The Role of Bone Morphogenetic Protein-4 in Mammalian Reproduction

By

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Declaration

I certify that the substance of this thesis has not already been submitted for any degree and is not being currently submitted for any other degree.

I certify that any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

Pradeep Singh Tanwar

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Publications arising from this thesis

Research papers

1. Tanwar PS and McFarlane JR. **Immunolocalization of Bone Morphogenetic Protein - 4 in reproductive organs of mice.** (Submitted to *Reproduction*).
2. Tanwar PS and McFarlane JR. **Immunolocalization of BMP-4 protein is developmentally regulated in mouse testis.** (Submitted to *Reproduction, Fertility and Development*).
3. Tanwar PS, O'Shea T and McFarlane JR. **Active immunization against BMP-4 decrease ovulation rate in ewes.** (Submitted to *Reproduction*).
4. Tanwar PS, O'Shea T and McFarlane JR. **In vivo evidence of role of Bone Morphogenetic Protein -4 in mouse ovary.** (Accepted by *Animal Reproduction*).

Conference abstracts

1. Tanwar PS and McFarlane JR. **In vivo evidence for a role of Bone Morphogenetic Protein -4 in ovarian function.** Proceedings of the Thirty Sixth Annual Conference of Australian Society for Reproductive Biology. 4-7 September 2005, Perth, WA, Australia (page 89).
2. Tanwar PS and McFarlane JR. **Bone Morphogenetic Protein-4 immunolocalization is developmentally regulated mice testes**. Proceedings of the Thirty Seventh Annual Conference of Australian Society of Reproductive Biology. 20-23 August 2006. Gold Coast, Queensland, Australia (page 47).
3. Tanwar PS, O'Shea T, Almahbobi G and McFarlane JR. **Vaccination against BMP-4 reduces ovulation rate in ewes.** Third Congress of the Australian Health

and Medical Research Congress. 26 November- 1 December 2006. Melbourne, Australia.

4. Tanwar PS and McFarlane JR. **Role of BMP-4 in early follicular development**. Proceedings of School of Biological, Biomedical and Molecular Sciences Postgraduate Conference. 10 February 2006. University of New England, Armidale, NSW, Australia (Page CL1)

Abbreviations

aa	Amino acids
ActR	activin receptor
ALK	activin receptor like kinases
AMHR	anti-mullerian hormone receptor
AMH	anti-mullerian hormone
ANOVA	analysis of variance
APS	3-aminopropyltriethoxysilane
BAMBI	BMP and activin membrane bound inhibitor
BCIP	5-bromo-4chloro-3-indolyl phosphate
BMPs	bone morphogenetic proteins
BMPR	BMP receptor
BRE-Luc	BMP responsive promoter constructs
cAMP	cyclic adenosine monophosphate
COC	cumulus oocyte complex
Co-smad	common smad
CL	corpus luteum
CYP11A1	cytochrome P450 side chain cleavage
CYP17	cytochrome P450 17 ά hydroxylase
CTGF	connective tissue growth factor
COX-2	cyclooxgenase 2
Dan	differential screening selected gene aberrative in neuroblastoma
dbcAMP	dibutyryl cyclic adenosine monophosphate
DNA	deoxyribonucleic acid
E2	estradiol
eCG	equine chorionic gonadotrophin
EGF	epidermal growth factor
ELISA	enzyme linked immunoabsorbent assay
FCA	Freunds complete adjuvant
FGF	fibroblast growth factor
bFGF	basic FGF

FIA	Freunds incomplete adjuvant
FSH	follicle stimulating hormone
FSH-RF	FSH-releasing factor
FSRP	follistatin related protein
GDF	growth differentiation factor
GDNF	glial cell derived neural cell factor
GnRH	gonadotrophin releasing hormone
GPI	glycosylphosphatidylinositol
HAS2	hyaluronan synthase 2
hCG	human chorionic gonadotrophin
HGF	hepatocyte growth factor
HOTT	human ovarian theca tumor cells
3βHSD	3β hydroxysteroid dehydrogenase
17βHSD	17β hydroxysteroid dehydrogenase
IGF	insulin-like growth factor
IU	international units
I-smad	inhibitory smad
Kd	dissociation constant
kDa	kilodalton
KGF	keratinocyte growth factor
KL	kit ligand
LH	luteinising hormone
LIF	leukemia inhibitory factor
MAPK	mitogen activated protein kinase
MH	MAD homologous region
MIS	mullerian inhibiting substance
mRNA	messenger RNA
MW	molecular weight
NPP	p-nitrophenyl phosphate, disodium salt hexahydrate
NRS	normal rabbit serum
oFSHbetaLuc	ovine FSHbeta promoter linked to a luciferase reporter gene
OSE	ovarian surface epithelium
P450scc	P450 cholesterol side chain cleavage
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PDE	phosphodiesterase
PDGF	platelet derived growth factor
PEG	polyethylene glycol
PGC	primordial germ cell
PMSG	pregnant mare serum gonadotrophin
PRDC	protein related to Dan and Cerberus
RGM	repulsive guidance molecule
RIA	radioimmunoassay
R-smad	receptor regulated smads
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
smurf	smad ubiquitination regulatory proteins
SPARC	secreted protein acidic and rich in cysteine
STAR	steroidogenic acute regulatory protein
SF-1	steroidogenic factor-1
TEMED	N,N,N',N'-tetramethylenediamine
TGF	transforming growth factor
TSC-36	TGF β stimulated clone-36
Tsg	twisted gastrulation
uPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
Vg-1	vegetalising factor-1
Wnt	wingless-type MMTV integration site family

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Abstract

The BMPs comprise the largest subgroup of the TGF-β superfamily. Various members of the BMP family have been shown to regulate mammalian folliculogenesis by affecting granulosa cell proliferation, steroidogenesis and by modulating the production of various endocrine factors such as activin A, follistatin, inhibin, estradiol and progesterone. *In situ* hybridization studies have shown expression of BMPR-IA, BMPR-IB and BMPR-II in the granulosa cells and oocyte of most of the follicles in the ovary suggesting that these cells have capacity to respond to BMP signaling. In addition, the mRNA for BMP-4 and BMP-7 has been in detected in the theca layer of rat follicles. In *in vitro* conditions, physiological concentrations of BMP-4 and BMP-7 enhanced and attenuated respectively, the stimulatory action of FSH on estradiol and progesterone production. The objective of the current study was to elucidate the role of BMP-4 in mammalian follicular development and spermatogenesis by using mouse and sheep as experimental models.

The transition of a primordial follicle to a primary follicle is an early step in folliculogenesis. All female mammals are born with a fixed stock of primordial follicles and exhaustion of that stock leads to menopause or infertility. Recently, several *in vitro* studies have indicated that BMP-4, BMP-7 and several other growth factors affect the transition of primordial to primary follicles. In this study passive immunization against BMP-4 was used to investigate the role of BMP-4 in this process in a prepubertal mouse model. After 7 days of treatment, the weight of anti BMP-4 treated ovaries was significantly lower than the ovaries from mice treated with non immune immunoglobulin (Ig). The number of primary follicles was lower and the numbers of primordial follicles were higher in anti BMP-4 treated ovaries compared to control ovaries. Treatment with PMSG showed no influence on the effects of anti

BMP-4 on ovary. Thus the results of our study indicate that *in vivo*, BMP-4 acts as a permissive factor in the transition of primordial to primary follicles.

In another experiment, BMP-4 protein in mouse ovary, oviduct and uterus was localized using immunohistochemistry and BMP-4 isoforms detected in mouse ovary by western blotting. In the ovary, BMP-4 protein was detected in all the stages of follicular development except primordial follicles. The intensity of staining was higher in healthy follicles than atrectic follicles, with significant staining in the corpus luteum. In the uterus, BMP-4 staining was limited to the blood vessels, endometrial gland and surface epithelium of endometrium. In the oviduct, BMP-4 was exclusively detected in epithelium and blood vessels. Different isoforms (apparent MW: 50, 35 and 15 kDa) of BMP-4 were detected in nonreducing and reducing condition in mouse ovary by western blot analysis.

In male mice, *in situ* hybridization studies have shown the presence of BMP-2, BMP-4, BMP-8A, BMP-8B in testis and BMP-4, BMP-7, BMP-8A in epididymis of mice. We localized BMP-4 protein in testis, epididymis, vas deferens, seminal vesicle and prostate of mice using immunohistochemistry and studied the role of BMP-4 in testosterone production by passively immunizing mice against BMP-4. In 1 week old mice, BMP-4 staining was not observed in the testis. At 2 weeks, BMP-4 specific staining was detected in spermatocytes. BMP-4 protein expression was also observed in spermatids of 4 wk and 7 wk old testis but was not detectable in testis from 9 wk old and adult mice. In contrast, BMP-4 specific staining was detected in epithelial cells of the epididymis at all the stages of testicular development. In the vas deferens, BMP-4 protein expression was limited to the luminal epithelial cells. BMP-4 specific staining was also observed in epithelial lining of the prostate gland. Treatment with anti BMP-4 decreased and increased testosterone secretion in prepubertal and adult mice, respectively. In addition, animals treated with anti BMP-4 and PMSG had lower testosterone

concentrations than PMSG treated animals. However, no difference in testosterone concentration was observed between anti BMP-4 plus PMSG and PMSG treated adult animals. These findings indicate that BMP-4 plays an important role in initiation phase of murine spermatogenesis and in the regulation of testosterone secretion.

Similar to the studies in mice, we investigated the expression of BMP-4 in ovary, oviduct, uterus, pituitary, testis and epididymis of fetal and adult sheep by immunohistochemistry and detected BMP-4 isoforms in ovine follicular fluid by western blotting. In fetal ovary, strong immunostaining was observed in pregranulosa cells, oocyte, primordial and primary follicle. In adult sheep ovary, strong immunostaining was observed in granulosa cells of preantral to late antral follicle stage of follicular development. BMP-4 expression was also detected in oocyte, corpus luteum, ovarian surface epithelium and, to lesser extent in theca cells but not in primordial and primary follicle. In oviduct, strong immunostaining was observed in epithelium and blood vessels in both fetal and adult sheep. In uterus, BMP-4 expression was observed in surface epithelium, circular muscles and blood vessels of fetus and also in epithelium of endometrial glands in adult sheep uterus. Strong immunostaining was observed predominantly in basophilic cells of adult sheep pituitary. Strong expression of BMP-4 was detected in epithelial cells of epididymis but no expression was detected at any stage of testicular development. Different isoforms (apparent MW: 100.4, 50.1, 42, 38.5, 33.5, 23.9 and 15.1 kDa) of BMP-4 were detected in nonreducing and reducing condition in ovine follicular fluid by western blot analysis. The differences in results of immunolocalization and western analysis studies in mice and sheep supports the view that this protein plays a different role in monoovular and polyovular species.

BMP-4 has been shown to affect steroidogenesis in sheep follicular cells in *in vitro* condition. However, role of this protein is not well understood in *in vivo* conditions,

particularly in species with low ovulation rate such as sheep. Therefore, we examined the role of BMP-4 in sheep reproduction by actively immunizing adult cyclic ewes against BMP-4 peptide. The effect of the active immunization against BMP-4 was observed on follicular development, ovulation rate and progesterone concentration. Active immunization against BMP-4 resulted in decreased ovulation rate without affecting follicular numbers (diameter $\geq$ 3mm). In addition, progesterone concentration in plasma of immunized animals was lower in luteal phase than control.

Overall, our studies have shown that BMP-4 plays an important role in follicular development, estrus, and spermatogenesis in mice and sheep.