# Three-Dimensional Image Analysis to Quantify the Temporo-Spacial Expression of Cellular Receptors

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***Abstract*—Ovarian folliculogenesis is primarily controlled by the action of gonadotropins namely follicle stimulating hormone (FSH) and luteinizing hormone (LH). Several reports indicated that the process of initial recruitment of primordial follicles to the growing follicles is not gonadotropin-dependent but Bone morphogenetic protein (BMP)-dependent. However, this has not been unequivocally confirmed. The aim of this study was to investigate the temporo-spacial protein expression of the BMP receptors 1B (BMPR1b), FSHR and LHR in several stages of follicle development. While the localization of all receptors was found in granulosa cell membrane of the follicles the temporal expression was varied. BMPR1b was expressed in all follicle stages, FSHR was detected in primary follicles onward and LHR was absent in both primordial and primary follicles but appeared in later stages. Quantitative analysis based on the intensity of fluorescent signals showed that the expression of BMPR1b, FSHR and LHR significantly ($p< 0.001$ $p< 0.0001$ $p< 0.0001$ respectively) increased with follicular development. We have concluded that the combination of sensitive immunofluorescence labeling and computerized 3D image analysis proves efficient tools for in situ detection and quantification of the expression of small amount of protein in a complex tissue structure.**

***Index Terms*—folliculogenesis, Bone morphogenetic protein, follicle stimulating hormone (FSH), luteinizing hormone (LH), 3D image analysis**

## I. Introduction

Ovarian folliculogenesis is the basis of the entire ovarian function including the production of mature oocytes ready for fertilization and the sexual hormones required for the development of female phenotypes. This process entails a series of chronological steps in which a growing follicle either matures to ovulation or dies by apoptosis [1]. The primordial follicle considered as the constructional units of female ovary. The initial transition of primordial follicles to growing primary follicles is the key limiting step in preserving or depletion of the female fertility reserve. It is well established that folliculogenesis is regulated by the gonadotropins FSH and LH, which are secreted by the pituitary gland [2]. In addition, complex autocrine and paracrine actions of several intra-ovarian factors such as the BMPs influence the action of gonadotropins [3]. For instance, it is now believed that the initial stage of follicle recruitment and development of primordial to primary follicles is independent of the gonadotropins [4] but rather associated with a specific spatial and temporal pattern of BMP expression [5].

However, such interplay between the gonadotropins and BMPs particularly in the initial recruitment of primordial

Follicles remain inconclusive. Because of the complex nature of the ovarian structure and the dynamics of folliculogenesis, most of the studies in relation to this issue have been conducted in vitro using molecular and biochemical approaches [6] or in situ microscopic analysis using manual subjective methods [7].

The use of new non-bias computerized technology in biomedical research has increased in the past decades. The computer-based method was introduced to provide accurate and reliable quantitative information as well as a non-time consuming tool [8]. This technology will eventually replace the conventional manual and subjective methods. In attempt to clarify the regulation of initial folliculogenesis; the present study demonstrates the interaction between BMP and gonadotropin signaling systems, using immunofluorescence detection and 3D image analysis of cell-membrane bound receptors of BMPs, FSH and LH in developing follicles of sheep ovaries.

## II. Materials and Methods

### A. Sample Collection and Histology

Sheep ovaries were collected from local abattoir and divided into 2 groups. One group was snap-frozen in liquid nitrogen and stored in -80 ℃ for further usage. The second group was fixed in 10% Neutral Buffered Formalin (NPF) and processed using Tissue-Tek VIP automatic tissue processor and embedded into paraffin wax. Tissue sections of 5 μm were cut, placed onto super frost slides (HD scientific supplies Pty Ltd, Australia) and stained with Hematoxylin and Eosin for morphological study to identify the different stages of follicular development.

### B. Immunoflourescent Labeling of BMPR1b, FSHR and LHR

Frozen tissues were partially embedded in OCT and 10 μm sections were prepared using a Cryostat (Carl Zeiss,

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Sydney, NSW, Australia) and fixed in 4% paraformaldehyde at 4℃ for 7 min. Indirect immunofluorescence labeling was performed as previously reported [9]. In brief, 4μg/ml of the primary antibodies was applied overnight at 4℃ in humidified chamber. The antibodies are (monoclonal) goat anti-BMPR1b (sc-5679), goat anti-FSHR (sc-7798) and goat anti-LHR (sc-26341), all from Santa Cruz Biotechnology, Santa Cruz, CA, USA. After a serial of washing a donkey Anti-goat second antibody conjugated with Alexa 488 (Molecular Probes, Australia) was added for 45 min in dark humidified chamber. Negative controls were performed by omitting the second antibodies for the presence of auto-fluorescent signals and non-specific binding. The sections were mounted using anti-Fade aqueous mounting medium containing 40, 60-diamidino-2-phenylindole (DAPI; Molecular Probes, NSW, Australia).

The sections were examined by Carl Zeiss semi-confocal fluorescent microscope equipped with CarlZeiss Digital Camera (200M Axiovert; Carl Zeiss, Sydney, NSW, Australia) and the images were captured using AsioVision 4.2.8 image analyzer software.

### C. 3D Image Analysis and Immuonoflourescent Intensity Quantification

For image acquiring, the exposure time was adjusted using control sections incubated with pre-immune serum in order to subtract auto-fluorescent and non-specific binding background. Surplus fluorescent signals, appeared after such subtraction, were considered specific binding, which were subjected for quantification study. Z-stalks of 10x1μm optical frames/sections, generated from the 10μm thick physical sections, were captured and compiled to generate 3D images. The 3D images were used to quantify the intensity of immuonoflourescent signals in the entire 10μm sections (Fig. 1).

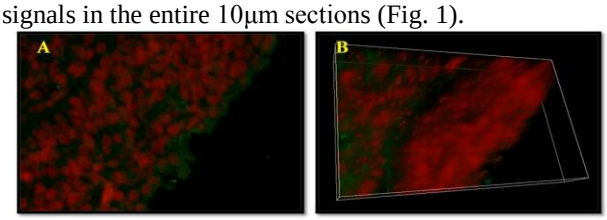


Figure 1. Granulosa cells from pre-ovulary follicles A. 2D image to determine the Localization and receptor expression B. 3D image for immuonoflourescent quantification. X40

### D. Statistical Analysis

Statistical analysis was performed using prism version 6 (Graph Pad Software, La Jolla, CA, USA). The results were analyzed using the mean and standard deviation of BMPR-1b, FSHR and LHR expression in four stages of follicular development and expressed as mean pixel/$\mu m^2$ ± SEM.

## III. Results

### A. Morphological Assessment

Four stages of follicular development have been identified and subjected to this study (Fig. 2A-D). Primordial follicles (Fig. 2A) consist of a primary oocyte surrounded by few spindle-shaped cells, granulosa cells (GCs). The primary follicle (Fig. 2B) initially consists of a primary oocyte surrounded by a complete layer of cuboidal GCs. The zona pellucid (ZP), a thick layer composed of glycoprotein and acid proteoglycans, forms between the oocyte and GCs. Once GCs proliferate and arrange into multiple layers the secondary follicle is formed (Fig. 2C). The Graafian follicle is characterized by a large, fluid-filled antrum and an eccentric oocyte (Fig. 2D).

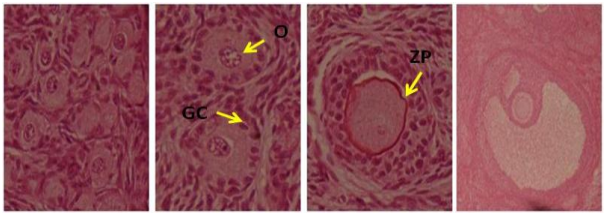


Figure 2. Four stages of follicle development A) Primordial follicle B) Primary follicle C) Secondary follicle D) pre-ovulatory follicle.

### B. Immuonoflourescent Localization of BMP1b, FSHR and LHR

Immunofluorescence microscopy revealed that the intensity of a positive immunolabelling of the membrane-bound receptors varies based on the stage of follicular development with the least signals captured in primordial follicles. BMPR1B was expressed in follicular cells of all stages (Fig. 3A-D). Immunolabelling of FSHR showed no staining in follicle cells of primordial follicles but expressed in primary follicles onward (Fig. 3E-H). LHR expression was absent in both primordial (Fig. 3 I) and primary follicles, which then expressed in later stages of follicular development (Fig. 3 I-L).

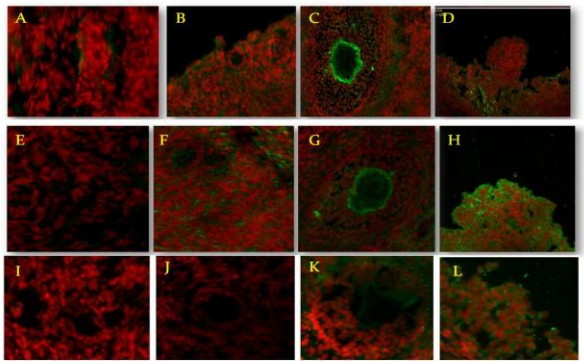


Figure 3. The expression of BMPR1b (A-D), FSHR (E-H) and LHR (I-L). A, E, I, primordial follicles; B, F, J, primary follicles; C, G, K, secondary follicles; D, H, L, Graafian follicles. Green color staining indicates a positive labeling of the receptors. E, I and J show negative staining of FSHR (E) and LHR (I, J). X40

### C. 3D Image Quantification of BMP1B, FSHR, and LHR

Expression during different follicular stages Quantitative analysis based on the intensity of fluorescent signals expressed in 3D images showed that the expression of BMPR1b, FSHR and LHR significantly ($p < 0.0001$) increases with follicular development (Fig. 4A-C). The intensity of labeling was higher for BMPR1b and lower for LHR. BMPR-1b expression was detected in the GCs in all follicular stages of follicular development (Fig.

4A). The FSHR expression was absent in primordial follicles but expressed in the rest stages of follicular development (Fig. 4B). LHR expression was absence in both primordial and primary follicular and expressed in the rest stages of follicular development (Fig. 4C).

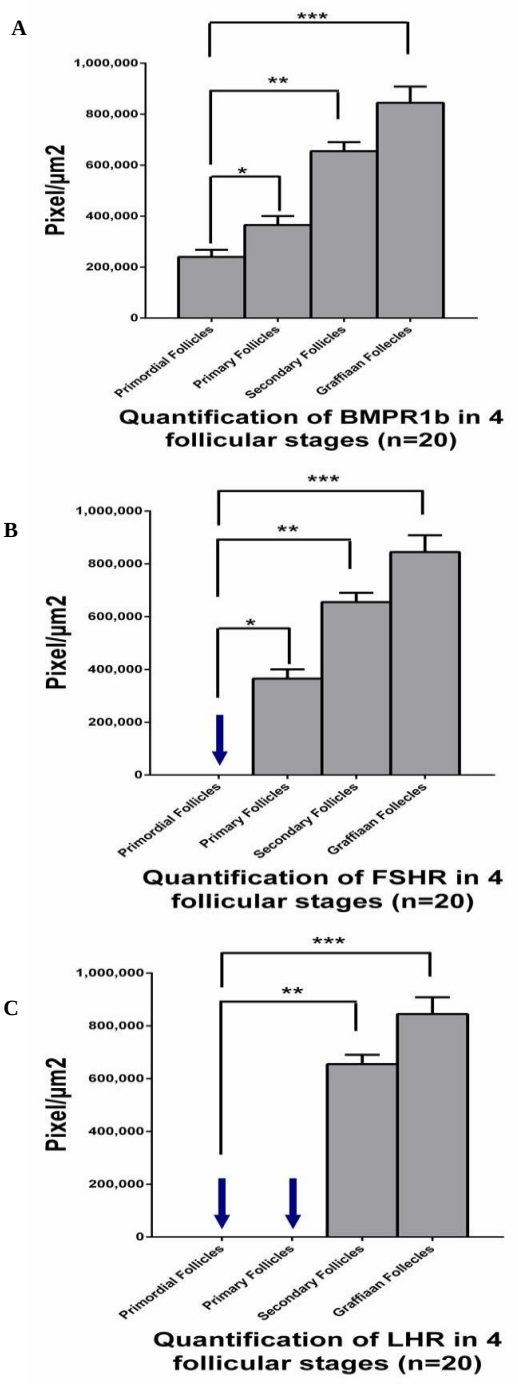


Figure 4. Showed the quantification of receptor expression (pixel/µm$^2$) in 4 stages of ovarian development. A) The BMPR1b expression B)FSHR expression C) LHR expression, number of follicles n=20.**: p<0**.001,** ***: p<0.0001

## IV. Discussion

This study demonstrates for the first time the temporo-spacial localization and quantification of BMPR1b, FSHR and LHR in sheep ovaries throughout the initial steps of folliculogenesis. The results indicate that gonadotropins are not involved in the recruitment of primordial follicles clarifying the ambiguity of the literatures [10], [11]. In addition, the in situ localization of these three receptors in the follicles across several stages of development unequivocally shows the interplay between these hormones and growth factors in the regulation of ovarian function. Traditionally, the detection and quantification of immuonoflourescent labeling is conducted by manual subjective methods presented in form of scores range from 0 (no staining) to 4 (intensive staining). This kind of studies may be subjected to bias and therefore non-reliable particularly for the purpose of statistical significance [8]. In addition, subjective approach is not efficient to detect and estimate a small amount of signals, such as within the cell membrane, within a complex structure. Furthermore, semi-quantitative method can only estimate signals emitted from the surface of a single section, which does not represent the actual amount of molecules in the 3D tissue structure. The present study provides alternative approach using computer-based quantitative analysis of 3D objects.

Ovarian folliculogenesis operates mainly under the control of FSH and LH; their levels are regulated by the hypothalamic-pituitary-gonadal axis [2]. However, it has been reported that normal follicle development occurs in mice with mutation in FSH β subunit and FSHR [12], suggesting that the regulation of initial follicle recruitment is gonadotropin-independent [13]. Instead, it is now believed that the initial recruitment of primordial follicles is a gonadotropin-independent process but controlled by intraovarian factors [14] such as the BMPs [15], yet other conflicting reports indicated otherwise [10], [16]

In this study, the absence of FSHR and LHR in primordial follicles unequivocally indicates that the early stage of follicular development is gonadotropin independent. However, the precautious expression of FSHR in primary follicles suggested that FSH may be involved indirectly by up-regulating the expression of BMPR1b [6].

## V. Conuclusion

The current study highlights the necessity and feasibility of using non-bias computerized method to detect and quantify the presence and distribution of a small quantity of molecules in a 3D complex structure such as the ovarian follicles. The outcome of the study is important in expanding our understanding of the role of BMPs in the regulation of ovarian functions.

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