list from the sample model, but they are present in the non filtered probe list. Only twenty-one probes discovered by the group model were not identified by the Sample model at the threshold FDR of 0.05. The list of all thirty three probes is shown in Table

13. It contains genes involved in biological processes such as cellular organization and biosynthesis, transport, and macromolecular metabolism.

The expression patterns identified by hierarchical clustering of the genes in the Group model list (HC1, 2 and 3, Figure 18) are similar to the five major gene expression clusters identified by the Sample model (Cluster 1-5, Figure 17). Many of the probes are common between the HCs from the group model and the clusters from the Sample model. Specifically, most of HC1 probes are also present in Cluster 1, with only five probes present in Cluster 3 and another six not present in the five clusters. About one quarter of the probes in HC2 are present in Cluster 1 (16 probes) and 3 (8 probes), but the others are not present in the five clusters. Half of the probes in HC3 are present in Cluster 4 (36 probes) and 5 (3 probes), while the other half are not present in the five clusters.

On the other hand, while Cluster 1, 3, 4, and 5 all have probes present in HC1-3, none of the Cluster 2 probes were present in HC1-3. This is because genes from Cluster 2 are up-regulated in only some 8.5 mm, one 9 mm and one >9 mm follicles (Figure 17). The group partition we used distributed these follicles within three groups. Consequently, the within-group gene variances increased, and Cluster 2 genes could not be identified by the Group model.

As shown in Table 7, out of 21 probes in Cluster 2, fifteen coded for MHC class I A and three probes code for MHC class I B, C, and G, respectively. These eighteen probes are only classified as four genes in the functional annotation. The loss of these four MHC class I genes doesn't remove the significant enrichment of the immune system genes (see discussion later). The previous discussion about macrophages in the Sample model is still valid as the macrophage-specific markers, the MHC class II genes (see

discussion later), are still retained. The last three probes code for prenylcysteine oxidase 1, ash2, and non-POU domain containing, octamer-binding proteins and they are all involved in primary metabolism and cellular metabolism. The loss of these three genes doesn't affect the main findings presented in the Sample model.

Overall, the discrepancies between probe lists of the Group and the Sample models are partially due to the large variance in gene expression between different samples within groups (Figure 17 and Figure 18), which lead to the smaller list of probes identified by the Group model. However, although the number of differentially expressed probes decreased in the Group model, the key findings identified by the sample model still remain:

1) The significant enrichment of the immune system genes

   Both model identified significant enrichments of the immune system genes. In addition to the more than one hundred probes for different immunoglobulin different heavy and light chains, there are more than 20 genes identified by both methods. All the 21 genes identified by the Group model (Figure 21) are also identified by the Sample model.

   Most importantly, a group of MHC class II genes, such as CD74 (invariant polypeptide of major histocompatibility complex, class II antigen-associated; a marker of macrophage (Wu et al. 2004)), MHC II DQ alpha I and MHC II DQ beta I are identified as significantly differentially expressed, which strongly support the previous hypothesis of the potential involvement of macrophages in the follicular selection process.

A group of interferon inducible genes, involved in immune response, is also identified as significantly enriched by both models. They are up-regulated in 8.5 mm follicles (Figure 21). These include interferon induced transmembrane protein 1 and 3 (IFITM1, IFITM3), interferon alpha inducible protein IFI-15K (G1P2) and IFI-6-16 (G1P3), interferon inducible guanylate binding protein 2 (GBP2), Myxovirus resistance 1 (MX1), and 2',5'-oligoadenylate synthetase 1 (OAS1).

2) The significant enrichment of the extracellular genes

   There is a significant enrichment of extracellular genes in both models. There are 10 extracellular space genes identified by the Group model and all of them are also identified by the Sample model. Most of them have a higher expression levels in the 8.5 mm and the >9 mm group follicles (Figure 22).

A comparison between the array data (from the Group model) and the real-time data when follicles are grouped on size indicates that out of fifteen genes, 11 of them can be regarded as having similar trends (MT1, MT2, HSD3B2, B2M, SCD, SerpinE1, CYP21A2, INHA, PKM2, SDC4, and THSB2) (Figure 23), while the other 4 are not in total agreement in the change trend (INHA, DEADC1, IGFBP2, and FST) (Figure 23).

In conclusion, our data strongly support the essential role of LH/LHR pathway on follicle development, the important function of the extracellular matrix and the associated cell receptors, and reveals a potentially important role for the immune system in follicle selection.

Table 1. Classification and characterization of small bovine follicles.

Follicle	Layers of granulosa cells	Follicle diameter (μm)	Oocyte diameter (μm)	Clearly defined theca internal
Primordial (type 1)	1 (flattened)	<40	29.7±0.3	-
Primary	1-1.5	40-80	31.1±0.4	-
Early pre-antral	2-3	81-130	49.5±2.4	-
Later pre-antral	4-6	131-250	68.6±2.8	+
Small antral	>6	250-500	92.9±4.5	++

Note: Classification and data taken from Braw-Tal and Yossefi (1997).

Table 2. The re-sequencing of 26 clones from the USDA BOV 1-5 library plates all confirmed their original EST identity.

Plate	Well	CloneID	EST Accession	Gene
R2-01 set1	K7	1BOV12L24	AW478104	Bos taurus similar to BH3 interacting domain death agonist (BID), transcript variant 2 (LOC510373), mRNA
R2-01 set1	P14	1BOV43A21	AW445511	major histocompatibility complex, class II, DQ
R2-02 set1	E9	1BOV51A5	AW446783	Complement factor I precursor (EC 3.4.21.45) (C3B/C4B inactivator)
R2-03 set1	M2	1BOV79N15	AW657981	guanine nucleotide binding protein, alpha
R2-04 set1	M14	1BOV90L5	AW659877	EEF1D protein
R2-07 set1	C5	1BOV121I6	BI541452	KIAA1539
R2-08 set1	O12	1BOV134D8	BI681401	Homo sapiens prostaglandin D2 synthase 21kD...
R2-09 set1	H1	1BOV141M24	BI774299	regulator of G-protein signaling 2, 24kDa
R2-10 set1	E7	1BOV145C3	AW307679	Homo sapiens AHNAK nucleoprotein (desmoyokin)
R2-12 set2	A7	2BOV30G10	AW483763	transmembrane 7 superfamily member 2
R2-14 set1	H12	2BOV68E8	BE808311	hydroxy-delta-5-steroid dehydrogenase, 3 beta-
R2-15 set1	N2	2BOV81H14	BF074547	NADH dehydrogenase (ubiquinone) 1 beta
R2-16 set1	H22	2BOV90C6	BI775194	CD68 antigen
R2-16 set1	H24	2BOV90C7	BI775266	Placenta-specific gene 8 protein (C15 protein)
R2-27 set1	G9	3BOV104J11	BM106534	phosphofructokinase, muscle
R2-28 set1	K11	3BOV116D22	BM255301	PREDICTED: Bos taurus similar to cingulin-like 1 (LOC514284
R2-28 set1	M14	3BOV119F11	BM255781	guanine nucleotide binding protein (G protein),
R2-28 set1	G15	3BOV115I4	BM254805	Homo sapiens pleiotrophin (heparin binding ...
R2-37 set1	O19	4BOV119K12	BI536775	tropomyosin 2 (beta) isoform 1
R2-38 set1	F3	4BOV137A23	BI539051	mitochondrial ribosomal protein S18C
R2-40 set2	E20	5BOV23B10	BE483370	immediate early response 3 interacting protein
R2-42 set1	J22	5BOV107E16	BF231079	beta-2-microglobulin precursor
R2-43 set1	D22	5BOV116E3	BE588535	cathepsin H
R2-43 set1	C2	5BOV112B14	BE846363	pregnancy-zone protein, also match partial to alpha-2-macroglobulin
R2-44 set1	H6	5BOV132F10	BE485662	unknown
R2-44 set1	G4	5BOV120K18	BE589401	unknown

Table 3. Clones picked from the bovine ovary cDNA libraries.
BLAST programs from NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) are used to find the highly matched bovine EST sequences (using "blastn") and human Reference mRNA (using "tblastx").

Plate	Well	CloneID	Highly matched Bovine EST	Human Gene
P_E1OV001	C7	E1OV001B4	AV615824	Homo sapiens tubulin, beta 6 (TUBB6), mRNA
P_E1OV001	E1	E1OV001C1	AV615891	Homo sapiens HLA-G histocompatibility antig...
P_E1OV001	G3	E1OV001D2	AV615975	Homo sapiens caveolin 1, caveolae protein, ...
P_E1OV001	A10	E1OV002A5	AV615772	Homo sapiens myeloid cell leukemia sequence...
P_E1OV001	G12	E1OV002D6	AV616005	Homo sapiens protein disulfide isomerase fa...
P_E1OV001	O18	E1OV002H9	AV616808	Homo sapiens cofilin 1 (non-muscle) (CFL1),...
P_E1OV001	F3	E1OV003C2	AV616880	Homo sapiens heterogeneous nuclear ribonucl...
P_E1OV001	F7	E1OV003C4	AV615936	PREDICTED: Homo sapiens similar to Ig heavy...
P_E1OV001	L19	E1OV003F10	AV616195	Homo sapiens decorin (DCN), transcript vari...
P_E1OV001	L17	E1OV003F9	AV616193	Homo sapiens inhibin, beta B (activin AB be...
P_E1OV001	B12	E1OV004A6	AV616322	Homo sapiens interferon induced transmembrane...
P_E1OV001	B16	E1OV004A8	AV616644	Homo sapiens tubulin, alpha 6 (TUBA6), mRNA
P_E1OV001	L14	E1OV004F7	BE484411	Homo sapiens similar to Ig heavy chain V...
P_E1OV005	E19	E1OV005C10	AV616567	Homo sapiens stress-induced-phosphoprotein ...
P_E1OV005	G13	E1OV005D7	BG692962	Homo sapiens alpha tubulin (K-ALPHA-1), mRNA
P_E1OV005	I11	E1OV005E6	AV616722	PREDICTED: Homo sapiens similar to Ig heavy...
P_E1OV005	M19	E1OV005G10	CK848121	Homo sapiens stathmin 1/oncoprotein 18 (STM...
P_E1OV005	N9	E1OV007G5	AV616920	Homo sapiens selenoprotein X, 1 (SEPX1), mRNA
P_E1OV005	P8	E1OV008H4	AV617023	Homo sapiens major histocompatibility complex.
P_E1OV009	A21	E1OV009A11	AV617055	matrix Gla protein
P_E1OV009	C19	E1OV009B10	BI539902	pyruvate kinase 3 isoform 1
P_E1OV009	O11	E1OV009H6	AV615760	Homo sapiens integrin beta 1 binding protein
P_E1OV009	A22	E1OV010A11	AW668976	
P_E1OV009	A6	E1OV010A3	AV617063	PREDICTED: Homo sapiens similar to prothymo...
P_E1OV009	C18	E1OV010B9	AV617157	Homo sapiens CD63 molecule (CD63), transcri...
P_E1OV009	G22	E1OV010D11	AV617326	Homo sapiens actin, alpha 2, smooth muscle,...
P_E1OV009	K20	E1OV010F10	AV617495	Homo sapiens alpha tubulin (K-ALPHA-1), mRNA
P_E1OV009	M6	E1OV010G3	AV617567	Homo sapiens CD74 molecule, major histocomp...
P_E1OV009	D21	E1OV011B11	AV617182	PREDICTED: Homo sapiens similar to Ig heavy...
P_E1OV009	H9	E1OV011D5	AV617335	Homo sapiens matrix Gla protein (MGP), mRNA
P_E1OV013	C22	E1OV014B11	BE588539	Homo sapiens ribosomal protein L13 (RPL13),...
P_E1OV013	B21	E1OV015A11	AV617749	Homo sapiens ribosomal protein L36a-like (R...
P_E1OV013	D13	E1OV015B7	AV617823	tropomyosin 2 (beta) isoform 1
P_E1OV013	J1	E1OV015E1	AV618063	Homo sapiens beta-2-microglobulin (B2M), mRNA
P_E1OV013	F24	E1OV016C12	BF073269	Homo sapiens tubulin, beta 2C (TUBB2C), mRNA
P_E1OV013	F18	E1OV016C9	AV617934	Homo sapiens similar to Ig heavy chain V...
P_E1OV013	H6	E1OV016D3	AV618010	PREDICTED: Homo sapiens similar to Ig heavy...
P_E1OV013	J18	E1OV016E9	AV618099	Homo sapiens ribosomal protein L13 (RPL13),...
P_E1OV013	N4	E1OV016G2	BE484411	Homo sapiens similar to Ig heavy chain V...
P_E1OV001	P6	E1OV004H3	--	
P_E1OV001	O12	E1OV002H6	--	
P_E1OV009	M16	E1OV010G8	--	
P_E1OV013	L19	E1OV015F10	--	

Table 4. Significant enrichment of biological processes in differentially expressed genes.

Data were obtained from the NIH-DAVID database by selecting GO Biological Process, level 4. Only selected biological processes with P-value less than 0.01 are shown. Percent refers to the percentage of genes involved in a given pathway among all the differentially expressed genes.

Term	Percent (%)	P value
antigen processing	1.50	2.40E-05
antigen presentation	1.70	2.00E-04
negative regulation of cellular physiological process	6.60	3.80E-04
cellular macromolecule metabolism	21.10	4.40E-04
regulation of cell size	2.50	7.80E-04
regulation of programmed cell death	4.10	1.50E-03
organelle organization and biogenesis	7.30	2.00E-03
regulation of cell cycle	4.80	3.70E-03
humoral immune response	2.20	9.10E-03
response to pest, pathogen or parasite	5.30	9.60E-03

Table 5. Significant enrichment of cellular components in differentially expressed genes.

Data were obtained from the NIH-DAVID database by selecting GO Cellular Component, level 2. Only selected cellular components with P-value less than 0.01 are shown. Percent refers to the percentage of genes located in a given cellular component among all the differentially expressed genes.

Term	Percent (%)	P value
extracellular region	5.40	4.30E-08
intracellular	50.30	6.00E-06
non-membrane-bound organelle	12.60	8.40E-04
ribonucleoprotein complex	5.40	1.90E-03
intracellular organelle	39.90	5.50E-03

Table 6. Extracellular matrix components that are differentially expressed among different follicle samples.

Accession	Gene name
BE753551	asporin (LRR class 1)
BF652958	collagen, type I, alpha 1
BG687794	collagen, type II, alpha 1
BF230262	collagen, type VI, alpha 3
BE589976	collagen, type XVII, alpha 1
BG689858	complement component 7
BE485617	connective tissue growth factor
BE479712	connective tissue growth factor
BG692089	decorin
AV616195	decorin (DCN)
BE480885	fibrillin 1 (Marfan syndrome)
BE588759	fibromodulin
BI535191	fibulin 1
BF193674	glypican-5
BE721926	glypican 6 precursor
BI681877	matrix Gla protein
AV617055	matrix Gla protein
AV617335	matrix Gla protein (MGP)
BE483482	periostin, osteoblast specific factor
BG692842	secreted protein, acidic, cysteine-rich (osteonectin)
BF231045	SPARC-like 1 (mast9, hevin)
BM253228	tenascin C (hexabrachion)
BI536820	thrombospondin 2 precursor
BE588788	tissue factor pathway inhibitor 2

Table 7. Gene list for clusters 1, 2 and 3.
These genes have relative high expression levels in 8.5mm follicles.

Bovine EST probe	Cluster	Gene Name
Response to biotic stimulus, immune response		
BE486958 and other 119 probes	1	Ig heavy chain V-II region
BE484536 and other 20 probes	1	Ig lambda chain V region
BI683121	1	immunoglobulin J polypeptide
BE487319	1	immunoglobulin kappa light chain
BG688986, BF231079, AV618063	1	beta-2-microglobulin precursor
BI899330, AV616322	3	interferon induced transmembrane protein 1
BE751955	3	interferon induced transmembrane protein 3 (1-8U)
BE757937	3	interferon, alpha-inducible protein (clone IFI-15K)
BE479755 and other 14 probes	2	major histocompatibility complex, class I, A (HLA-A)
BE480857	2	major histocompatibility complex, class I, B
AV617023	2	major histocompatibility complex, class I, C
BG692121	1	major histocompatibility complex, class II, DQ beta 2
BG690156	1	major histocompatibility complex, class II, DR alpha
BI682664	3	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
BI681428	3	ring finger protein 31
Response to biotic stimulus; Response to external stimulus; Response to stress		
AW659056, BE722088	3	2',5'-oligoadenylate synthetase 1
AV617567	1	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)
AV615891	2	HLA-G histocompatibility antigen, class I, G
BF074414	3	chemokine (C-X-C motif) receptor 4
BI682771	3	interferon, alpha-inducible protein (clone IFI-6-16)
BE589989	3	platelet-activating factor receptor
BF652994	3	signal transducer and activator of transcription 1, 91kDa
BE590193	3	BTG family, member 2
BE588838	3	protein S (alpha)
BE480885	1	fibrillin 1 (Marfan syndrome)
Primary metabolism, cellular metabolism		
BF230759	1	nudix (nucleoside diphosphate linked moiety X)-type motif 12
BE846316	2	non-POU domain containing, octamer-binding
BM089144	3	neuro-oncological ventral antigen 1
BE484321	1	glutamyl-prolyl-tRNA synthetase
BE590131	1	cysteinyl-tRNA synthetase
BE588535	1	cathepsin H
BG688750	2	prenylcysteine oxidase 1
BE588694	1	meningioma expressed antigen 5 (hyaluronidase)
BE681731	3	ubiquitin-conjugating enzyme E2L 6
BE588400	1	hect domain and RLD 2
BE588470	1	RAB25, member RAS oncogene family
BM086953	3	KIAA1404 protein

BI849485	1	zinc finger protein 462
BG690397	2	ash2 (absent, small, or homeotic)-like (Drosophila)
BE846068	3	ELK3, ETS-domain protein (SRF accessory protein 2)
BE481704	1	proteasome (prosome, macropain) 26S subunit, non-ATPase, 7 (Mov34 homolog)
BE845654	1	hypothetical protein FLJ23825
Transport, establishment of localization		
BF230436	1	Rhesus blood group, B glycoprotein
BE479346	1	solute carrier organic anion transporter family, member 2B1
BG688458	1	casein beta
Electrochemical potential-driven transporter activity, sugar transporter activity, nucleotide-sugar transporter activity		
BE487965	1	solute carrier family 35, member D2
Signal transduction, cell adhesion, cell motility		
BE588599	1	platelet/endothelial cell adhesion molecule (CD31 antigen)
Transferase activity, transferring one-carbon groups		
BE589261	1	protein arginine N-methyltransferase 7
Cation binding, polysaccharide binding, metal ion binding		
BE845486	1	follistatin-like 1
Other		
BG689591	1	similar to interleukin 32 isoform B isoform 1 [Bos taurus]
BE588542	1	similar to predicted CDS, reverse transcriptase family member (1O881)
BI774636	2	cytochrome oxidase subunit II
BM086834	3	similar to Interferon Sensitive Gene 12(a) protein
BE846379	1	Glycosylation-dependent cell adhesion molecule 1
BE478999	1	cell cycle progression 1
BG690578	1	ORM1-like 2 (S. cerevisiae)
BE488030	1	CCR4-NOT transcription complex, subunit 1
BI775266	1	placenta-specific 8
BE845831	1	responsive to centrifugal force and shear
BE478977	2	chromosome 14 open reading frame 31
BI682666	3	MANSC domain containing 1
BI976887	3	epithelial stromal interaction 1 (breast)
BE683743	3	DAMP-1 protein
BE755924	3	None
BE588335	1	None
BE589669	1	None

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Table 8. Gene list for clusters 4 and 5.
Genes in cluster 4 are mainly down-regulated at the 8.5mm follicles, while up-regulated at either 8 or 9mm follicles, and down-regulated at larger than 10mm follicles. Genes in cluster 5 are down-regulated at 8.5mm or larger follicles.

Bovine EST probe	Cluster	Gene Name
Cell organization and biogenesis		
AV616644	4	tubulin alpha 6
AW308044	4	enhancer of zeste homolog 2 (Drosophila)
AW652244	4	H2A histone family, member J
BE237555	4	H2A histone family, member V
BE479950	4	histone 1, H2ac
BE480595	4	H2A histone family, member Z
BE483306	4	nucleolar protein 5A (56kDa with KKE/D repeat)
BE684512	4	nephronophthisis 4
BE721392	4	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase 2
BE758025	4	profilin 2
BE809268	5	epithelial membrane protein 1
BE809708	4	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)
BF076713	4	histone 2, H2aa
BF076967	4	SET domain-containing protein 8
BG691573	4	high-mobility group 20B
BM956240	4	keratin 8
Cell proliferation		
AW659048	4	pituitary tumor-transforming 1
BE483388	4	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
BE681545	4	sperm associated antigen 5
BE685403	4	ubiquitin-conjugating enzyme E2C
BI538898	4	kinesin family member C1
BI682653	4	aurora kinase B
BI774026	4	enhancer of rudimentary homolog (Drosophila)
BI774847	4	baculoviral IAP repeat-containing 5 (survivin)
BI898467	4	mature T-cell proliferation 1
BI977151	4	septin 10
BM107139	4	G1 to S phase transition 1
BM107548	4	dUTP pyrophosphatase
BM285509	4	cyclin B1
BM286138	4	polo-like kinase 1 (Drosophila)
Cellular Metabolism		
BE481859	4	ubiquinol-cytochrome c reductase (6.4kD) subunit
BE681785	4	retinol dehydrogenase 11 (all-trans and 9-cis)
BF074547	4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa
BF604868	4	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like
Primary Metabolism		
BE664106	4	splicing factor, arginine/serine-rich 3
BE668678	4	stearoyl-CoA desaturase (delta-9-desaturase)
BE681761	4	forkhead box M1
BF654287	4	farnesyl-diphosphate farnesyltransferase 1
BF776331	4	heterogeneous nuclear ribonucleoprotein A2/B1
BI539902	4	pyruvate kinase, muscle

BI542071	4	phosphoglycerate dehydrogenase
BI773861	4	GLI-Kruppel family member HKR3
BI774997	4	TEA domain family member 4
BI776158	4	spermidine synthase
BI847256	4	splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)
BI849836	4	developmentally regulated GTP binding protein 1
Macromolecular Metabolism		
AV618099, BE588539	4	ribosomal protein L13
AW344540	4	makorin, ring finger protein, 2
AW426797	4	ribosomal protein S6 kinase, 90kDa, polypeptide 6
BE668167	4	cathepsin O
BE682785	4	mitochondrial ribosomal protein L34
BF077146	4	myo-inositol 1-phosphate synthase A1
BF077244	4	chaperonin containing TCP1, subunit 6A (zeta 1)
BI538315	4	proprotein convertase subtilisin/kexin type 7
BI774661	4	hypothetical protein MGC39558
BM257226	4	mitochondrial ribosomal protein S16
BM286560	5	lysyl oxidase-like 1
BM287471	4	receptor tyrosine kinase-like orphan receptor 2
Cell Adhesion		
AV615760, BI540727	4	integrin beta 1 binding protein 1
BE664746	4	plakophilin 3
BE683245	4	trophinin associated protein (tastin)
BI536820	5	thrombospondin 2
BI976428, BE681555	5	tumor necrosis factor receptor superfamily, member 12A
Blood Coagulation		
BE478875	5	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
BE752791	5	plasminogen activator, urokinase receptor
Signal Transduction		
AW336328	5	dickkopf homolog 3
BE667565	4	dickkopf-like 1
BF654618	4	purinergic receptor P2Y, G-protein coupled, 10
CK848121	4	stathmin 1/oncoprotein 18
Transport, Establishment of Localization		
AW658473	4	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5
BE667542	4	kinesin family member 20A
BM286486	4	NTF2-like export factor 1
Metal ion Binding, Cation Binding		
BE237335	5	MICAL-like 2
BE722262	4	zinc finger, DHHC domain containing 6
BF652740	4	deaminase domain containing 1
BI538026	4	S100 calcium binding protein A2
Response to Biotic Stimulus		
AW655090	4	sterile alpha and TIR motif containing 1
GTPase Activator Activity		
BI682370	4	hypothetical protein LOC257106
Purine Nucleotide Binding		
BF076846	5	nucleostemin

RNA Binding		
AW428160	4	heterogeneous nuclear ribonucleoprotein A/B
AW669019	4	heterogeneous nuclear ribonucleoprotein A3
Protein Binding		
BE664538	5	syndecan 4 (amphiglycan, ryudocan)
BE682031	4	BAI1-associated protein 1
BE682640	4	osteoclast stimulating factor 1
Chondroitin Sulfate Proteoglycan		
AW658172, BF776180	4	proteoglycan 1, secretory granule
Chondroitin / Heparan Sulfate Biosynthesis		
BI541727	Cluster4	carbohydrate (chondroitin) synthase 1
Others		
BE663402	4	Zwilch
BE667950	4	mannose-P-dolichol utilization defect 1
BE683388	4	hypothetical protein LOC339456
BE683880	4	trigger of mitotic entry 1
BE723695	4	hypothetical protein LOC84060
BE724209	4	hypothetical protein LOC23378
BF654381	4	SLAM family member 9
BF706803	4	MTB protein
BF773684	4	NONE
BF777150	4	hypothetical protein LOC55379
BI536312	4	ELOVL family member 6, elongation of long chain
BI537547	4	hypothetical protein LOC221908
BI681865	4	chromosome 13 open reading frame 3
BI898494	4	spindle pole body component 24 homolog
BM106356	4	hypothetical protein LOC154467
BM481505	4	NONE

Table 9. The expression values of the 271 probes identified in the Group model.

Bovine EST probe	Gene	G1 (8mm)	G2 (8.5mm)	G3 (9mm)	G4 (>9mm)
Hierarchical Cluster 1 (HC1)					
BE588535	cathepsin H	-0.507	0.907	-0.379	-0.021
BE480885	fibrillin 1 (Marfan syndrome)	-0.294	0.484	-0.240	0.049
BE845486	follistatin-like 1 precursor	-0.329	0.474	-0.267	0.122
BE484321	glutamyl-prolyl tRNA synthetase	-0.215	0.646	-0.273	-0.157
BE480680	HLA-DQB1	-0.264	0.480	-0.332	0.117
BF600849	HLA-DQB1	-0.324	0.461	-0.162	0.024
BE846205	C14orf124 protein	-0.365	0.515	-0.280	0.129
BE845654	hypothetical protein LOC284004	-0.516	0.950	-0.468	0.034
BG694188	immunoglobulin heavy chain	-0.692	1.171	-0.483	0.004
BE588685	immunoglobulin heavy chain	-0.446	1.034	-0.438	-0.151
BG692805	immunoglobulin heavy chain	-0.624	0.987	-0.436	0.073
BE845785	immunoglobulin heavy chain	-0.656	1.101	-0.524	0.079
BE845807	immunoglobulin heavy chain	-0.635	1.167	-0.722	0.190
BG688947	immunoglobulin heavy chain	-0.575	1.160	-0.711	0.126
BG689490	immunoglobulin heavy chain	-0.593	1.037	-0.490	0.046
BG692667	immunoglobulin heavy chain	-0.575	1.029	-0.476	0.022
BG693091	immunoglobulin heavy chain	-0.417	1.041	-0.636	0.012
BE476735	immunoglobulin heavy chain	-0.513	1.067	-0.601	0.047
BE481438	immunoglobulin heavy chain	-0.382	1.004	-0.680	0.058
BE588767	immunoglobulin heavy chain	-0.634	1.205	-0.692	0.120
BE588428	immunoglobulin heavy chain	-0.536	0.992	-0.508	0.053
BG691073	immunoglobulin heavy chain	-0.231	0.901	-0.595	-0.076
BG690425	immunoglobulin heavy chain	-0.535	0.949	-0.445	0.031
BE846341	immunoglobulin heavy chain	-0.344	0.832	-0.353	-0.135
BG690093	immunoglobulin heavy chain	-0.305	0.859	-0.383	-0.170
BG688426	immunoglobulin heavy chain	-0.554	0.811	-0.240	-0.018
BG688603	immunoglobulin heavy chain	-0.448	0.762	-0.270	-0.045
BG688447	immunoglobulin heavy chain	-0.513	0.821	-0.334	0.025
BE483986	immunoglobulin heavy chain	-0.471	0.827	-0.391	0.035
BE480644	immunoglobulin heavy chain	-0.383	0.732	-0.278	-0.071
BE488112	immunoglobulin heavy chain	-0.420	0.735	-0.248	-0.066
BE486699	immunoglobulin heavy chain	-0.321	0.677	-0.276	-0.080
BG690980	immunoglobulin heavy chain	-0.389	0.797	-0.445	0.036
BE482250	immunoglobulin heavy chain	-0.509	0.894	-0.404	0.020
BE485219	immunoglobulin heavy chain	-0.482	0.882	-0.413	0.013
BE482294	immunoglobulin heavy chain	-0.494	0.865	-0.328	-0.043
BE486159	immunoglobulin heavy chain	-0.495	0.733	-0.360	0.122
BE486845	immunoglobulin heavy chain	-0.428	0.734	-0.333	0.027
BE486616	immunoglobulin heavy chain	-0.316	0.642	-0.302	-0.023
BE481202	immunoglobulin heavy chain	-0.481	0.714	-0.299	0.067
BG691437	immunoglobulin heavy chain	-0.423	0.680	-0.273	0.016
BE588753	immunoglobulin heavy chain	-0.385	0.718	-0.365	0.032
BE589350	immunoglobulin heavy chain	-0.461	0.844	-0.401	0.017
BE483110	immunoglobulin heavy chain	-0.340	0.809	-0.431	-0.038
BE484612	immunoglobulin heavy chain	-0.350	0.714	-0.360	-0.003
BE480610	immunoglobulin heavy chain	-0.392	0.805	-0.333	-0.080
BE487609	immunoglobulin heavy chain	-0.236	0.686	-0.408	-0.042

BE482913	immunoglobulin heavy chain	-0.477	0.622	-0.203	0.058
BE487460	immunoglobulin heavy chain	-0.233	0.613	-0.280	-0.100
BE485907	immunoglobulin heavy chain	-0.385	0.722	-0.323	-0.013
BE485128	immunoglobulin heavy chain	-0.245	0.644	-0.259	-0.140
BE483154	immunoglobulin heavy chain	-0.362	0.584	-0.183	-0.039
BG688420	immunoglobulin heavy chain	-0.357	0.605	-0.198	-0.050
BE487664	immunoglobulin heavy chain	-0.300	0.494	-0.194	0.000
BE479990	immunoglobulin heavy chain	-0.334	0.516	-0.183	0.001
BE476895	immunoglobulin heavy chain	-0.300	0.571	-0.206	-0.066
BE480012	immunoglobulin heavy chain	-0.296	0.527	-0.169	-0.063
BE484508	immunoglobulin heavy chain	-0.255	0.594	-0.265	-0.073
BE488140	immunoglobulin heavy chain	-0.232	0.550	-0.248	-0.071
BE487595	immunoglobulin heavy chain	-0.289	0.616	-0.255	-0.072
BM106851	immunoglobulin heavy chain	-0.228	0.461	-0.150	-0.083
BE485851	immunoglobulin heavy chain	-0.232	0.609	-0.184	-0.193
BE845758	immunoglobulin heavy chain	-0.235	0.504	-0.135	-0.134
BE483310	immunoglobulin heavy chain	-0.196	0.450	-0.147	-0.107
BE480296	immunoglobulin heavy chain	-0.269	0.483	-0.138	-0.076
BE478888	immunoglobulin heavy chain	-0.179	0.510	-0.215	-0.117
BE480565	immunoglobulin heavy chain	-0.273	0.546	-0.150	-0.123
BE477214	immunoglobulin heavy chain	-0.285	0.531	-0.139	-0.107
BE482299	immunoglobulin heavy chain	-0.259	0.480	-0.080	-0.141
BE590178	immunoglobulin heavy chain	-0.862	1.002	-0.533	0.393
BE484411	immunoglobulin heavy chain	-0.590	0.985	-0.459	0.064
AV617934	immunoglobulin heavy chain	-0.630	0.982	-0.574	0.221
BE484411	immunoglobulin heavy chain	-0.404	0.816	-0.394	-0.018
BE589807	immunoglobulin heavy chain	-0.238	0.478	-0.258	0.018
BE484289	immunoglobulin heavy chain	-0.480	0.723	-0.312	0.068
AV617182	immunoglobulin heavy chain	-0.662	0.970	-0.562	0.253
AV615936	immunoglobulin heavy chain	-0.668	0.911	-0.435	0.192
AV618010	immunoglobulin heavy chain	-0.370	0.679	-0.377	0.068
BE476453	immunoglobulin light chain	-0.451	0.972	-0.599	0.078
BE488014	immunoglobulin light chain	-0.515	0.999	-0.511	0.026
BG691510	immunoglobulin light chain	-0.468	0.920	-0.586	0.134
BE485669	immunoglobulin light chain	-0.473	0.829	-0.341	-0.015
BE476331	immunoglobulin light chain	-0.419	0.861	-0.469	0.027
BE485662	immunoglobulin light chain	-0.518	0.787	-0.325	0.056
BF077033	immunoglobulin light chain	-0.374	0.613	-0.379	0.139
BG693446	immunoglobulin light chain	-0.347	0.482	-0.148	0.013
BE484536	immunoglobulin light chain	-0.359	0.518	-0.254	0.095
BE482455	immunoglobulin light chain	-0.365	0.586	-0.241	0.020
BG689524	immunoglobulin light chain	-0.286	0.540	-0.340	0.086
BI682771	interferon induced 6-16 protein	-0.293	1.080	-0.722	-0.065
BE757937	interferon, alpha-inducible protein	-0.637	0.835	-0.339	0.142
BE721138	lung cancer-related protein 8	-0.219	0.813	-0.275	-0.319
BI682666	MANSC domain containing 1	-0.494	0.636	-0.332	0.190
BI682664	myxovirus resistance protein 1	-0.548	0.948	-0.577	0.178
BE721081	NONE	-0.058	0.790	-0.541	-0.191
BG690578	ORMDL2	-0.387	1.114	-0.658	-0.069
BI775266	Placenta-specific gene 8 protein (C15 protein) (BM-004).	-0.338	0.877	-0.379	-0.160
BE481704	proteasome 26S non-ATPase subunit 7	-0.272	0.468	-0.189	-0.008
BE845831	responsive to centrifugal force and shear	-0.386	0.526	-0.187	0.047
BE683743	similar to DAMP-1 protein	-0.359	0.680	-0.432	0.111

BE588335	T cell receptor alpha (TCRA) gene, J segments and C region	-0.485	0.770	-0.176	-0.109
BE666586	tumor necrosis factor, alpha-induced protein 6	-1.559	0.817	-0.110	0.853
Hierarchical Cluster 2 (HC2)					
AW659056	2',5'-oligoadenylate synthetase 1	-0.440	0.410	-0.150	0.180
BE590166	5'-3' exoribonuclease 2	-0.217	-0.051	-0.186	0.454
BE476803	acyl-coA synthetase medium-chain family member 1	0.544	0.052	-0.728	0.132
BI849595	ADP-ribosylation factor GTPase activating protein 3	-0.433	0.061	0.008	0.364
BI683248	apolipoprotein D	-0.360	0.229	-0.312	0.443
BF074358	apolipoprotein E	-0.227	0.044	-0.140	0.323
AV618063	beta-2-microglobulin (B2M)	-0.302	0.447	-0.219	0.074
BE488030	CCR4-NOT transcription complex, subunit 1	-0.197	0.366	-0.098	-0.071
AV617567	CD74 molecule	-0.395	0.512	-0.416	0.300
BF776113	chemokine (C-X-C motif) ligand 12	-0.273	0.284	-0.202	0.191
BF074414	chemokine (C-X-C motif) receptor 4 isoform b	-0.143	0.405	-0.220	-0.042
BI538351	chromosome 21 open reading frame 70	-0.463	0.097	-0.038	0.404
BI849864	complement component 1, q subcomponent, beta polypeptide	-0.270	0.385	-0.264	0.149
BG691712	cyclin D3	-0.033	0.286	-0.261	0.007
BE590131	cysteinyl-tRNA synthetase isoform c	-0.284	0.383	-0.176	0.077
BI898472	DiGeorge syndrome critical region gene 2	-0.135	0.237	-0.352	0.251
BE480038	eukaryotic translation initiation factor 4b	-0.368	0.070	0.064	0.233
BF776059	eukaryotic translation initiation factor 5B	-0.259	-0.014	-0.167	0.440
BG691487	family with sequence similarity 32, member A	-0.487	0.182	0.065	0.239
BE589461	fatty acid synthase	-0.391	0.400	-0.183	0.173
BI682632	flotillin 1	-0.332	0.134	0.016	0.182
BF651289	glutathione S-transferase A1	-0.557	0.303	-0.095	0.349
BF193674	glypican-5	-0.242	0.111	-0.292	0.423
AV617625	golgi autoantigen, golgin subfamily b, macrogolgin (with transmembrane signal), 1	-0.354	0.557	-0.457	0.254
BI680361	growth arrest and DNA-damage-inducible, beta	-0.307	-0.035	-0.013	0.354
BI542011	guanylate binding protein 2,	-0.266	0.331	-0.182	0.117
AV617887	heat shock 90kDa protein 1, beta	-0.309	0.288	-0.181	0.202
BE588400	hect domain and RLD 2	-0.237	0.380	-0.219	0.077
BG692668	HLA-DQA2	-0.386	0.565	-0.416	0.237
BI898324	HLA-DQB1	-0.347	0.465	-0.327	0.209
BE845645	HLA-DQB1	-0.314	0.594	-0.523	0.243
BE751050	HLA-DQB1	-0.193	0.440	-0.343	0.096
BM285794	HLA-DQB1	-0.219	0.579	-0.439	0.079
BE757086	hypothetical protein FLJ32115	-0.375	0.029	-0.028	0.373
BI683121	IGJ	-0.240	0.337	-0.301	0.204
BI849376	immunoglobulin heavy chain	-0.208	0.402	-0.088	-0.106
BE478635	immunoglobulin heavy chain	-0.175	0.400	-0.146	-0.079
BI975985	immunoglobulin heavy chain	-0.039	0.408	-0.284	-0.085
BE487402	immunoglobulin heavy chain	-0.235	0.295	-0.073	0.012
BE485765	immunoglobulin heavy chain	-0.209	0.312	-0.067	-0.036
BF776346	immunoglobulin kappa constant	0.193	0.133	-0.498	0.172
BG691879	immunoglobulin kappa constant	0.269	0.088	-0.548	0.192
BE478211	immunoglobulin light chain	-0.291	0.402	-0.186	0.075
BF889951	immunoglobulin light chain	-0.210	0.397	-0.101	-0.086

AV615748	insulin-like 3 (Leydig cell)	-0.826	0.287	0.017	0.522
BM088711	integrin, beta 5	-0.528	0.133	0.128	0.266
AV616322	interferon induced transmembrane 1	0.020	0.446	-0.496	0.030
BI899330	interferon induced transmembrane 1	0.227	0.321	-0.462	-0.087
BE751955	interferon-induced transmembrane protein 3	-0.143	0.593	-0.509	0.059
BG691149	invariant gamma chain	-0.275	0.429	-0.297	0.142
AW483972	kynurenine aminotransferase III	-0.314	-0.154	0.197	0.272
BI775407	limitrin	-0.125	0.463	-0.359	0.021
AV617065	limitrin	-0.500	0.320	-0.188	0.369
AV618233	lin-7 homolog C (C. elegans)	-0.725	0.112	0.122	0.492
BM287881	LR8 protein	-0.984	0.342	-0.076	0.718
BM286284	Neural proliferation differentiation and control protein-1 precursor	-0.305	0.278	-0.186	0.213
BE485871	NONE	-0.290	-0.119	-0.097	0.506
AV616853	NONE	-0.351	0.219	-0.078	0.210
BE589669	NONE	-0.326	0.424	-0.187	0.089
BE667982	nth endonuclease III-like 1	0.249	0.001	-0.361	0.111
BI539645	nucleoside-diphosphate kinase 1 isoform a	-0.241	0.345	-0.178	0.073
BM258220	olfactomedin-like 2B	-0.325	0.153	-0.136	0.308
BF775923	peptidyl-prolyl isomerase G (cyclophilin G)	-0.296	0.140	-0.205	0.361
AV616507	phenylalanine-tRNA synthetase	-0.850	0.300	-0.046	0.596
AW654433	placenta-specific 8	-0.129	0.312	-0.209	0.025
BI681401	prostaglandin D2 synthase 21kDa (brain)	-0.307	0.455	-0.396	0.248
BI774890	proteasome beta 8 subunit isoform E2 proprotein	0.002	0.233	-0.335	0.100
BE589261	protein arginine N-methyltransferase 7	-0.198	0.349	-0.150	-0.001
BE588470	RAB25, member RAS oncogene family	-0.199	0.419	-0.180	-0.040
BI538822	regulatory solute carrier protein family 1	-0.331	-0.074	0.167	0.238
BE588842	rho/rac guanine nucleotide exchange factor 2	-0.168	0.386	-0.181	-0.037
AV617663	ribosomal protein S14 (RPS14)	-0.370	0.039	0.157	0.174
AV616842	ribosomal protein S3 (RPS3)	-0.765	0.228	0.090	0.447
AV617187	ribosomal protein S3A (RPS3A)	-0.306	0.049	-0.117	0.374
BF651339	secreted and transmembrane 1 precusor	-0.210	0.158	-0.229	0.281
AW656093	secretory granule, neuroendocrine protein 1 (7B2 protein)	-0.321	0.035	0.091	0.195
BF652994	signal transducer and activator of transcription (STAT1)	-0.093	0.302	-0.228	0.019
AV617693	similar to CG33196-PB	-0.610	0.429	0.023	0.158
BI535705	solute carrier family 26, member 11	-0.326	0.087	-0.002	0.240
BE479346	solute carrier organic anion transporter family,	-0.173	0.358	-0.198	0.013
AW483763	transmembrane 7 superfamily member 2	-0.452	0.130	-0.393	0.715
BG691599	tryptophanyl-tRNA synthetase	0.034	0.284	-0.269	-0.048
AW656034	tubulin tyrosine ligase-like family, member 3	-0.432	0.118	0.034	0.280
BM258429	TYRO protein tyrosine kinase binding protein	0.030	0.296	-0.436	0.110
BE588662	ubinuclein 1	0.077	0.241	-0.296	-0.023
BE681731	ubiquitin-conjugating enzyme E2L 6 isoform 1	-0.380	0.406	-0.255	0.230
AV616044	UP|RL31_HUMAN (P12947) 60S ribosomal protein L31, complete	0.096	0.269	-0.342	-0.023
AV615752	vimentin (VIM)	-0.789	0.247	0.040	0.502
BI774882	yippee-like 3 (Drosophila)	-0.338	0.309	-0.105	0.134
BI849485	zinc finger protein 462	-0.122	0.278	-0.254	0.098
Hierarchical Cluster 3 (HC3)					
BG692962	alpha tubulin (K-ALPHA-1)	0.204	0.004	0.140	-0.348

BE589170	alpha tubulin (K-ALPHA-1)	0.283	-0.104	0.081	-0.261
BI682653	aurora kinase B	0.375	-0.152	-0.034	-0.188
BI774847	baculoviral IAP repeat-containing protein 5	0.397	-0.333	0.066	-0.129
BG687761	basic leucine zipper and W2 domains 2	-0.400	0.000	0.559	-0.158
BE722024	BUB1 budding uninhibited by benzimidazoles 1	0.337	-0.184	-0.061	-0.092
BF890328	C3orf40	0.349	-0.102	-0.208	-0.039
BI541727	carbohydrate (chondroitin) synthase 1	0.348	-0.196	-0.040	-0.112
AW415374	chaperonin containing TCP1, subunit 2	0.288	-0.137	0.067	-0.218
BF077244	chaperonin containing TCP1, subunit 6A isoform	0.300	-0.116	0.058	-0.242
AW336260	chemokine (C-C motif) ligand 2	0.260	0.144	-0.072	-0.332
BI681504	chemokine (C-C motif) ligand 2	0.518	0.293	-0.241	-0.570
BI681865	chromosome 13 open reading frame 3	0.383	-0.126	-0.038	-0.219
BE749546	collectin sub-family member 12 isoform I	0.545	-0.197	-0.078	-0.270
BM285509	cyclin B1	0.477	-0.254	0.134	-0.358
BI542071	D-3-phosphoglycerate dehydrogenase (3-PGDH)	0.603	-0.215	-0.107	-0.281
BF652740	deaminase domain containing 1	0.446	-0.187	-0.018	-0.240
BE667565	dickkopf-like 1 (soggy) precursor	0.458	-0.163	-0.003	-0.292
BM107548	dUTP pyrophosphatase	0.371	-0.155	-0.024	-0.192
BE479792	erythrocyte membrane protein band 4.1-like 2	0.081	0.206	0.187	-0.474
BI976407	family with sequence similarity 64, member A	0.456	-0.163	-0.002	-0.291
BE681555	Fibroblast growth factor-inducible immediate-early response protein 14	0.999	-0.323	0.256	-0.932
BI976428	Fibroblast growth factor-inducible immediate-early response protein 14	0.712	-0.241	0.174	-0.645
BI774293	FLJ13111 protein	0.330	-0.085	-0.034	-0.212
BF654618	G-protein coupled purinergic receptor P2Y10	0.387	-0.192	-0.058	-0.137
BE480595	H2A histone family, member Z	0.477	-0.164	0.045	-0.357
BI681634	high-mobility group nucleosomal binding domain	0.332	-0.107	0.032	-0.257
AW315322	homer homolog 1 (Drosophila)	0.038	0.235	0.204	-0.477
AV617709	Hypothetical protein FLJ12541	0.356	-0.206	0.093	-0.243
BE723695	hypothetical protein LOC84060	0.425	-0.243	-0.039	-0.144
AV617933	immunoglobulin heavy chain	0.080	0.219	0.207	-0.506
BE809708	Importin alpha-2 subunit	0.376	-0.222	0.054	-0.209
BM956240	keratin 8	0.431	-0.200	-0.029	-0.203
BF605392	keratin 8 (KRT8)	0.438	-0.276	0.034	-0.197
BE667542	Kinesin family member 20A (Rabkinesin-6)	0.513	-0.311	0.141	-0.343
BI538898	kinesin family member C1	0.556	-0.085	-0.268	-0.204
BI682096	lamin B receptor	0.337	-0.018	-0.103	-0.217
BI849746	lamin B1	0.374	-0.234	-0.134	-0.005
BE668652	lysyl oxidase preproprotein	0.259	-0.450	0.333	-0.142
AW344540	makorin, ring finger protein, 2	0.461	-0.090	-0.243	-0.128
BE682031	membrane associated guanylate kinase, WW and PDZ	0.454	-0.270	0.074	-0.258
BE685559	metallothionein 1B (functional)	0.199	0.450	0.266	-0.915
BI535388	metallothionein 2A	0.160	0.431	0.162	-0.753
AW670008	metallothionein 3 (growth inhibitory factor (neurotrophic))	0.232	0.179	0.239	-0.650
BE590057	MFGE8 protein	-0.256	-0.345	0.784	-0.184
BM106648	MHC class I-like family A1	0.465	-0.493	0.094	-0.066
BE684989	mitochondrial carrier protein MGC4399	0.352	-0.185	-0.053	-0.114

BE681545	mitotic spindle coiled-coil related protein	0.375	-0.359	0.139	-0.155
BI680630	NOL5A protein	0.404	-0.025	-0.131	-0.248
BI681072	NONE	0.047	0.211	0.168	-0.426
AW313891	NONE	-0.045	-0.117	0.436	-0.274
BE666643	Nucleolar phosphoprotein Nopp34	0.367	-0.078	-0.151	-0.138
BE483306	nucleolar protein 5A	0.502	-0.159	-0.131	-0.212
BF230164	oxoglutarate (alpha-ketoglutarate) dehydrogenase	-0.193	-0.349	0.356	0.186
BM286138	polo-like kinase	0.425	-0.234	-0.010	-0.181
BI682370	Rho GTPase activating protein 30	0.320	-0.276	0.062	-0.105
AV618082	ribosomal protein SA	0.349	-0.139	-0.053	-0.157
BE667735	sec13-like protein isoform 2	0.424	-0.218	-0.103	-0.104
AW659048	Securin (Pituitary tumor-transforming protein 1)	0.433	-0.174	-0.013	-0.247
AV616920	selenoprotein X, 1 (SEPX1)	0.622	-0.460	-0.061	-0.100
BE478875	SERPINE1 (plasminogen activator inhibitor-1)	0.452	-0.186	0.030	-0.296
BM031483	similar to proliferating cell nuclear antigen	0.406	-0.130	-0.219	-0.056
BI776158	spermidine synthase	0.478	-0.147	-0.099	-0.232
AV617667	SPFH domain family, member 2 isoform 1	0.205	-0.170	0.292	-0.327
BI898494	spindle pole body component 24 homolog	0.469	-0.191	-0.021	-0.257
BE664106	splicing factor, arginine/serine-rich 3	0.420	-0.208	-0.102	-0.109
BE668678	stearoyl-CoA desaturase	0.339	-0.149	0.026	-0.216
BG688965	thymidylate synthetase	0.376	-0.082	-0.090	-0.204
BI848473	Transcription factor jun-B, complete	0.313	-0.017	0.104	-0.400
BE683880	trigger of mitotic entry 1	0.354	-0.213	0.053	-0.194
BE683245	Trophinin-associated protein (Tastin) (Trophinin-assisting protein)	0.469	-0.255	0.037	-0.251
AV616644	tubulin, alpha 6 (TUBA6)	0.343	-0.145	0.054	-0.252
AW356068	tubulin, beta 2A (TUBB2A)	0.371	-0.104	-0.046	-0.222
BE685403	ubiquitin-conjugating enzyme E2C isoform 1	0.393	-0.259	0.125	-0.260
BI774661	UDP-GalNAc:betaGlcNAc beta	0.370	-0.092	-0.086	-0.192
BI682243	v-maf musculoaponeurotic fibrosarcoma oncogene homolog f (avian)	0.390	-0.117	-0.029	-0.244
BE722262	Zinc finger DHHC domain containing protein 6 (Zinc finger protein 376)	0.313	-0.286	0.105	-0.133
BE663402	Zwilch	0.365	-0.192	-0.137	-0.036

Table 10. Enrichment of biological processes and cellular components in the differentially expressed gene list.
Data were obtained from the NIH-DAVID database by selecting GO Biological Process, level 4 and GO Cellular Components, level 2. GO terms with P-value less than 0.05 were shown.

Gene Ontology	Term	Percent (%)	P value
GOTERM_BP_4	immune response	13.94%	1.72E-04
GOTERM_BP_4	organelle organization and biogenesis	10.91%	6.46E-04
GOTERM_BP_4	mitotic cell cycle	4.85%	0.0018
GOTERM_CC_2	non-membrane-bound organelle	14.55%	0.014
GOTERM_CC_2	extracellular space	6.06%	0.015

Table 11. Genes used for qRT-PCR analysis.

Gene	GenBank Accession	Forward Primer	Reverse Primer	Size
Control				
ACTB	NM173979	cgccatggatgatgatattgc	aagccggccttgcacat	66
GAPDH	U85042	aaggccatcaccatcttcca	actacatactcagcaccagcatcac	74
PFKM	BM106534	ggtgcctgccgctgact	tgacactgccctcaacaccat	64
CGNL1	BM255301	tcgcggctaaccgaaatg	tgggcaccgaggagaagag	71
Positive Controls				
MT1	BE685559	ccagggctgtgtctgcaaa	ctattgacaactagggcaggttctc	84
MT2A	BI535388	accctcgccatcctttgc	atccatggcgagctgaactg	63
Hormone response and steroidogenesis				
LHCGR[1]	U20504	tgggacaacgctgatttcc	gcgtcgttgtgcatcttctc	66
AMH[2]	NM173890	cggtccatctctctctctgg	aaggctgaggtgctgaagac	96
CYP19A1[1]	U18447	ccatctgtgctgattccatca	cacatagcccaagtcattgca	71
CYP21A2	BI682708	gcatcccgacctgttcatg	gcttaatgatggcgagatgagtt	73
HSD3B2	BE808311	agagaccatcatgaacgtcaatgt	acgctggcctggacaca	69
INHA	BI680983	agatgtctcccaggccatc	ggctggaacacatacgtgaa	105
INHBA	DY052988	gccgaaatgaatgaacttatgga	cttcctggctgtgcctgatt	72
FST	BF774514	gccagagctgcaagtccagta	gagctgcctggacagaaaaca	72
IGF system				
IGF1[2]	BM288554	ctgcttttgtgatttcttgaagca	agcaagcacagggccagata	81
IGF2[2]	BM483253	accctccagtttgtctgtgg	cggaagcaacactcttcca	101
IGFBP2	AF074854	caagggtggcaaacatcac	gacctggtccaattcctgct	94
PAPPA[1]	AF421141	cttcaacactccatacaataacttcatg	gacctgattgggtgtgaagga	76
Cluster 1				
B2M	BF231079	ccgagtgaaacacgttactttgg	cctcgatggtgctgcttaca	75
Cluster 4				
PKM2	BC102826	caccggcatcatctgtacca	ttccagacttaatcatctccttcaatg	74
SCD	AF188710	ggagtcaccgaacctacaaagc	ggtgttgccaatgatcaggaa	62
DEADC1	BF652740	cgtggagccctgtatcatgtg	ccatagacaaccagtgggattct	66
Cluster 5				
THSB2	BI536820	tgctgttacccagacacctcagt	tcagggataggaggtctgctaga	72
SDC4	CK980315	tccgctccctgtcttctca	cggatcttcccagaagtgatg	72
SERPINE1	BE478875	ccttgtagtctcagcccgaatg	ccgcaccacgaagaggaa	70

Note: 1. Genes not present on the array but checked with qRT-PCR.
2. Genes presented on the array but with close-to-background intensities.

Table 12. Genes identified by PTM with similar expression profiles to the LHR gene.

Gene	KEGG-pathway
farnesyl diphosphate synthase; farnesyl-diphosphate farnesyltransferase 1; isopentenyl-diphosphate delta isomerase; squalene epoxidase	Biosynthesis of steroid
cytochrome P450, family 21, subfamily A, polypeptide 2 (CYP21A2); 3β-HSD (HSD3B2)	C-21 steroid hormone metabolism
glutathione S-transferase A1; glutathione S-transferase A4; glutathione S-transferase M3; glutathione S-transferase M4	Glutathione metabolism
cytochrome p450, family 4, subfamily f, polypeptide 2	arachidonic acid metabolism,
sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (tm) and short cytoplasmic domain, (semaphorin) 5b	axon guidance
cyclin-dependent kinase inhibitor 1c (p57, kip2)	cell cycle
complement component 4 binding protein, alpha	complement and coagulation cascades
acyl-CoA synthetase short-chain family member 2; phosphoglycerate mutase 2 (muscle)	glycolysis / gluconeogenesis
aldehyde dehydrogenase 1 family, member a1	retinol metabolism
flotillin 1	insulin signaling pathway
mitogen-activated protein kinase 1	MAPK signaling pathway
apolipoprotein E	neurodegenerative disorders
ATP synthase, H+ transporting, mitochondrial f1 complex, delta subunit	oxidative phosphorylation; ATP synthesis
branched chain aminotransferase 2, mitochondrial	valine, leucine and isoleucine degradation
putative nuclear protein orf1-fl49	
protease, serine, 2 (trypsin 2)	
chromosome 8 open reading frame 72	
KIAA0258	
brain expressed x-linked 2	
sodium channel, voltage-gated, type ix, alpha	
scavenger receptor class b, member 1	
LR8 protein	
ferredoxin 1	
otospiralin	
protease, serine, 3 (mesotrypsin)	
timp metallopeptidase inhibitor 2	
family with sequence similarity 76, member b	
family with sequence similarity 32, member a	
troponin i type 3 (cardiac)	
transmembrane 7 superfamily member 2	
solute carrier family 35, member f5	

Table 13. Probes that were differentially expressed between different groups but not in the Sample model 976 probe list.

Bovine EST probe	Gene Name
Cell organization and biogenesis	
BE589170*	alpha tubulin (K-ALPHA-1), mRNA
BE722024*	BUB1 budding uninhibited by benzimidazoles 1
BI681634*	high-mobility group nucleosomal binding domain
BF605392	keratin 8 (KRT8), mRNA
BI680630*	NOL5A protein (OTTHUMP00000030037).
BE588842	rho/rac guanine nucleotide exchange factor 2
AW656034	tubulin tyrosine ligase-like family, member 3
AW356068*	tubulin, beta 2A (TUBB2A), mRNA
Transport	
BE749546	collectin sub-family member 12 isoform I
BE684989*	mitochondrial carrier protein MGC4399
BI538822	regulatory solute carrier protein family 1
BE667735	sec13-like protein isoform 2
BI535705	solute carrier family 26, member 11
Macromolecule metabolism	
BE667982	nth endonuclease III-like 1
BF775923	peptidyl-prolyl isomerase G (cyclophilin G)
Cellular localization	
AW656093	secretory granule, neuroendocrine protein 1 (7B2 protein)
Cell cycle	
AW415374*	chaperonin containing TCP1, subunit 2
BG691712*	cyclin D3
BI539645*	nucleoside-diphosphate kinase 1 isoform a
Cellular metabolism	
BE476803	acyl-coa synthetase medium-chain family member 1
BI774293	FLJ13111 protein
BE588662	ubinuclein 1
Structural molecule activity	
BI849746	lamin B1
BI682096	lamin B receptor
Immune	
BI975985	immunoglobulin heavy constant alpha 1
BE487402*	immunoglobulin heavy constant alpha 1
BE485765	immunoglobulin heavy constant alpha 1
Other	
BF890328	C3orf40
BI976407	family with sequence similarity 64, member A
AV617709	hypothetical protein FLJ12541
AV617667	SPFH domain family, member 2 isoform 1
AW313891	

Note: probes labeled with * were identified by Sample model with FDR 0.05 but not passing the 1.4 fold filtering.

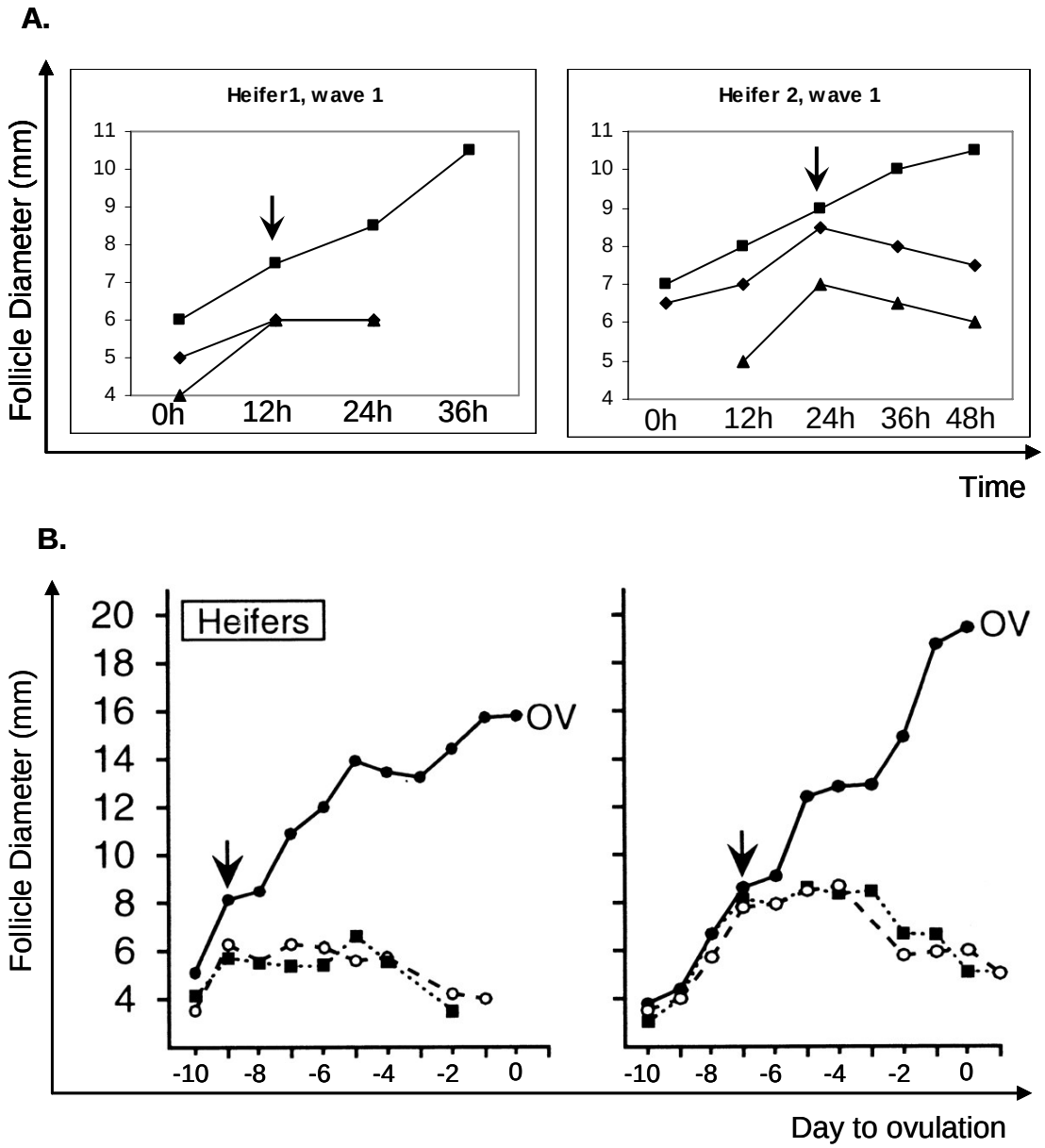


Figure 1. Profile of the three largest follicles of the first wave (A) and the ovulatory wave (B) in different individuals.

The arrow indicates the estimated time of deviation. Figure 1A is based on the data from our lab. Figure 1B is adapted from Ginther et al.(2001).

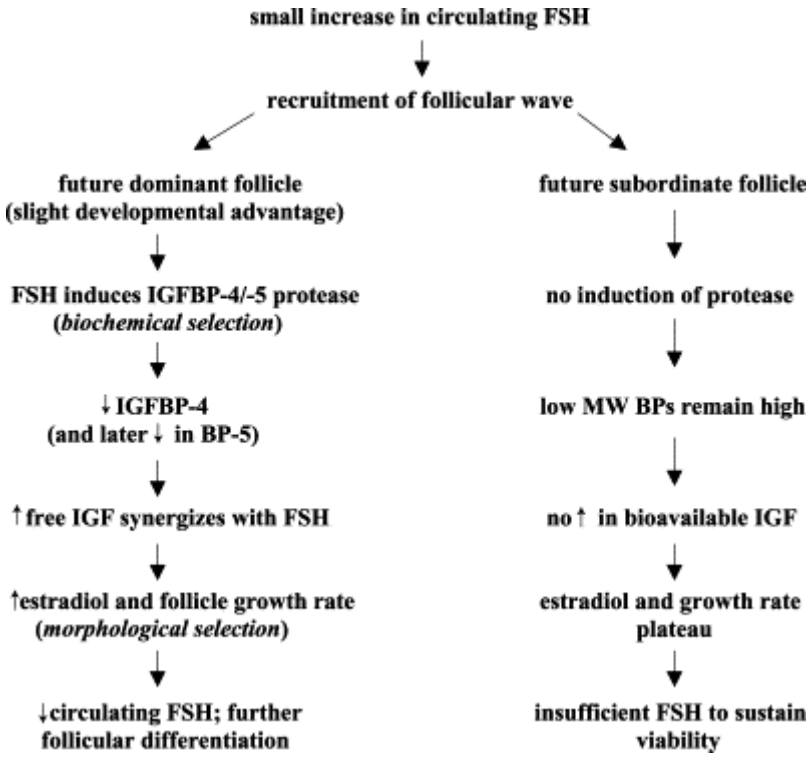


Figure 2. Suggested sequence of events during selection of the dominant follicle in cattle.

The picture is taken from Fortune et al., 2004. As described by Fortune et al., this model suggests that a critical event of "biochemical selection" is the induction by FSH of a protease for IGFBP-4 in one follicle of a cohort and that biochemical selection quickly leads to "morphological selection" and further differentiation of the dominant follicle.

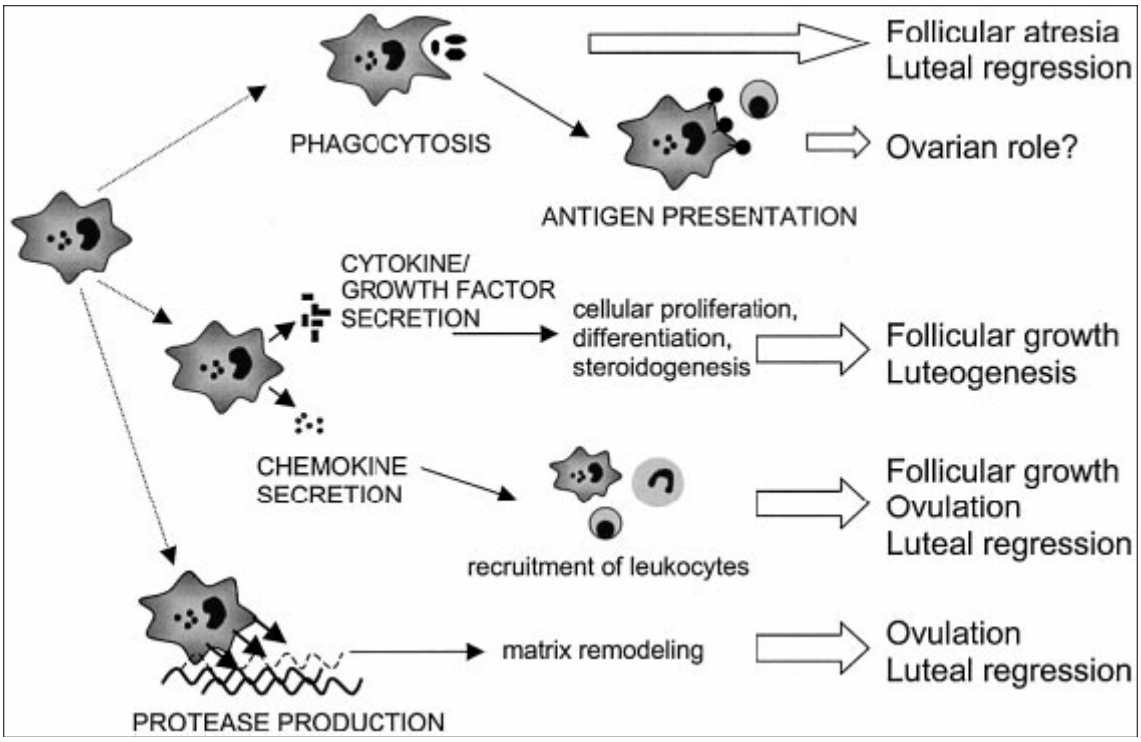


Figure 3. Macrophage involvement in ovarian function.
The picture is taken from Wu et al. 2004.

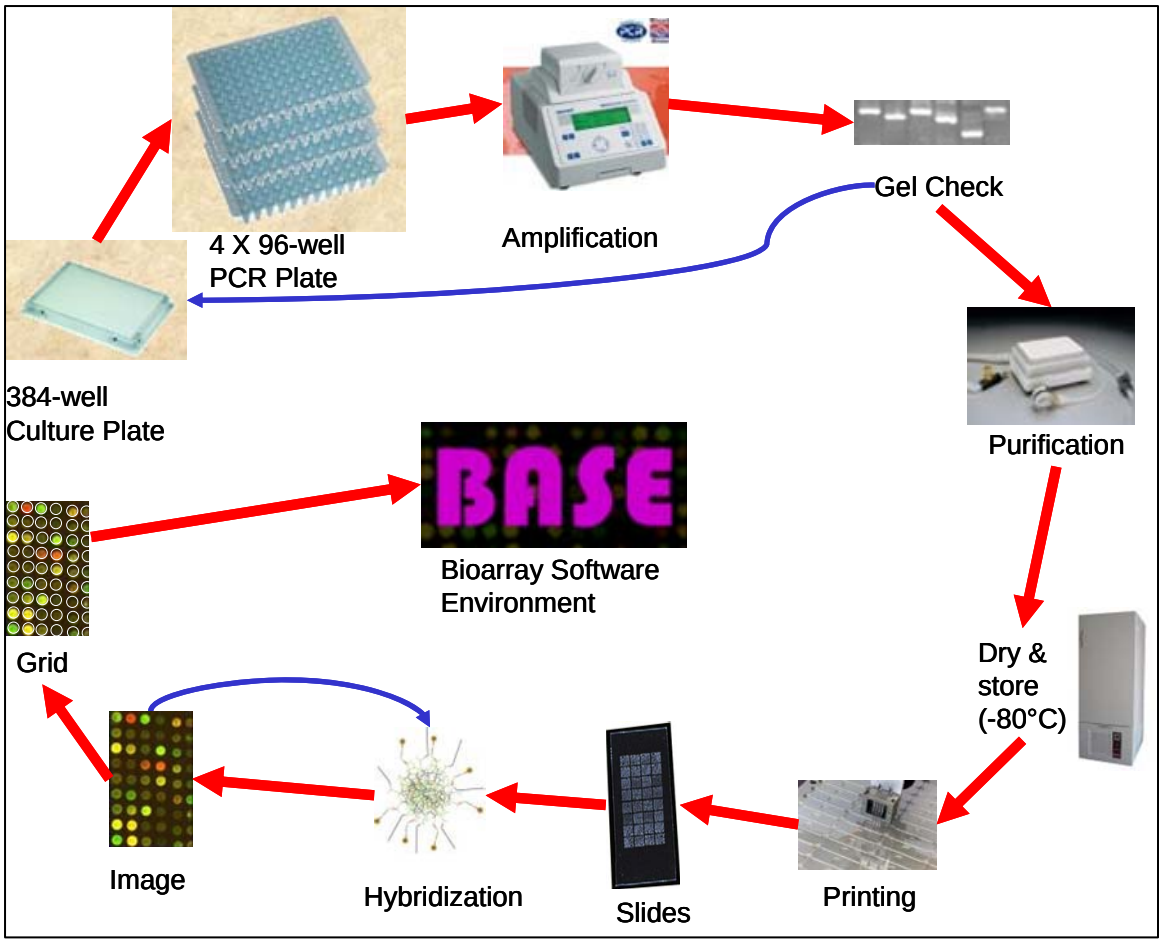


Figure 4. The process of array construction, hybridization and data collection.

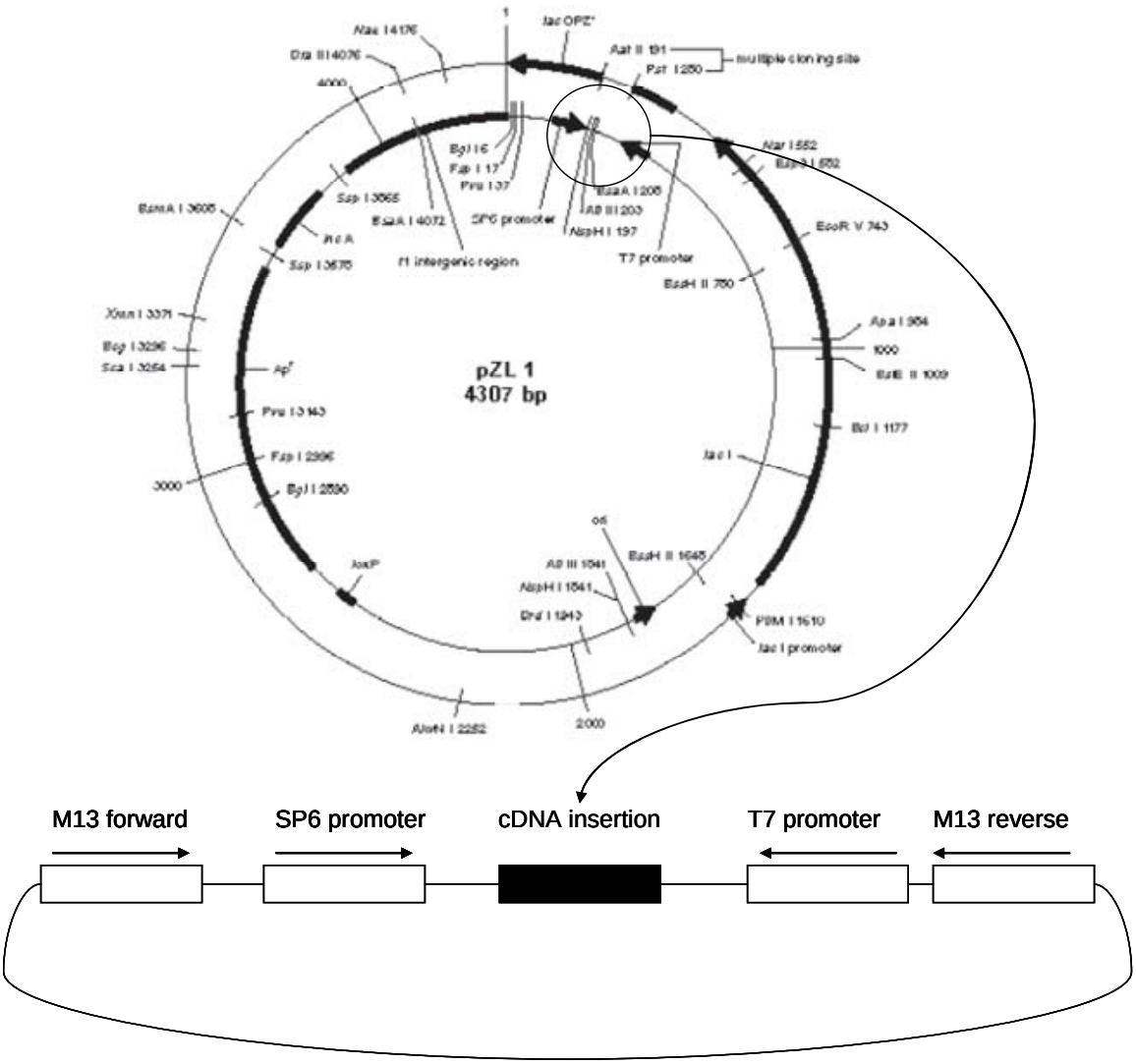


Figure 5. Map of pZL1 showing the M13 forward and reverse primers and T7/SP6 primer pairs.
The picture is modified from the vector map from www.invitrogen.com.

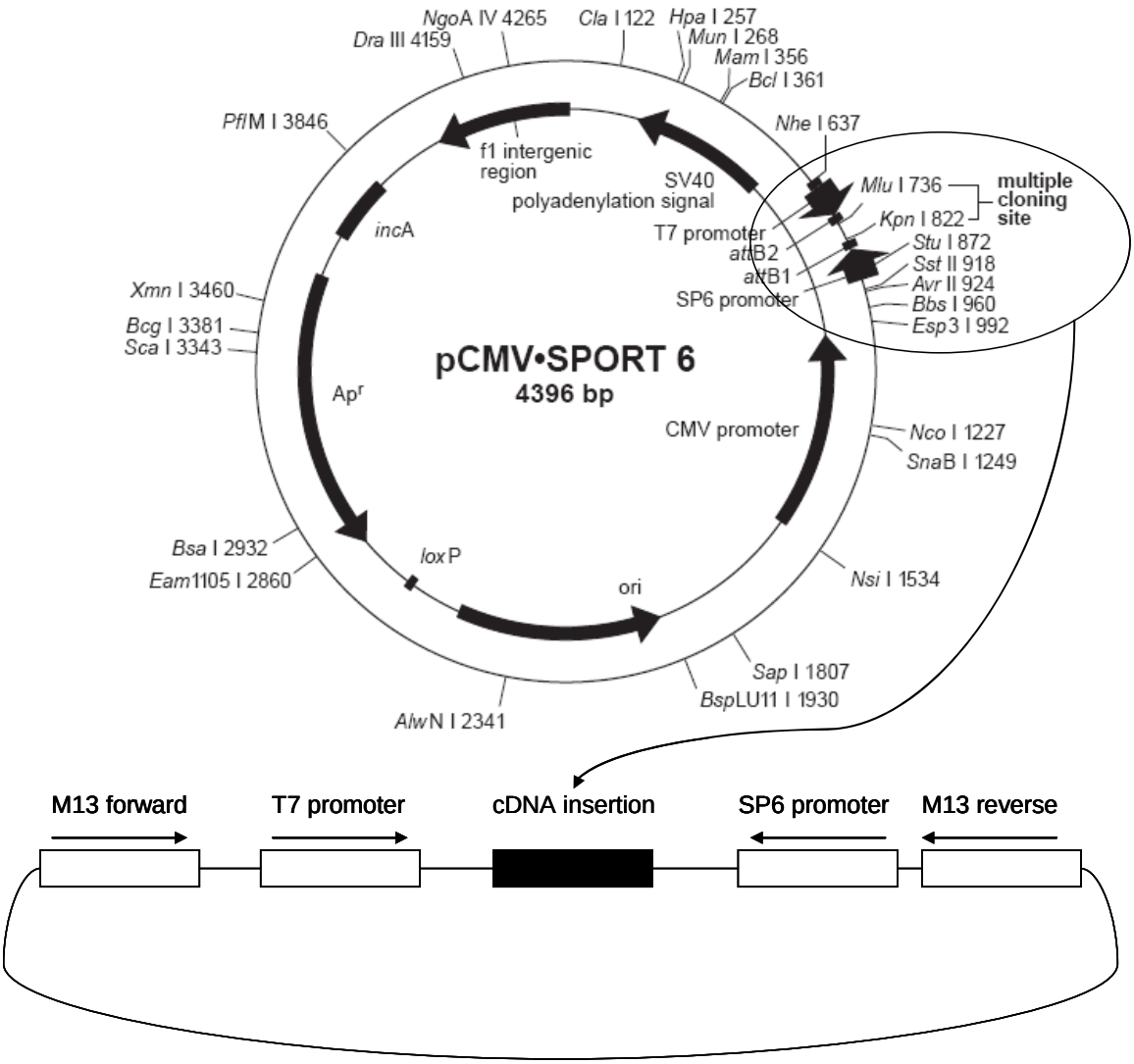


Figure 6. Map of pCMV·SPORT6 showing the M13 forward and reverse primers and T7/SP6 primer pairs.
The picture is modified from the vector map from www.invitrogen.com.

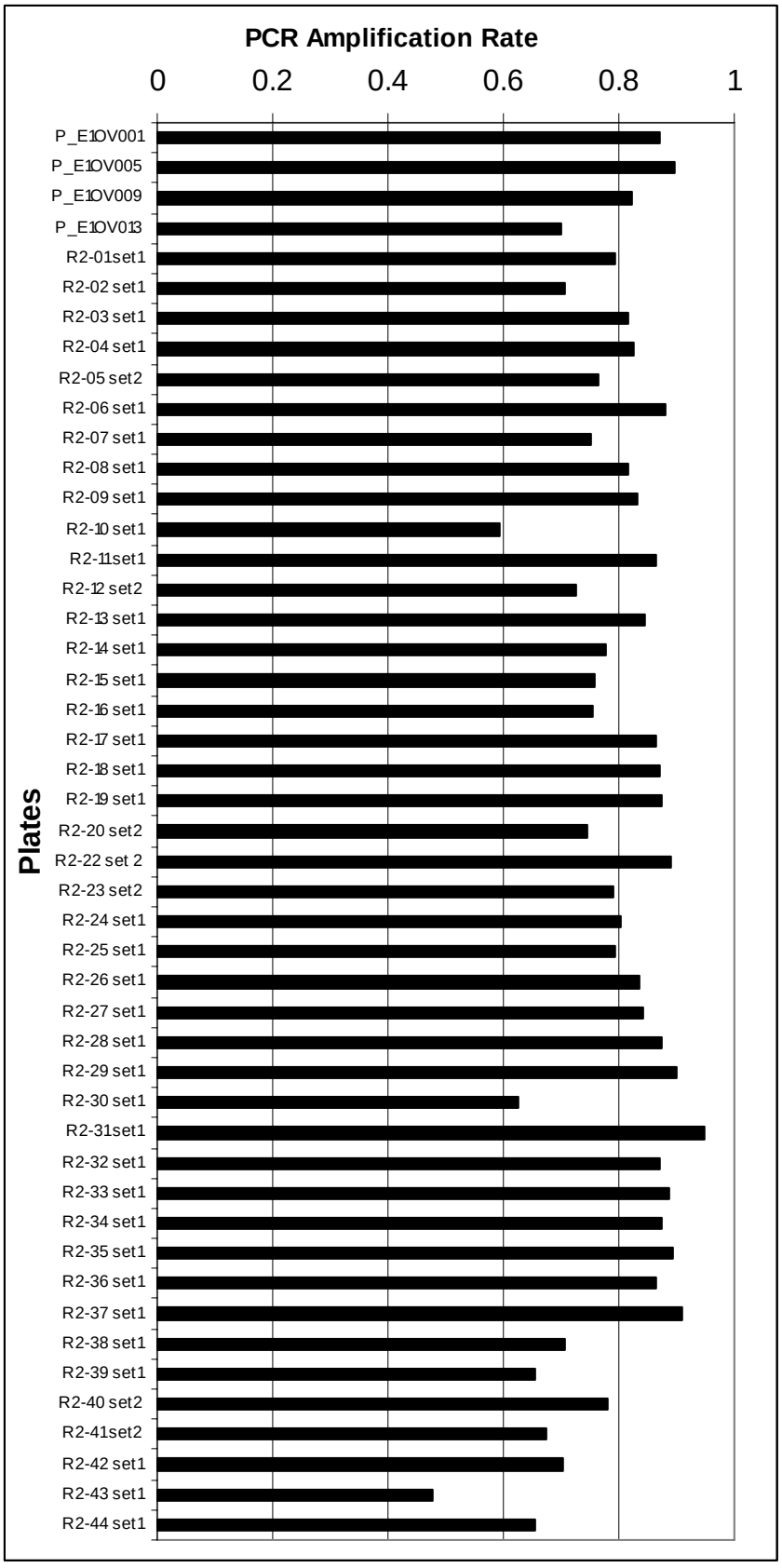


Figure 7. The PCR amplification rate for all the 384-well culture plates.

A

B)

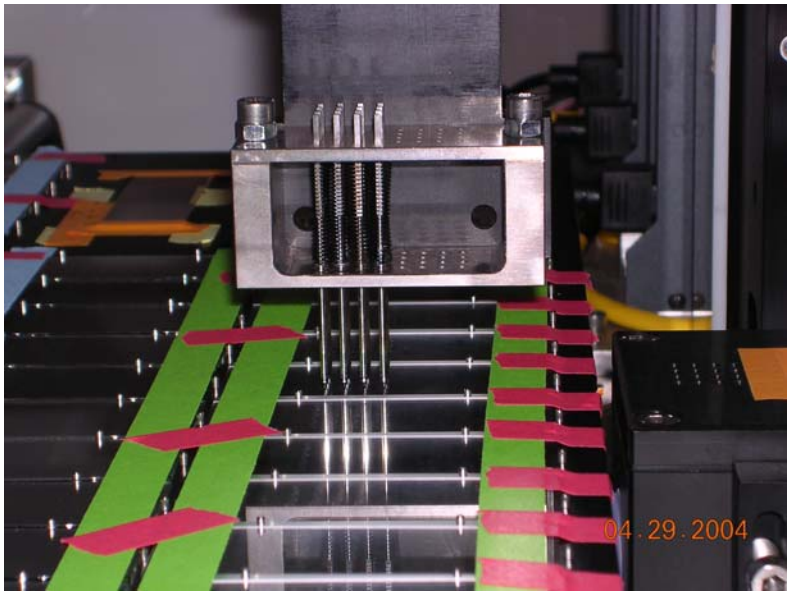


Figure 8. A) The arrayer assembled in our lab and B) the printing head, which can hold up to 32 pins, in a 4×8 format.
Also showing in B is the tape used to stabilize the slides. Pictures were taken by Yi Zhuang.

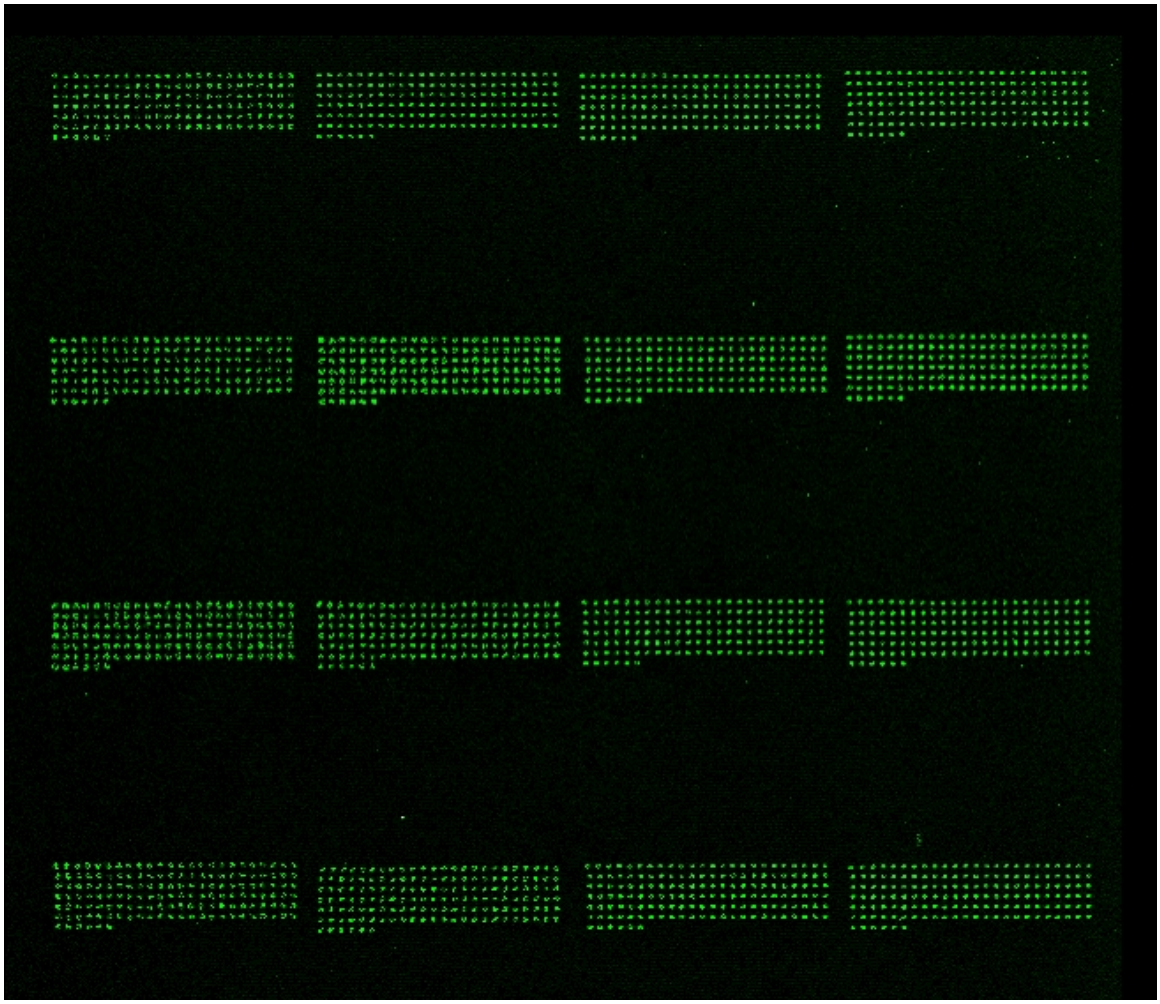


Figure 9. Test print showing the uniform morphology of the spots printed by each pin.

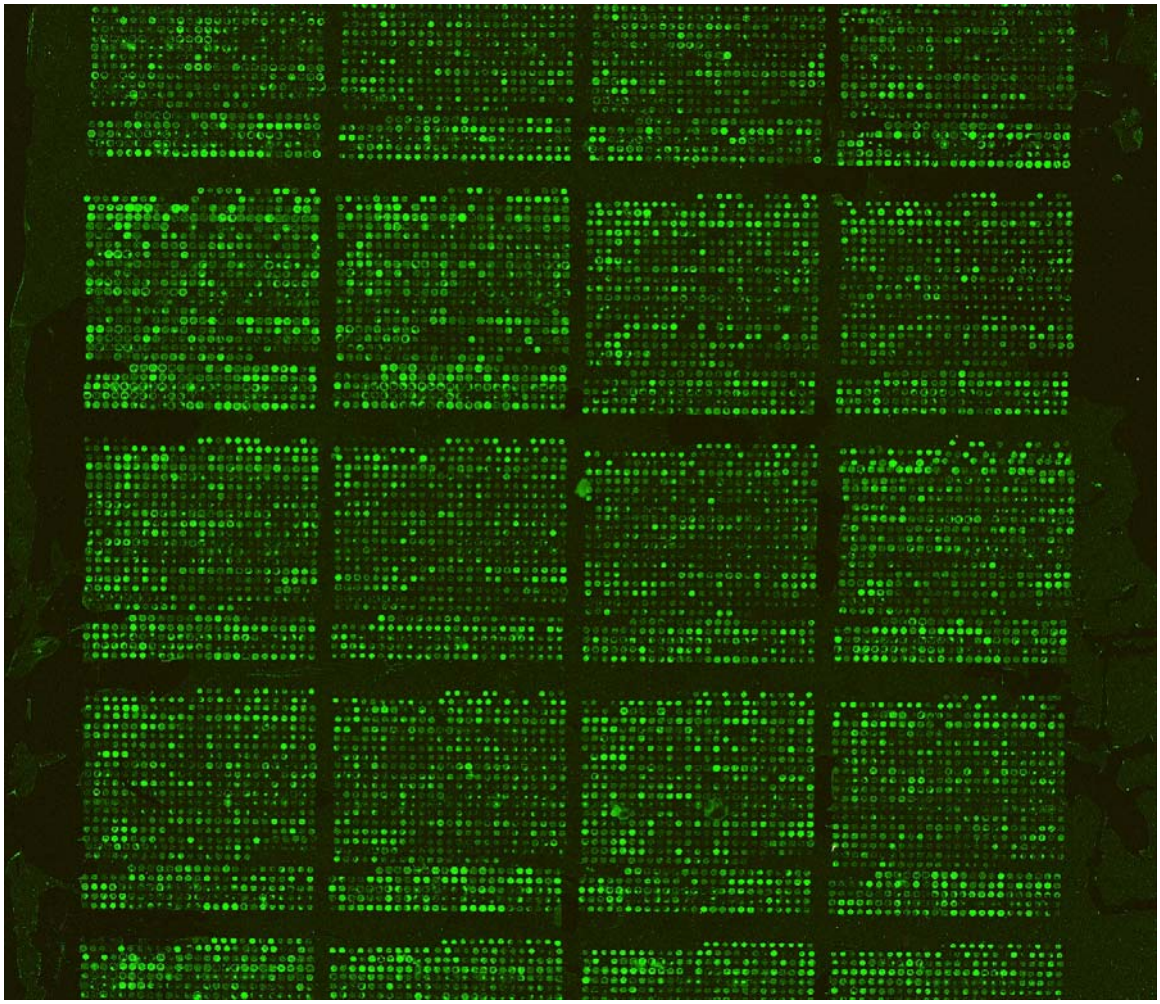


Figure 10. Partial image of a bovine 18K array after 19 months of storage.
The array is printed on poly-L-lysine slide and is hybridized with one sample labeled with cy3. This hybridization was done by Raja Sekhar Settivari.

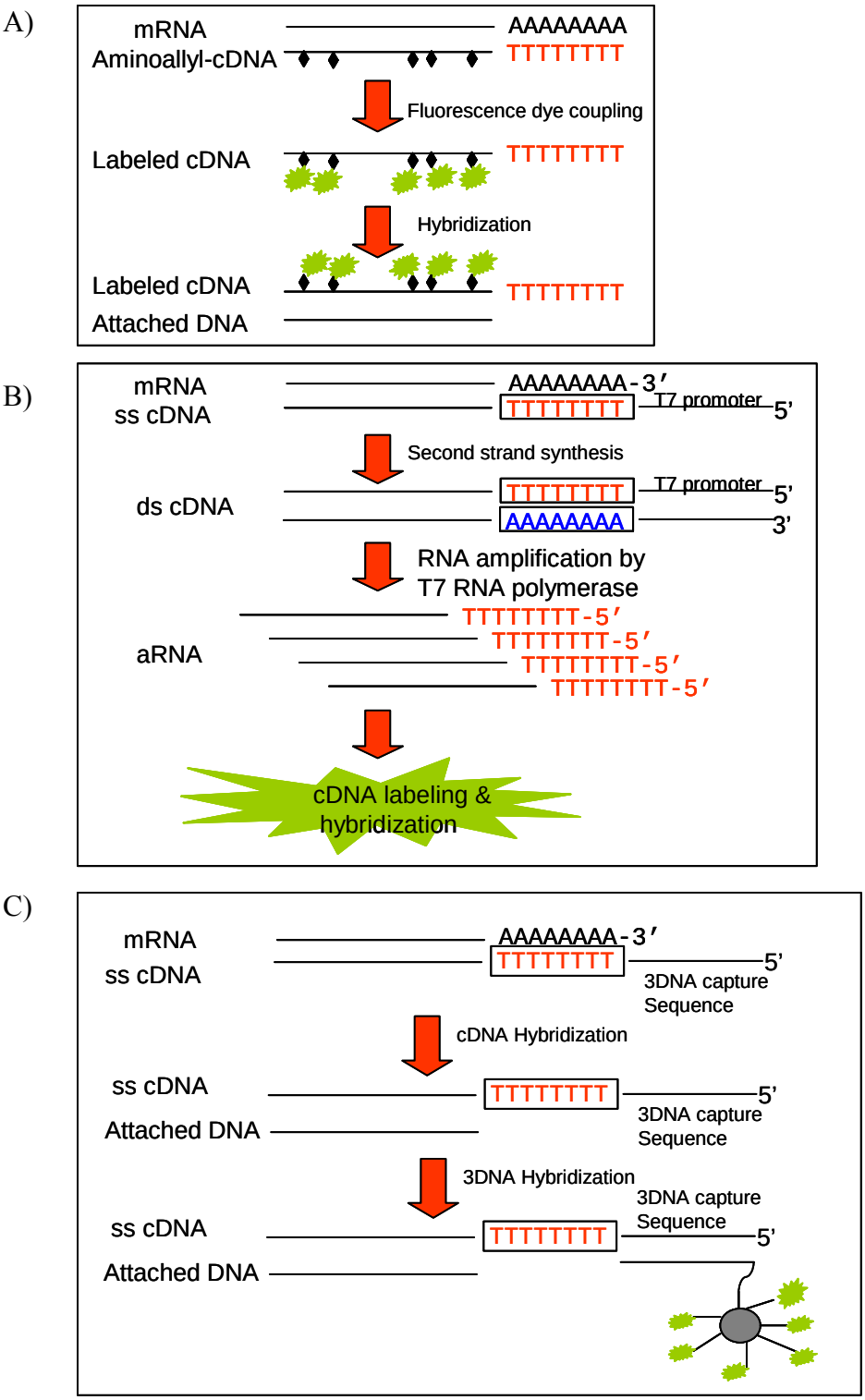


Figure 11. Different labeling and hybridization strategies.
A) Indirect labeling with aminoallyl dUTP, B) RNA amplification following by cDNA labeling, C) 3DNA multiple labeling. Attached DNA refers to the DNA probe on the array. A) and B) are modified from Richter et al., 2002. C) is drawn according to 3DNA manual (http://www.genisphere.com/).

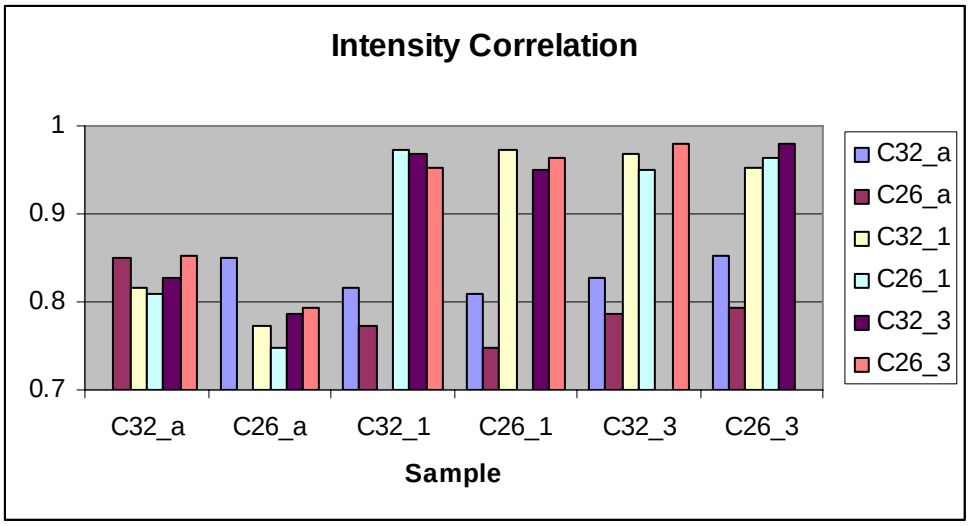


Figure 12. The intensity correlation of all the genes on the array between different samples.
C32_a and C26_a are the data for sample C32 and C26 from aRNA amplification method. C32_1 and C26_1 are the data for sample C32 and C26 from 3DNA labeling system with 1µg of total RNA. C32_3 and C26_3 are the data for sample C32 and C26 from 3DNA labeling system with 3µg of total RNA. The array used in this study is the bovine 1.6K array.

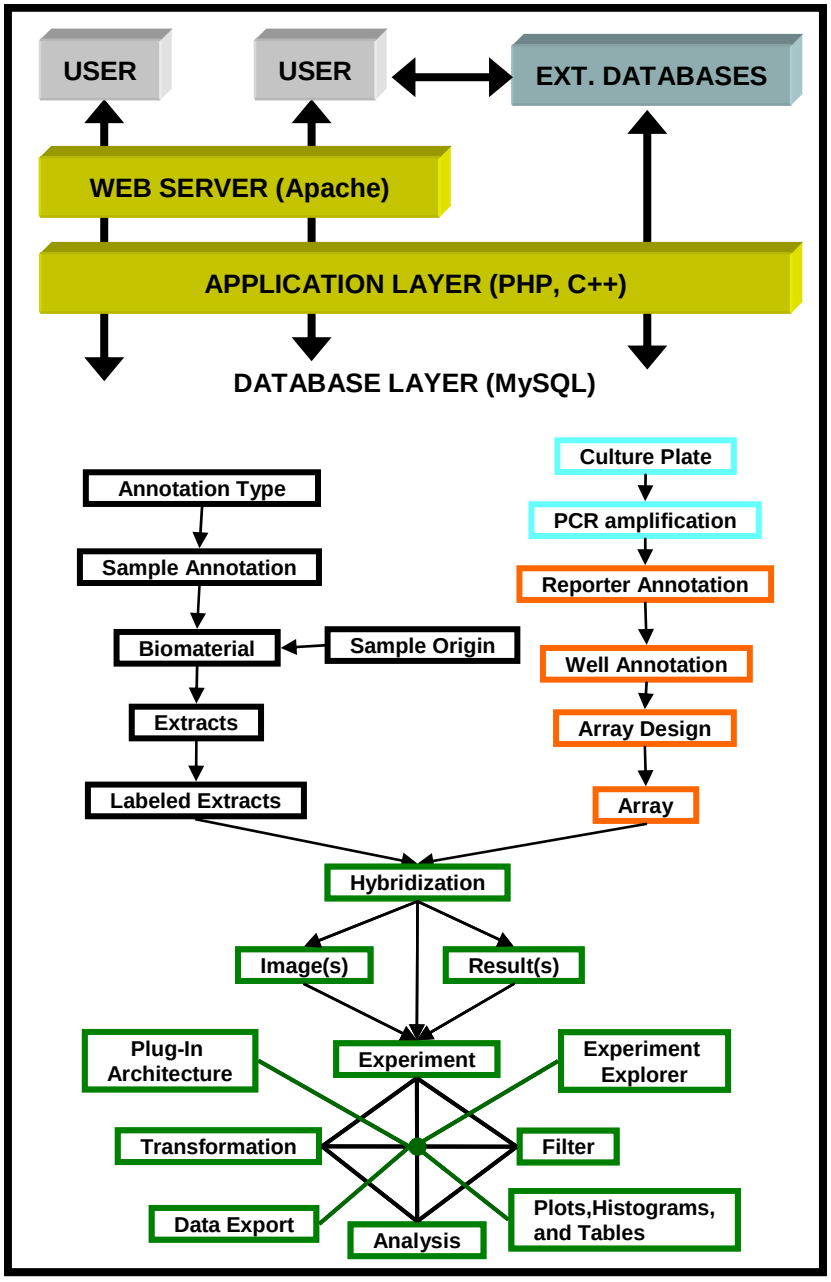
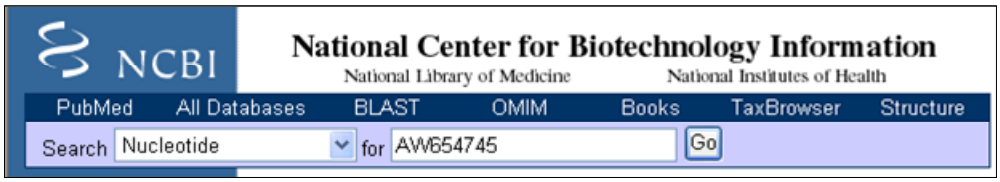
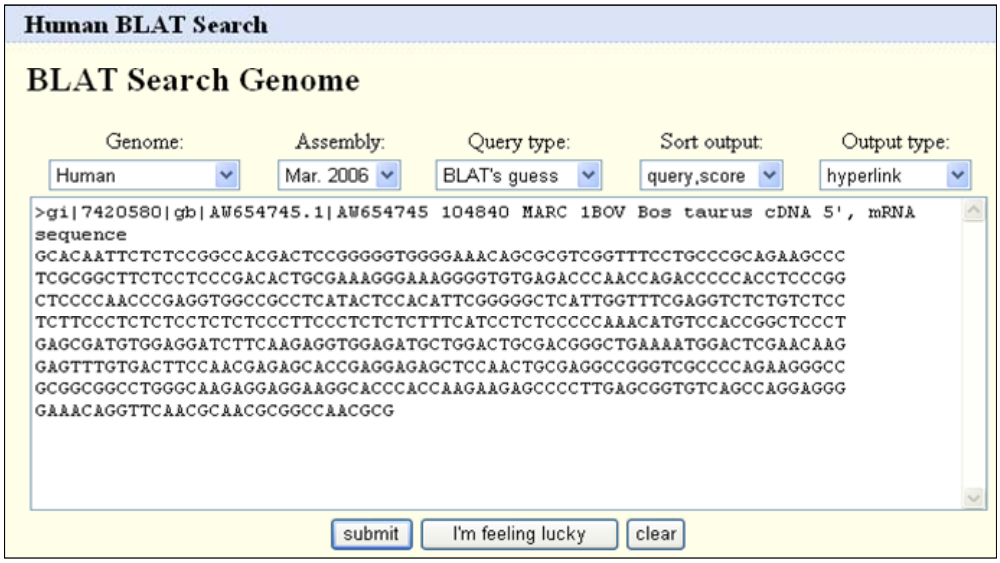


Figure 13. Simplified schematic overview of BASE database structure.
Users interact with BASE server through web browser. Sample information is in black boxes. Array information is in orange box. PCR information is in light blue box and is optional. Hybridization and data analysis are in green boxes. The picture is modified from an image obtained from BASE web site (http://base1.thep.lu.se/).

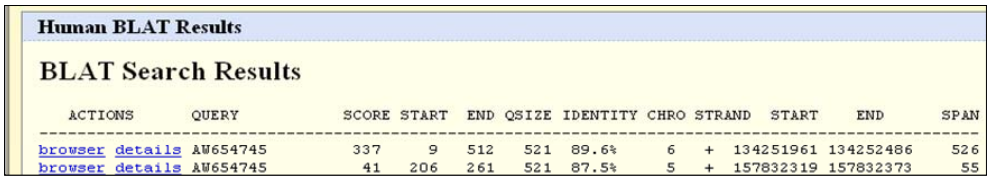
1. Get the sequence from NCBI



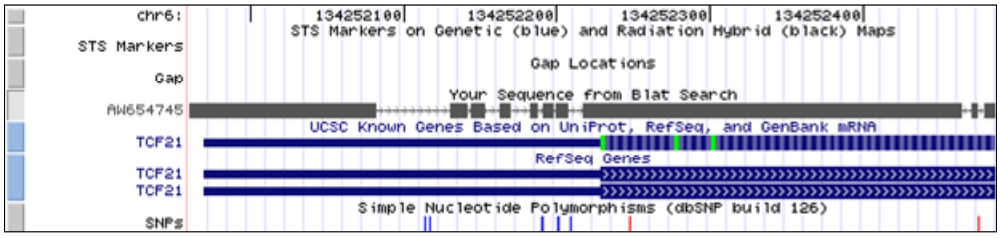
2. Copy the sequence and paste it into the UCSC BLAT window, against human genome sequence.



3. In the result page, check the "score" and "span" to determine if the match is good.



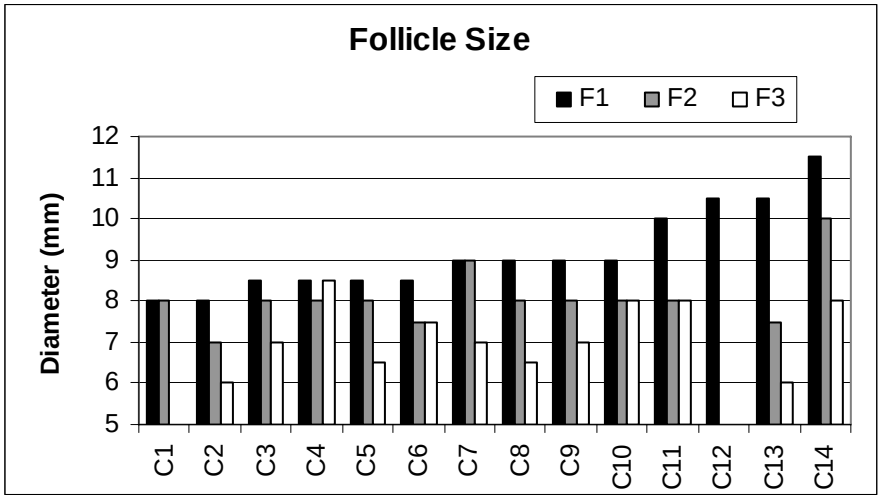
4. If a good match is found, click on "browser" to check the details



5. Extract gene information

Figure 14. Example illustrating how to run a BLAT search on the UCSC webserver. Screenshots are taken from NCBI (http://www.ncbi.nlm.nih.gov/) and UCSC (http://genome.ucsc.edu/).

15A).



15B).

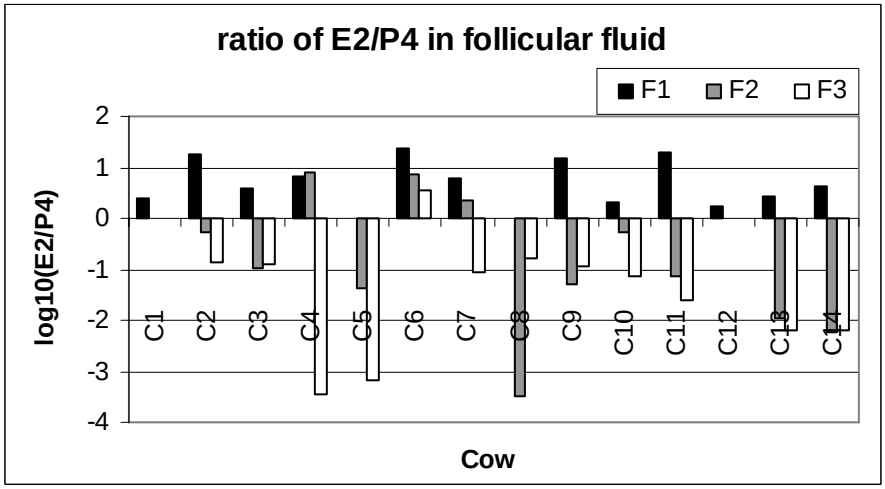


Figure 15. The diameter size and E2/P4 ratio of the 1st, 2nd and 3rd (F1, F2 and F3, if available) largest follicles of the 14 cows used for the microarray experiment and qRT-PCR.

A) follicle diameter, with follicles from each cow plotted side by side. B) corresponding ratio of E2/P4 of each follicle. The ratios are expressed in log10 scale. The data for F1 in cow 5 and 8 are missing as the follicles were broken before we could collect the follicular fluid.

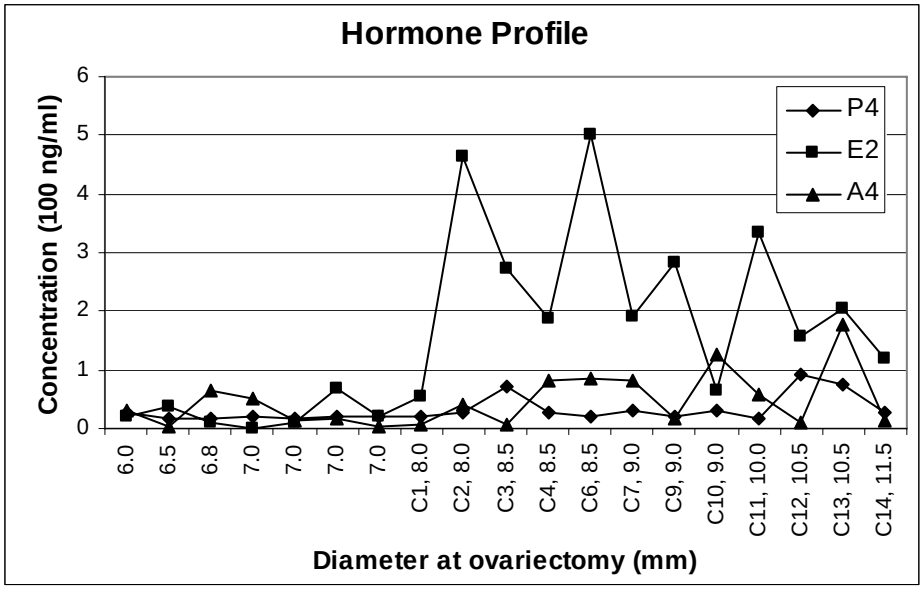
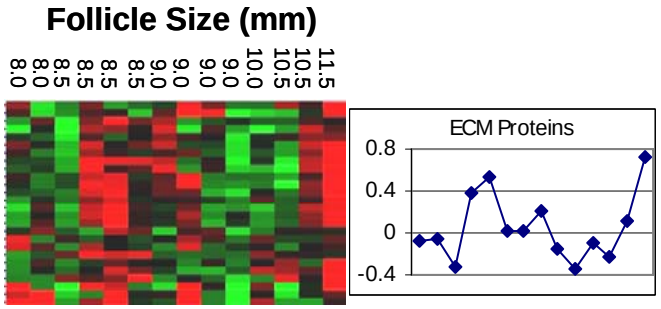


Figure 16. The intrafollicular concentration of Estradiol (E2), Progesterone (P4) and Androgen (A4) of the largest healthy follicles from each cow.
Data for two follicles (8.5mm and 9mm in diameter) is missing as their follicular fluids are unavailable).

17A)



17B)

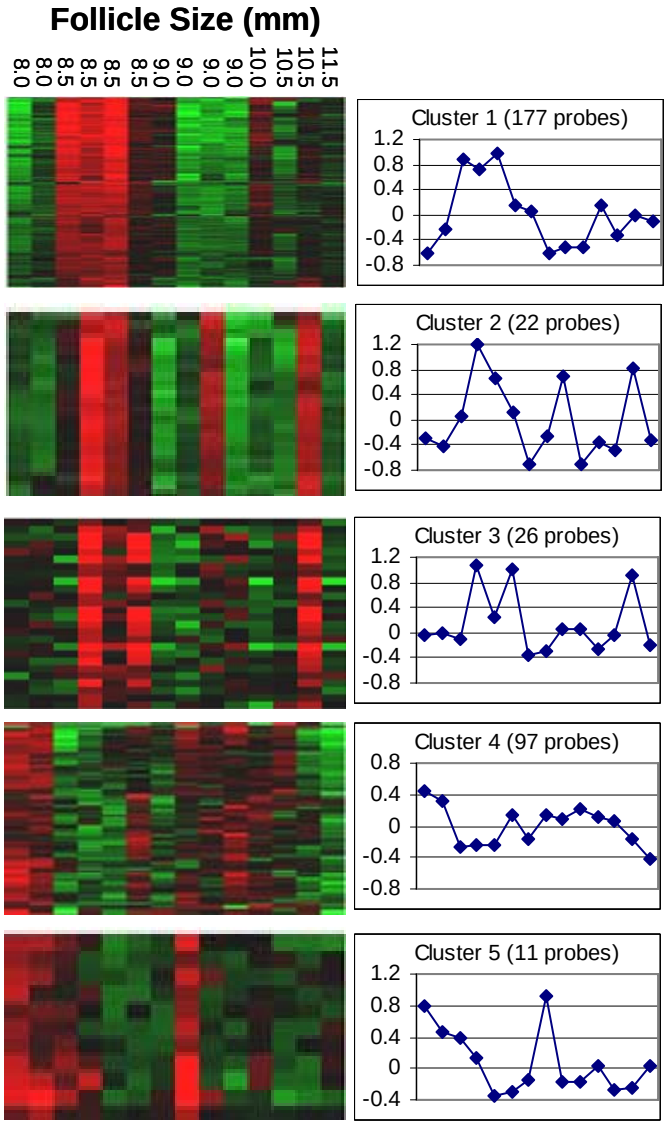


Figure 17. - Expression profiles of differentially expressed genes ifentified by the sample model

A) Genes whose products are located in the extracellular matrix compartment. In the cluster image (left side), each column represents one follicle sample with the indicated size and each row represents one probe. The expression values are log2 transformed and mean-centered-to-zero for each probe. Red color indicates a relative higher expression level and green color indicates a relative lower expression level. The graph on the right shows the median expression values of all probes in the cluster for each follicle sample. **B**) Five clusters of genes with similar expression profiles were identified using hierarchical clustering and self-organizing maps algorithms.

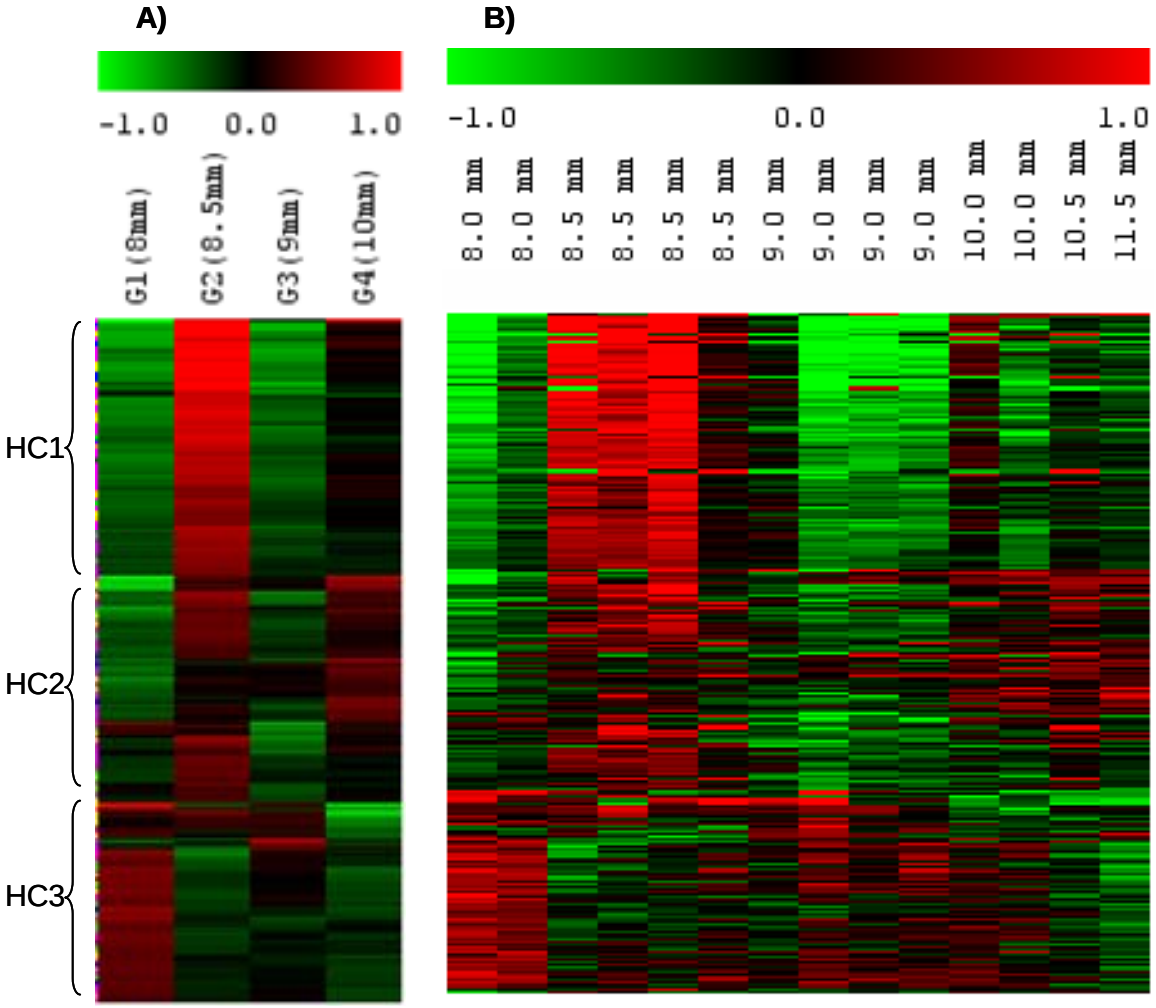


Figure 18. The expression patterns of 271 probes identified by the Group model.

A) The expression pattern of the group effect for the 271 probes.

B) The expression pattern of the sample effect for the 271 probes from the Sample model. The probes in the two figures are shown in the same order.

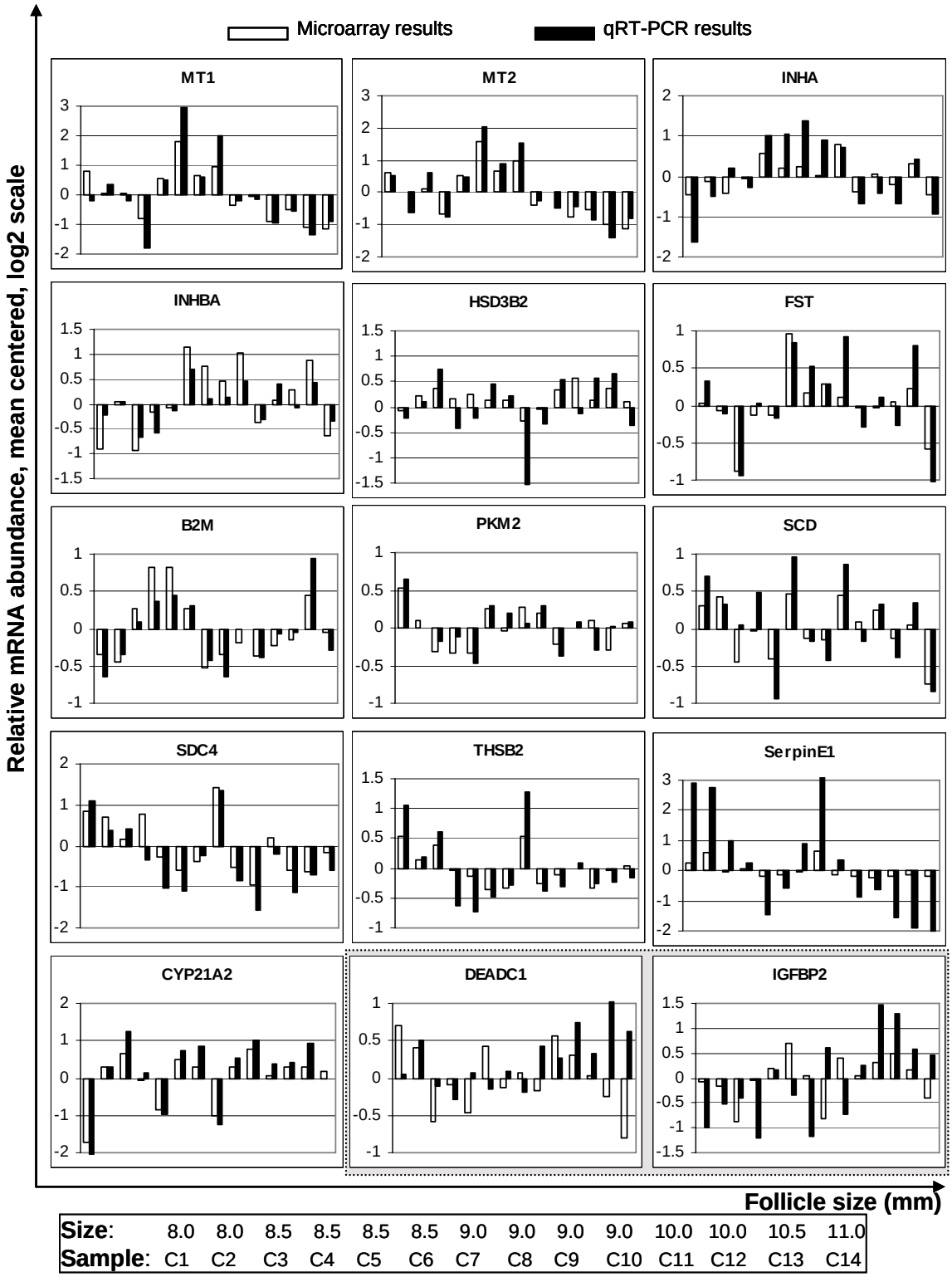


Figure 19. Comparison of microarray and qRT-PCR results.
The values were mean centered for array and qRT-PCR respectively, showing relative abundances. All the values are in log2 scale. The first 13 genes are generally consistent in the direction of expression changes between array and qRT-PCR results while the last two genes (DEADC1 and IGFBP2) are not.

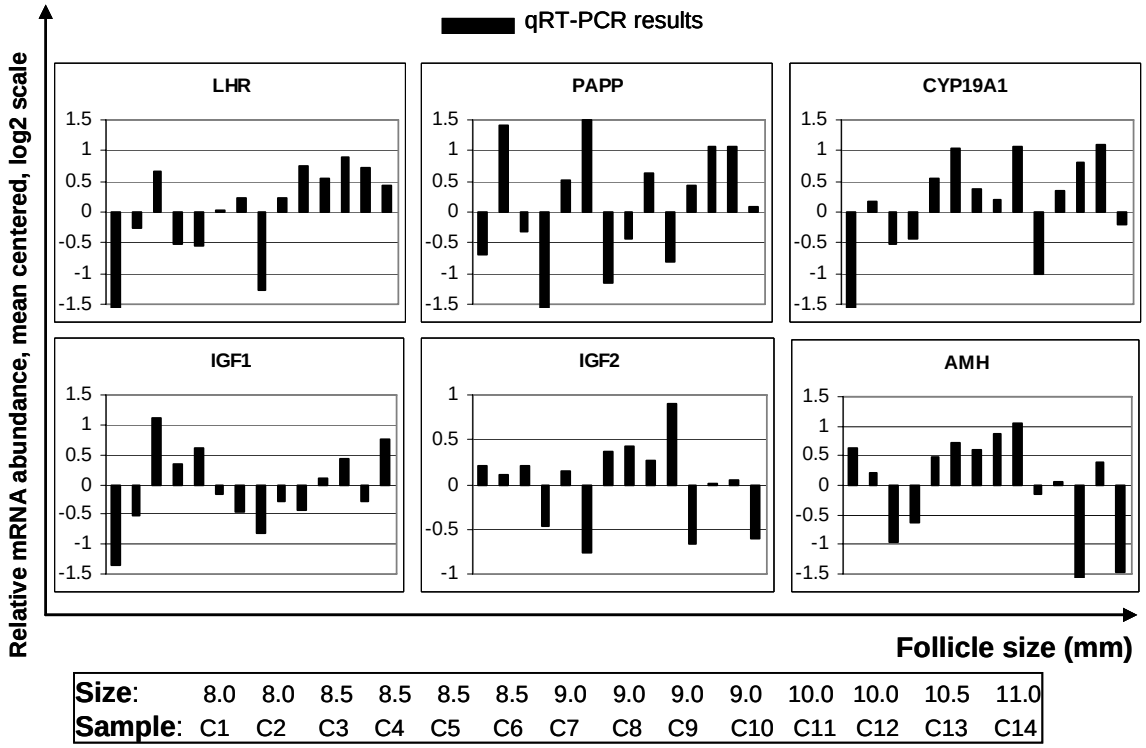


Figure 20. qRT-PCR results of six genes without measurements from the microarray experiment.
The values are mean centered and in log2 scale.

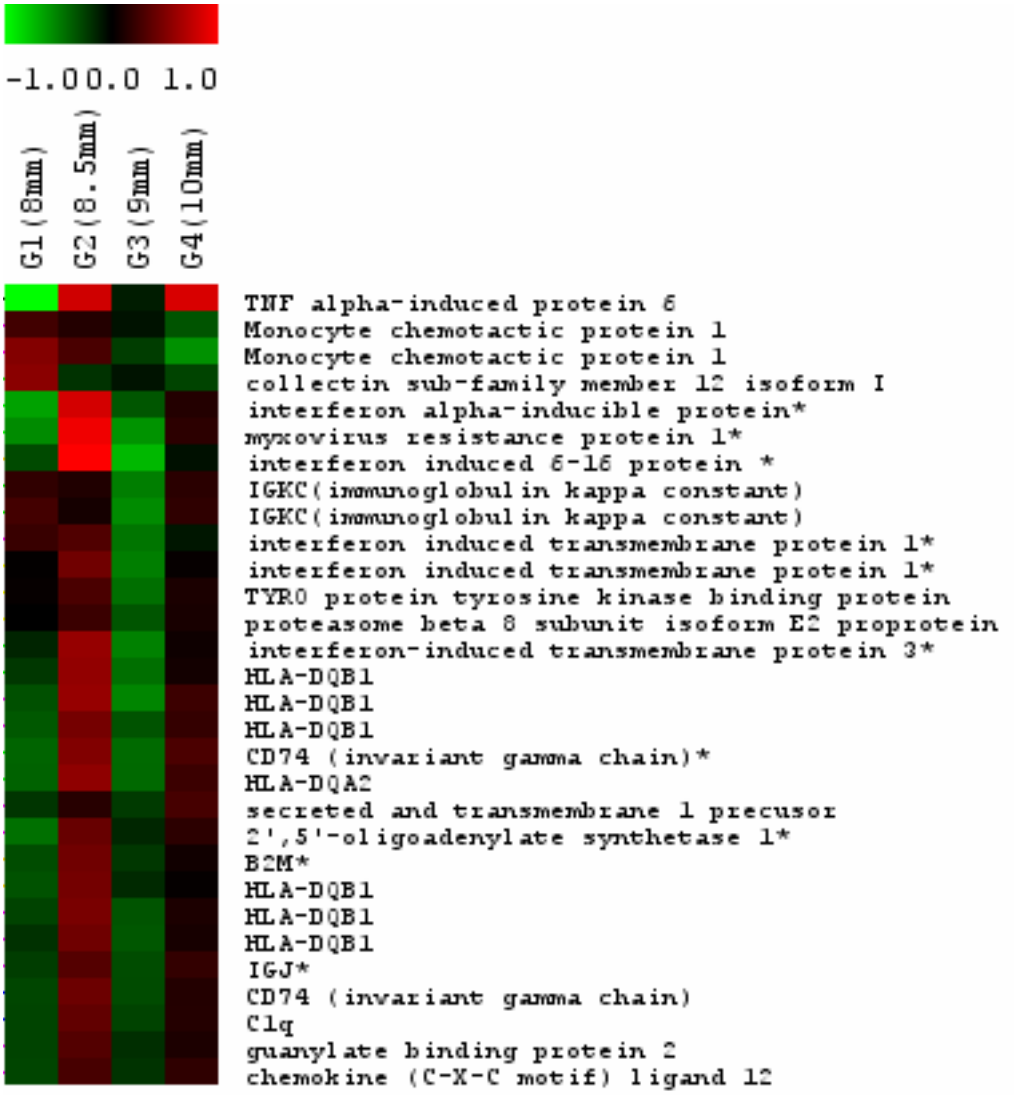


Figure 21. The expression profile of the immune system genes identified by the Group model.

Probes labeled with * were also present in the Cluster 1 and 3 of the Sample model. Some genes are represented by multiple probes.

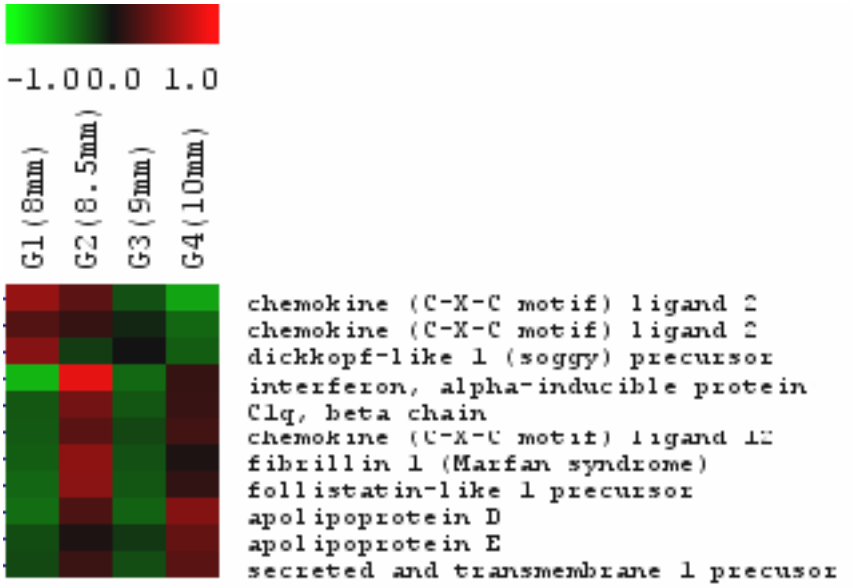


Figure 22. Extracellular space genes identified by the Group model.

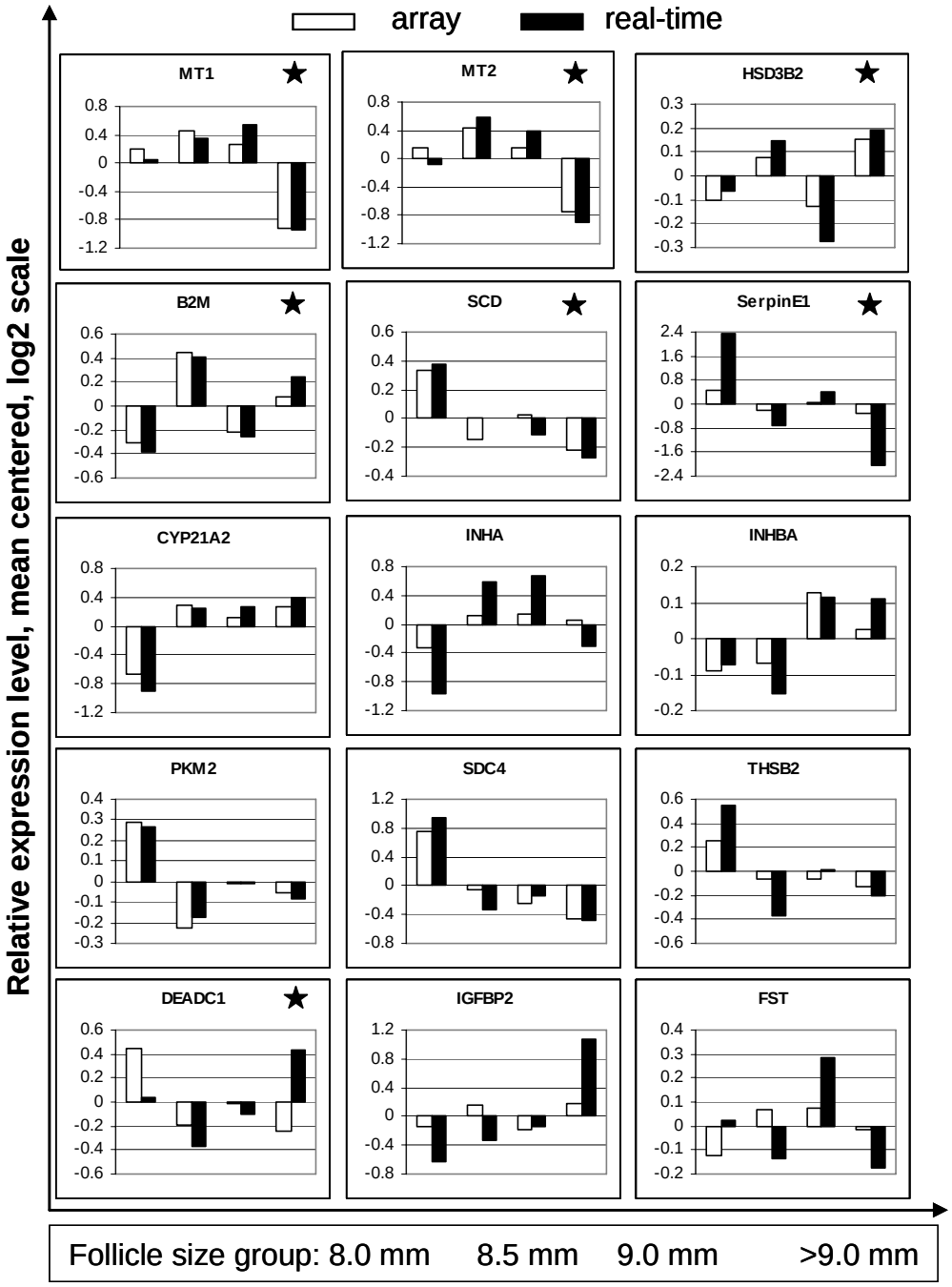


Figure 23. Comparison of microarray and real-time results while follicles are grouped on size.

The microarray data are from the Group model. The real-time data are the means of each group. All the data are in log2 scale and mean-centered to 0. The black star indicates the genes are siginificantly differentially expressed in the Group model.

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APPENDIX

BASE 1.2.17 installation and maintenance

A. Install of BASE1.2.17

This document contains the instruction of install a new BASE server (1.2.17) on Red Hat Enterprise WS 4.0 and move data from an old version (1.2.15) to the new version. All passwords are removed for security reason.

Check all the required software

- gnuplot pass
- ghostscript (pstopnm, pstopdf) pass
- netpbm (libnetpbm, pnmscale, pnmcut, etc.) pass
- libpng (libpng, pnmtopng, pngtopnm) pass
- libtiff (tiffsplit), libjpeg, libz pass
- GSL pass
- libgd pass
- bash, binutils, awk, sed, GNU make pass

Apache installation pass

Identify "httpd.conf" location: /etc/httpd/conf/httpd.conf

Turn on httpd in “service” and save.

MySQL installation pass

Identify “my.cnf” location: /etc/my.cnf

Turn on mysql in “service” and save.

PHP installation pass

Identify version: 4.3.9

Identify “php.ini” location: /etc/php.ini

(by use command “shell>php –i >phpinfo.html” then check “phpinfo.html”)

Check PHP is compiled with all the required software pass

(at least --with-gd --with-png --with-jpeg --with-zlib)

Create users

Create user “base”, belong to group “base”

User:**base**

Pass: **password1**

Create user “basejob”, belong to group “base”

User: **basejob**

Pass: **password1**

Set up the machine hostname

1. in "**network**", choose "**device-eth0**", then choose "**automatically obtain IP address settings with DHCP**" and input hostname "amadea.asrc.agri.missouri.edu"
2. choose "**automatically obtain DNS information from provider**"
3. Now de-choose "**automatically obtain IP address**" by choose "**Statically set IP address**"

   Address: 128.206.129.206

   Subnet Mask: 255.255.255.0

   Default Gateway Address: 128.206.129.254

Then, set up DNS:

Hostname: amadea.asrc.agri.missouri.edu

Primary DNS: 128.206.10.3

Secondary DNS: 128.206.10.2

Domain name: dhcp.missouri.edu

Set up MySQL

1. set up grant table

   This is only for a new machine because it only requires to be set up once.

   Make sure MySQL is running, then type the following command (use "slocate" to find its position):

   Shell>./mysql_install_db

2. create "root" account for mysql and set password

shell> ./mysqladmin –u root –h localhost –p ‘password2’

shell> ./mysqladmin –u root –h amadea.asrc.agri.missouri.edu –p ‘password2’

3. edit “my.cnf”

   add line: “skip-networking

   query_cache_limit = 30k

   query_cache_size = 30M

   query_cache_type = 1”

4. Now logout with “root”, login with “base”

   Shell> mysql –h localhost –u root –p

   Enter password

   Mysql> create database base;

   Mysql> create database basedynamic;

   Mysql> GRANT ALL on base.* to base@localhost IDENTIFIED by ‘pasword1’

   Mysql> GRANT ALL on basedynamic.* to base@localhost;

Change httpd

1. open httpd.conf
2. change the apache running user to “**base**”, group “**base**”
3. Add line “AcceptPathInfo on” somewhere (anywhere, as long as not in some block)

4. change "DocumentRoot" to "/usr/local/base/www" (2 places, one is the definition, the other is the following directory description)

change php.conf

This is still the change of httpd.conf. But in Apache 2, they put some configurations in other files under "conf.d" instead of a big httpd.conf.

1. DirectoryIndex index.html index.phtml
2. AddType application/x-httpd-php .php .php3 .phtml

Change PHP.ini

1. check (default, noting to change)

   off (register_globals, magic_quotes_runtime, magic_quotes_gpc, and display_errors)

   on (short_open_tag, log_errors, file_upload)
2. post_max_size 150M
3. upload_max_filesize 150M
4. memory_limit 512M

BASE

1. unzip BASE

   shell> tar zxpf base-1.2.17.tar.gz

   enter the base/sql directory and run "base_mysql.sql" by

   shell> mysql –u base –p base <base_mysql.sql

to create tables in database “base”.

2. Install BASE

   Shell> ./configure --prefix=/usr/local/base --with-jobrunner-user=basejob --with-jobrunner-group=base

   Shell> make

   Shell> su

   enter password

   Shell> make install

3. change the configuration file “config.inc.php” in “/usr/local/base”

   enter password, contact information and server name, etc.

4. now copy the backup data from the old version (1.2.15). It contains 3 folders, “data”, “base” and “basedynamic”.

5. Replace the “data” folder in “/usr/local/base/” with the “data” folder from backup.

6. Replace the “base” and “basedynamic” folder in “/var/lib/mysql/” with the “base” and “basedynamic” folder from backup.

7. change the ownership of “base” and “basedynamic” under “/var/lib/mysql” to mysql by:

   shell> cd /var/lib/mysql

   shell> chown –R mysql base

   shell> chgrp –R mysql base

   shell>chown –R mysql basedynamic

   shell>chgrp –R mysql basedynamic

   Also need to change the mod of the files to “660”.

Shell> cd base

Shell>chmod 660 *

Shell> cd ../basedynamic

Shell>chmod 660 *

8. The "data" directory under "/usr/local/base" should have ownership and group of "base", if not, change as exampled above.
9. By now, the data should be ready. Run the following command to migrate database:

   Shell> cd /usr/local/base/bin

   Shell> ./dbmigrate_1.2_to_latest.php

   Shell> ./refreshQuotaUsage.php

   Shell> ./checkDatabase.php

   Everything should be OK.
10. start base

    shell> ./startBase.php
11. Now put "base.server" to "etc/init.d" so it can automatically start when computer is on.

    Shell> cp base.server /etc/init.d/

    Shell> chkconfig

Open a browser and BASE should run. Cheers!

B. Base Data Backup:

1. Login as “root”
2. Enter the Base bin directory

   [~, root]cd /usr/local/base/bin

   [~, bin]./backup.sh

   The computer will take ~20 minutes to create the backup folder, which is located under **“/usr/local/base/backup”**. The folder name is something like “yyyy-mm-dd”, the date you run the backup. It will back up all the data as well as the tables in MySQL database (“base” and “basedynamic”).

   During Backup, Base will be temperately shutdown. It will automatically start after backup is finished.

3. Connect the USB external harddrive to the computer. Check out the drive name through “Application→System tools→Hardware browser”. Normally it is sdb1. Notice that sda1 is for the internal harddrive.

   Mount the USB harddrive to /mnt/usb as following:

   [~, ~]mount /dev/sdb1 /mnt/usb

   Now you should be able to read and write the external hard drive through “/mnt/usb”.

4. Backup the folder to a new USB harddrive and all the work is done.

   This can be done by following steps:

[~, root]cd /usr/local/base/backup

[~, backup]ls

You should see a folder named “2006-~-~” (the date you run the backup). I will use 2006-09-10 as an example.

[~, backup]nohup cp –R 2006-09-10 /mnt/usb &

After type in the above command, you can log out. It takes several hours to transfer the file to the external hard drive.

BASE data recovery is similar to the data migration described in the installation of BASE. Just copy the 3 folders (data, base, basedynamic) back and make necessary modifications.

VITA

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