

# **The Role of Bone Morphogenetic Protein-4 in Mammalian Reproduction**

**By**

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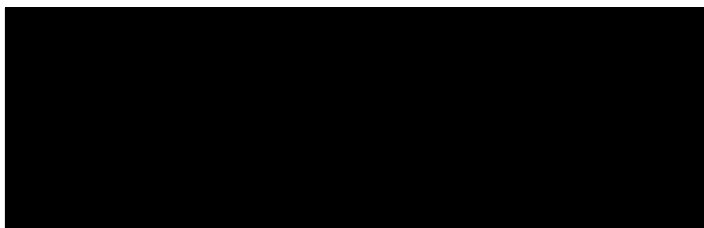
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# Declaration

*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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# Table of Contents

ACKNOWLEDGEMENTS .....	II
LIST OF CONTENTS.....	III
PUBLICATIONS ARISING FROM THIS THESIS.....	VI
ABBREVIATIONS.....	VIII
LIST OF FIGURES.....	XI
LIST OF TABLES.....	XVII
ABSTRACT .....	XVIII
<b>CHAPTER 1: GENERAL INTRODUCTION .....</b>	<b>1</b>
1. 1. FOLLOWING ARE THE SOME OF THE IMPORTANT MEMBERS OF THE BMP FAMILY – ..	3
1.1. 1. <i>BMP-1</i> .....	3
1.1. 2. <i>BMP-2 (BMP-2A or BMP-2- <math>\alpha</math>)</i> .....	4
1.1. 3. <i>BMP-4 (BMP-2B or BMP-2-<math>\beta</math>)</i> .....	4
1.1. 4. <i>BMP-5</i> .....	6
1.1. 5. <i>BMP-6 (Vgr-1)</i> .....	8
1.1. 6. <i>BMP-7 (Osteogenic protein-1 or OP-1)</i> .....	8
1.1. 7. <i>BMP-15 (GDF-9B)</i> .....	9
1.1. 8. <i>GDF-9</i> .....	11
1. 2. BMP SIGNALING .....	14
1.2. 1. <i>Receptors</i> .....	17
1.2. 2. <i>SMAD proteins</i> .....	22
1. 3. FACTORS AFFECTING BONE MORPHOGENETIC PROTEINS SIGNALING.....	27
1.3. 1. <i>Intracellular factors</i> .....	27
1.3. 2. <i>Extracellular factor</i> .....	29
1. 4. LOCAL REGULATORY FUNCTIONS OF BMPs IN REPRODUCTION.....	36
1.4. 1. <i>Pituitary</i> .....	36
1.4. 2. <i>Ovary</i> .....	38
1.4. 3. <i>Granulosa cells</i> .....	41
1.4. 4. <i>Thecal cells</i> .....	45
1.4. 5. <i>Primordial germ cell (PGC)</i> .....	46
1.4. 6. <i>Oocyte and oocyte derived factors</i> .....	49
1.4. 7. <i>Sheep breeds with a high ovulation rate</i> .....	52
1.4. 8. <i>BMPs and Testis</i> .....	58
1.4. 9. <i>Embryo development</i> .....	59
1. 5. AIM OF THE PROJECT.....	61
<b>CHAPTER 2: MATERIAL AND METHODS.....</b>	<b>62</b>
2. 1. ANTISERUM PRODUCTION .....	62
2.1. 1. <i>Immunization of sheep</i> .....	62
2.1. 2. <i>Enzyme-linked immunoabsorbent assay (ELISA)</i> .....	62
2.1. 3. <i>Antibody purification from sheep plasma</i> .....	64

2.1. 4. Biotinylation of antibody.....	65
2. 2. IMMUNOHISTOCHEMISTRY .....	66
2.2. 1. Tissue preparation, embedding and sectioning.....	66
2.2. 2. Immunostaining.....	66
2. 3. WESTERN ANALYSIS .....	68
2. 4. IMAGE PROCESSING .....	69
2. 5. ANIMAL ETHICS .....	69
2. 6. STATISTICS .....	69
<b>CHAPTER 3: <i>IN VIVO</i> EVIDENCE OF ROLE OF BMP-4 IN THE MOUSE</b>	
<b>OVARY .....</b>	<b>70</b>
3. 1. INTRODUCTION .....	70
3. 2. MATERIALS AND METHODS .....	73
3.2. 1. Reagents and hormones .....	73
3.2. 2. Animals.....	73
3.2. 3. Histological analysis and follicle counting.....	73
3. 3. RESULTS .....	75
3.3. 1. Effect of anti BMP-4 on ovarian weight.....	75
3.3. 2. Effect of anti BMP-4 treatment on follicular development.....	75
3. 4. DISCUSSION .....	80
<b>CHAPTER 4: IMMUNOLOCALIZATION OF BMP- 4 IN REPRODUCTIVE</b>	
<b>ORGANS OF MICE .....</b>	<b>83</b>
4. 1. INTRODUCTION .....	83
4. 2. MATERIAL AND METHODS.....	89
4.2. 1. Tissues and sections preparation.....	89
4.2. 2. Enzyme-linked immunoabsorbent assay (ELISA) .....	89
4.2. 3. Immunohistochemistry .....	89
4.2. 4. Western blotting .....	89
4. 3. RESULTS .....	90
4. 4. DISCUSSION .....	96
<b>CHAPTER 5: IMMUNOLOCALIZATION OF BMP-4 PROTEIN IS</b>	
<b>DEVELOPMENTALLY REGULATED IN MICE TESTES.....</b>	<b>101</b>
5. 1. INTRODUCTION .....	101
5. 2. MATERIALS AND METHODS .....	105
5.2. 1. Tissues and section preparation.....	105
5.2. 2. Passive immunization against BMP-4.....	105
5.2. 3. Immunohistochemistry .....	105
5.2. 4. Testosterone assay.....	106
5. 3. RESULTS .....	107
5.3. 1. BMP-4 immunolocalization in testis, epididymis, vas deferens, prostate and seminal vesicles.....	107
5.3. 2. Passive immunization against BMP-4 and testosterone secretion.....	107
5. 4. DISCUSSION .....	114
<b>CHAPTER 6: IMMUNOHISTOCHEMICAL LOCALIZATION OF BMP-4</b>	
<b>PROTEIN IN THE REPRODUCTIVE AXIS OF SHEEP .....</b>	<b>118</b>

6. 1. INTRODUCTION .....	118
6. 2. MATERIAL AND METHODS .....	121
6.2. 1. <i>Tissue and section preparation</i> .....	121
6.2. 2. <i>Immunohistochemistry</i> .....	121
6.2. 3. <i>Western blotting</i> .....	122
6. 3. RESULTS .....	123
6. 4. DISCUSSION .....	131
<b>CHAPTER 7: ACTIVE IMMUNIZATION AGAINST BMP-4 DECREASES OVULATION RATE IN EWES.....</b>	<b>138</b>
7. 1. INTRODUCTION .....	138
7. 2. MATERIAL AND METHODS.....	141
7.2. 1. <i>Animals</i> .....	141
7.2. 2. <i>Active immunization of ewes against BMP-4</i> .....	141
7.2. 3. <i>Determination of antibody titer and cross reactivity</i> .....	142
7.2. 4. <i>Determination of Progesterone concentration</i> .....	142
7. 3. RESULTS .....	144
7.3. 1. <i>Antibody titers</i> .....	144
7.3. 2. <i>Effect of active immunization on ovulation rate and follicle number</i> .....	144
7.3. 3. <i>Effect of active immunization on progesterone concentration</i> .....	145
7. 4. DISCUSSION .....	151
<b>CHAPTER 8: GENERAL DISCUSSION.....</b>	<b>154</b>
<b>REFERENCES .....</b>	<b>160</b>
<b>APPENDIX .....</b>	<b>213</b>

# 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

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

<b>FIA</b>	Freunds incomplete adjuvant
<b>FSH</b>	follicle stimulating hormone
<b>FSH-RF</b>	FSH-releasing factor
<b>FSRP</b>	follistatin related protein
<b>GDF</b>	growth differentiation factor
<b>GDNF</b>	glial cell derived neural cell factor
<b>GnRH</b>	gonadotrophin releasing hormone
<b>GPI</b>	glycosylphosphatidylinositol
<b>HAS2</b>	hyaluronan synthase 2
<b>hCG</b>	human chorionic gonadotrophin
<b>HGF</b>	hepatocyte growth factor
<b>HOTT</b>	human ovarian theca tumor cells
<b>3<math>\beta</math>HSD</b>	3 $\beta$ hydroxysteroid dehydrogenase
<b>17<math>\beta</math>HSD</b>	17 $\beta$ hydroxysteroid dehydrogenase
<b>IGF</b>	insulin-like growth factor
<b>IU</b>	international units
<b>I-smad</b>	inhibitory smad
<b>Kd</b>	dissociation constant
<b>kDa</b>	kilodalton
<b>KGF</b>	keratinocyte growth factor
<b>KL</b>	kit ligand
<b>LH</b>	luteinising hormone
<b>LIF</b>	leukemia inhibitory factor
<b>MAPK</b>	mitogen activated protein kinase
<b>MH</b>	MAD homologous region
<b>MIS</b>	mullerian inhibiting substance
<b>mRNA</b>	messenger RNA
<b>MW</b>	molecular weight
<b>NPP</b>	p-nitrophenyl phosphate, disodium salt hexahydrate
<b>NRS</b>	normal rabbit serum
<b>oFSHbetaLuc</b>	ovine FSHbeta promoter linked to a luciferase reporter gene
<b>OSE</b>	ovarian surface epithelium
<b>P450sc</b>	P450 cholesterol side chain cleavage
<b>PBS</b>	phosphate buffered saline

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

## List of Figures

**Figure 1. 1.** Comparison of the amino acid sequence of BMP-4 in Chicken (*Gallus gallus*), Dama dama (Fallow deer/ *Cervus dama*), Human (*Homo sapiens*), Mouse (*Mus musculus*), Rabbit (*Oryctolagus cuniculus*), Rat (*Rattus norvegicus*) and African clawed frog (*Xenopus laevis*). Identical aa are indicated with (\*) while differences in aa are indicated with (.) or (:). (Human protein reference database [www.hprd.org](http://www.hprd.org)). \_\_\_\_\_ 7

**Figure 1. 2.** Mechanism of BMP signaling revealing multiple levels of regulation, including : 1) secreted extracellular antagonists that bind to their cognate BMPs and prevent receptor binding; 2) signaling from preformed heteromeric complex of type I and type II BMP receptors in which ligand binding results in activation of smads-1/-5 pathway, which can regulated by inhibitory smad-6 and -7, smad binding proteins, Ski and Tob, and ubiquitination and degradation by smurf-1 and -2; 3) signaling from ligand induced heteromeric complexes of type-I and type-II BMP receptor, which results in the activation of a p38 MAPK pathway; and 4) nonsignaling BMP pseudoreceptors, BAMBI. Reproduced from (Canalis et al., 2003). \_\_\_\_\_ 16

**Figure 1. 3.** Phylogenetic relationship of paralogous TGF- $\beta$ / GDF/ BMP ligands, as well as characterized receptors and signaling pathways for individual ligands. The alignment of 35 TGF $\beta$ -related ligands was performed using the C-terminal region containing the cystine knot structure, starting from the first invariant cysteine residue. Based on published literature, the type II and type I receptors as well as the intracellular signaling smad proteins for individual ligands are listed. Dashed lines indicate orphan ligands under investigation. Reproduced from (Mazerbourg et al., 2005). \_\_\_\_\_ 20

**Figure 1. 4.** Members of the activin/TGF- $\beta$  receptor serine/threonine kinase receptor superfamily. Left: dendrogram representing the different type I and type II receptors. Right: graphic representation of the structure of the TGF- $\beta$  receptor superfamily members. Number in parenthesis (right side) represents the number of amino acids for each receptor. The percentages of homology in the ligand binding domain and in the cytoplasmic kinase domain are indicated inside the sequence of the receptors and are relative to the ALK4/ ActR-IB. The signal peptide sequence and the transmembrane domain of the receptors are represented in black, and the N-glycosylation sites on the extracellular domain are marked as follows (Y). The horizontal dotted line in the center

separates the type I receptors from the type II receptors. MIS, mullerian inhibiting substance. Reproduced from (Lebrun et al., 1996). \_\_\_\_\_ 22

**Figure 1. 5.** The smad family. Listed members are from vertebrates unless otherwise indicated. Vertebrate smads are highly conserved between human and *Xenopus*. The dendrogram indicates the relative level of amino acid sequence identity between vertebrate smads. The highly conserved MH1 and MH2 are indicated. Receptor regulated smads are directly phosphorylated by TGF- $\beta$  family type I receptors, and this phosphorylation allows association with a collaborating smad (co-smad). Antagonistic smads inhibit this smad activation process. Reproduced from (Massague, 1998). \_\_\_\_\_ 25

**Figure 1. 6.** Smad domains and their functions. In the basal state, smads form homooligomers and remain in an inactive state through an interaction between the MH1 and MH2 domains. Receptor-regulated smads interact with activated type I receptors via the MH2 domain and become activated by receptor mediated phosphorylation at the C-terminal SS(V/M)S motif. In the activated state, smads associate with smad-4 and with the DNA-binding protein by the MH2 domain. The MH1 domain of some smads also participates in DNA binding, and the MH2 domain participates in transcriptional activation. MAP kinases phosphorylate some smads in their linker region, inhibiting smad accumulation in the nucleus. Reproduced from (Massague, 1998). \_\_\_\_\_ 26

**Figure 1. 7.** A model of the two-cell, two gonadotrophin theory of follicular estrogen biosynthesis. The diagram combines known events taking place in the granulosa and theca-interstitial cells: (+) stimulatory; (-). Reproduced from (Erickson et al., 1985). \_\_\_\_\_ 45

**Figure 2. 1.** This graph shows the binding of anti-BMP-4 antibody to BMP-4 peptide in competition with increasing amount of BMP-2 and BMP-4 peptide. The amounts of BMP-2 and BMP-4 peptide used to compete with BMP-4 peptide coated on plate are indicated on X-axis. \_\_\_\_\_ 64

**Figure 3. 1.** The in vivo effects of anti BMP-4 treatment on ovarian weight in the presence or absence of eCG. The data are shown as mean  $\pm$  SEM for individual ovaries of five mice of each treatment group repeated 3 times. Different lettered subscripts represent significant differences in ovarian weight of each treatment group ( $P < 0.0001$ ). \_\_\_\_\_ 77

**Figure 3. 2.** The effects of anti BMP-4 treatment on follicular development in the presence or absence of a low dose of eCG. The data are displayed as percent of total follicle number per ovary in each treatment group. The data are shown as mean  $\pm$  SEM for five mice in each treatment group repeated 3 times ( $P < 0.0001$ ). \_\_\_\_\_ 78

<b>Figure 3. 3.</b> Representative ovarian sections from pubertal mouse ovaries after 7 days of treatment with anti BMP-4, eCG with or without anti BMP-4 or non immune serum. Each panel displays a partial view of ovarian section of each treatment group at 200X magnification. (A) Ovarian section from anti BMP-4 treated group (B) Ovarian section from control group (C) ovarian section from anti BMP-4 with eCG treated group (D) Ovarian section from eCG treated group. Primordial follicle (pr), Primary follicle (pa), Developing follicle (de)	79
<b>Figure 4. 1(A).</b> Expression of BMP-4 protein in primary (Pra), preantral (Pan) and antral (An) but not in primordial (Pri) follicle of mouse ovary (200 X)	92
<b>Figure 4. 2(B).</b> Expression of BMP-4 of tertiary follicle (Granulosa cells: G; Theca cells: T; Surface epithelium: SE; Stromal tissue: S; Oocyte:O) (200 X)	92
<b>Figure 4. 3(C).</b> Expression of BMP-4 in granulosa and theca cells of tertiary follicle (Granulosa cells: G; Theca cells: T) (1000X)	92
<b>Figure 4. 4(D).</b> Expression of BMP-4 in atretic follicle (Granulosa cells: G; Theca cells: T; Surface epithelium: SE; Oocyte: O) (200X)	93
<b>Figure 4. 5(E).</b> Localization of BMP-4 in corpus luteum (400X)	93
<b>Figure 4. 6(F).</b> Expression of BMP-4 in oviduct (Epithelium: Ep; Connective tissue: Ct; Blood vessels: Bv) (200X)	93
<b>Figure 4. 7(G).</b> BMP-4 protein localization in uterus (Blood vessels: Bv; Surface epithelium of endometrium: Sep; Endometrial gland: Eg )(200X)	94
<b>Figure 4. 8(H).</b> Negative control (200X)	94
<b>Figure 4. 9.</b> Western blot analysis of mouse ovary for detection of BMP-4. The samples were subjected to 12.5% SDS-PAGE under non-reducing (lane A) and reducing conditions (lane B). The membranes were treated with equal concentration of anti BMP-4 antibody (lane A and B) and non specific purified sheep Ig (lane C and D). The approximate molecular weight of bands detected is shown.	95
<b>Figure 5. 1(A).</b> BMP-4 protein was localized in epithelium of epididymis, while no expression was observed in testis at 1 wk of age (100 X) (E: epididymis; T: testis).	109
<b>Figure 5. 2(B).</b> BMP-4 protein was detected in pachytene spermatocytes at 2 wk of age (200 X)	109
<b>Figure 5. 3(C).</b> Expression of BMP-4 protein was detected in spermatocytes at 4 wk of age (200 X)	109
<b>Figure 5. 4(D).</b> BMP-4 protein was detected in spermatocytes and some interstitial cells (arrow) at 7 wk of age (200 X)	110

- Figure 5. 5(E).** No expression of BMP-4 protein was observed in testis of 9 wk old and adult mice (200 X) \_\_\_\_\_ 110
- Figure 5. 6(F).** BMP-4 protein expression in epithelium of epididymis was observed throughout postnatal development (200.X) \_\_\_\_\_ 110
- Figure 5. 7(G).** BMP-4 protein was detected in luminal epithelium of vas deferens (400 X) \_\_\_\_\_ 111
- Figure 5. 8(H).** Expression of BMP-4 protein was observed in epithelium of prostate gland (200 X) \_\_\_\_\_ 111
- Figure 5. 9.** Effect of anti BMP-4, antiBMP-4 and eCG, eCG and non immune serum treatment on testicular testosterone concentration of prepubertal mice. Distinct letters above bars represents statistically significant differences between groups ( $P < 0.05$ ). 112
- Figure 5. 10.** Effect of anti BMP-4, antiBMP-4 and eCG, eCG and non immune serum treatment on testicular testosterone concentration of adult mice. Distinct letters above bars represents statistically significant differences between groups ( $P < 0.05$ ). \_\_\_\_\_ 113
- Figure 6. 1(A).** Expression of BMP-4 protein in fetal ovary (Oocyte: Oc; Pregranulosa cells: Pg; Basal membrane: Bm; Stroma: S; Primordial: Pri and Primary follicles: Pra) (400X). \_\_\_\_\_ 125
- Figure 6. 2(B).** No expression of BMP-4 in primordial and primary follicles of adult ovary (Primary follicles: Pra) (400X). \_\_\_\_\_ 125
- Figure 6. 3(C).** Expression of BMP-4 in preantral follicle (Pan) of adult ovary (Granulosa cells: G; Theca cells: T; Blood vessels: Bv; Oocyte: O)(400X). \_\_\_\_\_ 125
- Figure 6. 4(D).** Expression of BMP-4 in large antral follicle of adult ovary (400X)\_\_ 126
- Figure 6. 5(E).** Localization of BMP-4 in ovarian surface epithelium (OSE) of adult ovary (Ovarian surface epithelium: OSE; Primordial: Pri )(400X) \_\_\_\_\_ 126
- Figure 6. 6(F).** Expression of BMP-4 in corpus luteum in adult ovary (400X) \_\_\_\_\_ 126
- Figure 6. 7(G).** BMP-4 protein localization in fetal oviduct (BMP-4 protein expression in adult oviduct is similar to fetal oviduct. Fig. not shown) (Epithelium: Epi; Blood vessels: Bv) (400X) \_\_\_\_\_ 127
- Figure 6. 8(H).** Expression of BMP-4 in fetal uterus (Blood vessels: Bv; Surface epithelium of endometrium: Sep) (200X) \_\_\_\_\_ 127
- Figure 6. 9(I).** BMP-4 expression in adult sheep uterus (Surface epithelium of endometrium: Sep; Endometrial gland: Eg) (200X) \_\_\_\_\_ 127

- Figure 6. 10(J).** Hematoxylin and Eosin stain section of adult sheep pituitary, arrowhead indicate basophilic cells while arrow indicate acidophilic cells (same section was used in immunostaining; 1000X) \_\_\_\_\_ 128
- Figure 6. 11(K).** BMP-4 expression in pituitary gland, arrowhead showing staining in basophilic cells while arrow indicate acidophilic cells (1000X) \_\_\_\_\_ 128
- Figure 6. 12(L).** BMP-4 expression in epididymis (Basal cell: Ba)(400X) \_\_\_\_\_ 128
- Figure 6. 13(M).** Lack of expression of BMP-4 in testis (200X) \_\_\_\_\_ 129
- Figure 6. 14(N).** Negative control (200X). \_\_\_\_\_ 129
- Figure 6. 15.** Western blot analysis of ovine follicular fluid for detection of BMP-4. The samples were subjected to 12.5% SDS-PAGE under non-reducing (lane A and C) and reducing conditions (lane B and D). The membranes were treated with equal concentration of anti BMP-4 antibody (membrane 1) and non specific purified sheep Ig (membrane 2). The approximate molecular weight of bands detected is shown. \_\_\_\_\_ 130
- Figure 7. 1.** This graph shows the binding of plasma collected from BMP-4 immunized and control ewes to 100 ng of rbBMP-4 protein. Plasma from BMP-4 immunized ewes showed significant binding with BMP-4 peptide while no binding to BMP-4 peptide was observed with plasma collected from control ewes..... 145
- Figure 7. 2.** Plasma from different treatment groups was tested for reactivity with BMP-4 peptide. .... 146
- Figure 7. 3.** Plasma from different treatment groups was tested for cross reactivity with BMP-2 peptide. No cross reactivity was detected to BMP-2 peptide. .... 147
- Figure 7. 4** Mean of number of CL and follicles observed at laparoscopic examination of ewes immunized against BMP-4 and controls. Number of corpus lutea was significantly lower in ewes immunized against BMP-4 than in controls ( $P < 0.05$ ). No significant difference was observed between numbers of follicles in different treatment groups ( $P > 0.05$ )..... 148
- Figure 7. 5** Mean concentration of progesterone in plasma of ewes immunized against BMP-4 and control. Progesterone concentration was lower in luteal phase of the estrous cycle in immunized ewes than in controls ( $P < 0.05$ ), but no difference in progesterone concentration was observed in follicular phase of estrous cycle ( $P > 0.05$ ). \_\_\_\_\_ 149



# List of Tables

<b>Table 1. 1.</b> <i>The mutant phenotype of some of the members of the TGF <math>\beta</math> superfamily</i>	12
<b>Table 1. 2.</b> <i>Mammalian TGF <math>\beta</math> super-family receptors and their alternative names</i>	17
<b>Table 1. 3.</b> <i>The mutant phenotype of some of the receptors of the TGF <math>\beta</math> family</i>	19
<b>Table 1. 4.</b> <i>The mutant phenotype of intracellular signaling molecules of TGF-<math>\beta</math> receptors family</i>	24
<b>Table 1. 5.</b> <i>Identified major genes affecting ovulation rate in sheep. Reproduced from (Fabre et al., 2006).</i>	56
<b>Table 4. 1.</b> <i>The schematic representation of different BMP-4 isoforms (S1: first cleavage site, S2: second cleavage site, NG: non glycosylated, G: glycosylated). (The molecular weights of some isoforms are calculated on the basis of previously reported studies and only represent full glycosylation forms of BMP-4).</i>	88

# Abstract

The BMPs comprise the largest subgroup of the TGF- $\beta$  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 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 atretic 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.