Expression of Growth Differentiation Factor-9 In the Developing Ovary and In Nonovarian Tissues

Jessica L. Bowser, Sowmya Srikanthan, William J. Hendry and Jeffrey V. May

Department of Biological Sciences, Wichita State University, Wichita, Kansas 67260, U.S.A

INTRODUCTION

During mammalian ovarian organogenesis, the massive loss of oogonia via apoptosis is attenuated by the association of germ cells with somatic mesoepithelial cells of the gonadal ridge to form primordial follicles. It is suspected that the germ cell actively participates in this process via the production of local regulatory factors. Growth differentiation factor-9 (GDF-9), a member of the TGF-ß superfamily, has been shown to be essential for normal follicular development beyond the primary follicle stage in rodents and humans and a key factor for primordial follicle formation in sheep, bovine, and hamsters [1-4]. Several TGF-β family members have been shown to have highly specific and restrictive tissue distributions; most likely reflecting their roles as developmental regulators. GDF-9 has long been reported as an oocyte-specific factor since it was first identified by the use of degenerate primers in 1992 [5]. Early analysis of GDF-9 mRNA demonstrated the exclusive nature of its expression in the mammalian ovary, specifically in the oocyte. Furthermore, GDF-9 has been shown to mimic oocytes *by* stimulating cumulus expansion and development *in vitro* [6]. GDF-9 is essential for the normal progression and development of follicle development in the mammalian ovary. Few studies have addressed the possibility of GDF-9 expression in non-ovarian tissues [7]. Our lab discovered post-reproductive (>60 weeks of age) transcriptional expression of GDF-9 in the hamster ovary. The appearance of this oocyte-specific factor at a period following follicle exhaustion within the hamster ovary, lead us to examine non-ovarian tissues for expression of GDF-9.

METHODS AND MATERIALS

RNA Isolation and RT/PCR

Syrian Golden Hamsters were purchased from Harlan Spague Dawley and maintained according to the guidelines of the Institutional Animal Care and Use Committee (IACUC). At varying ages (neonatal to adult), animals were randomly selected, anesthetized and terminated by CO_2 asphyxiation and cervical dislocation. The upper region of the reproductive tract containing the upper uterine horn, oviduct, ovary, and bursa was removed and the ovaries were rapidly isolated under a dissecting microscope. Tissues from other organs were then collected using segregated instruments, presoaked in 70% ethanol. Ovarian and non-ovarian samples designated for analysis by RT/PCR were snap frozen by on dry ice, and stored at -80C. Ovaries destined for protein analysis, via immunohistochemistry, were fixed in 4% paraformaldehyde and sent to Histo-Scientific Research Laboratories Inc. (Woodstock, VA) for embedding, sectioning and staining.

White Blood Cell Isolation

Golden hamsters of various ages were anesthetized with CO_2 and terminated by cervical dislocation. Using an 18 gauge/3cc syringe containing 20 µl of 0.5 M EDTA, serum was extracted from the right atrium of the heart. Whole blood samples were centrifuged and the buffy coat removed and stored at -80C for RNA isolation.

DNA sequencing

PCR products were extracted from agarose gels and purified using a QIAEX Gel extraction Kit. Products were forwarded to the University of Kansas Medical School Biotechnology Facility in Kansas City, KS., for sequencing.

Immunohistochemistry

Ovarian sections displaying several stages of follicle formation were subjected to immunohistochemical procedures (Methods in Molecular Biology, Volume 34). Anti-rat GDF-9 monoclonal antibody, courtesy of Dr. Aaron Hsueh (Stanford University) was used. The

GDF-9 antibody was detected using a peroxidase substrate kit. (NOVA RED, Vector Labs.

RESULTS

Hamster ovaries at 1 and 3 days of age are characterized by oogonial mitosis. The ovary is comprised of large numbers of oogonia contained in nests that have been thought to play a key role in the protection of the germ cells until they associate with somatic cells. The expression of GDF-9 mRNA is clearly evident at this early developmental time point (data not shown). At 5 days of age, oocytes begin to undergo the process of apoptosis. Such action results from the failure of the oocyte to associate with nearby somatic cells. By day 9, follicular development is well underway. Expression of GDF-9 mRNA is highly visible at this stage in primordial, primary and secondary follicles (Biology of Reproduction, Proceedings of the 38th Annual Meeting of the Society for the Study of Reproduction, July 24-27, 2005, Abstract 489).

Expression of GDF-9 mRNA in reproductively senescent ovaries (data not shown) caused our lab to explore the possibility of GDF-9 expression in non-ovarian tissues. All tissues isolated from 235 day-old hamsters showed a high level of GDF-9 expression (Fig. 1). Similar results were observed at all ages beginning with neonatal day 2 up to 2 years of age (data not shown). Due to the high degree of vascularity of the organs examined, we suspected that GDF-9 mRNA expression could be due to the presence of GDF-9 in white blood cells. RT/PCR of total RNA isolated from hamster whole blood white blood cell fractions clearly indicated the presence of GDF-9 mRNA (Fig.2). DNA sequencing of the PCR products revealed a 91% and 80% homology to mouse and human GDF-9, respectively. Immunohistochemical analysis of the ovary indicated a positive signal for GDF-9 protein in the highly vascularized corpus luteum in addition to a strong signal emanating from oocytes (Fig.3)

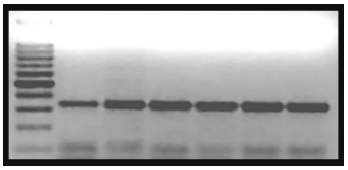

Figure 1: GDF-9 mRNA expression. Day 235 hamster tissues; ovary, liver, kidney, spleen, testis, and uterus.

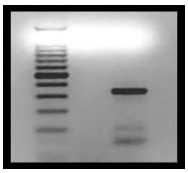

Figure 2: GDF-9 mRNA expression in hamster white blood cell fractions

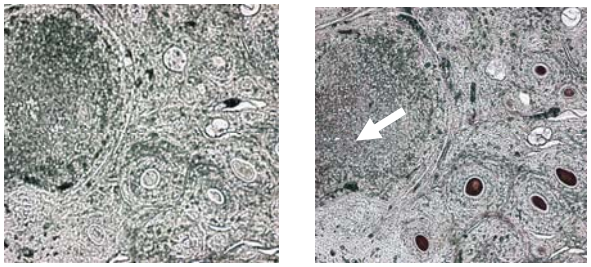

Figure 3: Immunohistochemical detection of GDF-9 expression in hamster ovaries. Left image depicts non-immune control. GDF-9 protein expression is highly evident in the oocytes of the follicles (right image). Expression can be seen in the interior portion of the corpus luteum as well (white arrow).

DISCUSSION

The expression of GDF-9 in the neonatal ovary has been reported to occur in rodents beginning with the development of primary follicles. GDF-9 gene knockout in mice results in the failure of follicles to develop beyond the primary stage, but has no effect on primordial follicle formation. GDF-9 mRNA is expressed at an even earlier stage, before primordial follicle formation, suggesting that GDF-9 is an important regulator during organization and development of follicles at all stages.

Our results strongly suggest that non-ovarian expression of GDF-9 mRNA in various tissues is likely due to the presence of white blood cells. In support of this premise, we have found that saline perfusion of the kidney results in a 33% decline in GDF-9 mRNA expression relative to non-perfused tissue. As of yet, the identity of the blood cells responsible for GDF-9 expression is unknown. The identity of these cells may predict the cellular role of GDF-9.

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